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Chiral alcohol production by NADH-dependent phenylacetaldehyde reductase coupled with *in situ* regeneration of NADH

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Phenylacetaldehyde reductase (PAR) produced by styrene-assimilating *Corynebacterium* strain ST-10 was used to synthesize chiral alcohols. This enzyme with a broad substrate range reduced various prochiral aromatic ketones and β-ketoesters to yield optically active secondary alcohols with an enantiomeric purity of more than 98% enantiomeric excess (e.e.). The *Escherichia coli* recombinant cells which expressed the *par* gene could efficiently produce important pharmaceutical intermediates; (*R*)-2-chloro-1-(3-chlorophenyl)ethanol (28 mg·mL^{-1}) from *m*-chlorophenacyl chloride, ethyl (*R*)-4-chloro-3-hydroxy butanoate) (28 mg·mL^{-1}) from ethyl 4-chloro-3-oxobutanoate and (*S*)-*N*-*tert*-butoxycarbonyl(Boc)-3-pyrrolidinol from *N*-Boc-3-pyrrolidinone (51 mg·mL^{-1}), with more than 86% yields. The high yields were due to the fact that PAR could concomitantly reproduce NADH in the presence of 3–7% (v/v) 2-propanol in the reaction mixture. This biocatalytic process provided one of the best asymmetric reductions ever reported.

Keywords: phenylacetaldehyde reductase; NADH regeneration; chiral alcohol; asymmetric reduction; *Corynebacterium* sp.

Enantioselective organic synthesis is in great demand for pharmaceuticals, agricultural chemicals and liquid crystals. Routes to obtaining optically pure compounds include enantiomer separation from a racemic mixture, derivation of natural substances, and asymmetric synthesis. Chiral metal complexes have been successfully used as catalysts in a number of cases of enantioselective synthesis [1,2]. However, in many reactions, difficulties remain in attaining sufficient optical purity and practical usage. To overcome the disadvantages of conventional processes, biocatalytic transformation systems using microorganisms and enzymes have been applied to the asymmetric synthesis of optically active substances. Asymmetric reduction is one of the most promising processes, because there is no loss of substrate compared with racemic separation using hydrolases. The reduction of the 4-substituted acetophenones by Baker's yeast gave the corresponding (*S*)-alcohols with an enantiomeric excess (e.e.) purity of 82–96% [3]. Acetone powder of the fungus *Geotrichum candidum* cells gave (*S*)-alcohols from acetophenone derivatives with an optical purity of 99% e.e. [4,5]. However, such a microbial process is not yet of practical use because of the low product accumulation.

Oxidoreductase have been used directly in the preparation of chiral alcohols with NAD^+-dependent ADHs from yeast, horse liver [6], *Candida parapsilosis* [7] and *Pseudomonas* sp. [8], and with $NADP^+$-dependent ADHs from *Thermoanaerobium brockii* [9] and *Lactobacillus kefir* [10], aldehyde reductase from *Sporobolomyces salmonicolor* (EC 1.1.1.2) [11], and carbonyl reductase (EC 1.1.1.184) from *Candida magnoliae* [12]. However, they have the disadvantages of a narrow substrate specificity, insufficient stereospecificity and sensitivity to organic solvents. Furthermore, to overcome the biocatalytic reduction it is important to regenerate NAD(P)H. Although there have been many efforts to reproduce NADH with coupling systems using formate/formate dehydrogenase, the enzyme's high cost and low activity [13] has precluded general usage. Recently, Shimizu *et al.* [14] and Kataoka *et al.* [15] reported a recombinant enzyme system consisting of aldehyde reductase of *S. salmonicolor* or carbonyl reductase of *C. magnoliae* coupled with an NADPH regenerating system comprising glucose dehydrogenase in a water/organic solvent two-phase system. They have succeeded in the accumulation of (*R*)- and (*S*)-4-chloro-3-hydroxybutanoates ethyl esters from the corresponding ketone.

We have also found a novel NADH-dependent reductase, named phenylacetaldehyde reductase (PAR), in a styrene-assimilating *Corynebacterium* strain [16–20]. Its primary structure shows a 29% identity to *Sulfolobus solfataricus* ADH, 27% to *Candida parapsilosis* ADH, 26% to *Bacillus stearothermophilus* ADH, and 26% to *Escherichia coli* ADH. Actually, the enzyme contains 2 mol of zinc per mol of subunit, and is considered as a new member of zinc-containing medium-chain alcohol dehydrogenase [19,20]. This enzyme shows a broad substrate range and catalyzes the reduction of not only various aryl ketones but also 2-alkanones [18]. Its physical properties, substrate- and stereospecificities [17,18] are rather different from those of

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Abbreviations: ADH, alcohol dehydrogenase; Boc-PN, *N*-Boc-3-pyrrolidinone; Boc-PL, *N*-Boc-3-pyrrolidinol; *m*-CPC, *m*-chlorophenacyl chloride; CCE, 2-chloro-1-(3-chlorophenyl) ethanol; ECOB, ethyl 4-chloro-3-oxobutanoate; ECHB, ethyl 4-chloro-3-hydroxybutanoate; e.e., enantiomeric excess; KPB, potassium phosphate buffer; PAR, phenylacetaldehyde reductase; IPTG, isopropyl thio-β-D-galactoside.

Enzymes: NAD^+-dependent alcohol dehydrogenase (EC 1.1.1.1); $NADP^+$-dependent alcohol dehydrogenase (EC 1.1.1.2); carbonyl reductase (EC 1.1.1.184); phenylacetaldehyde reductase (EC 1.1.1.-).

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previously reported general NAD^+-dependent ADH (EC 1.1.1.1) [21], NAD^+/$NADP^+$-dependent aryl-alcohol dehydrogenases (EC 1.1.1.90 and 91) [22,23] and $NADP^+$-dependent aldehyde and carbonyl reductases [14,15]. However, the physicochemical properties of PAR are quite similar to those of NAD^+-dependent carbonyl reductase from *Rhodococcus erythropolis* [24].

In this report, we describe the practical application of recombinant *E. coli* cells expressing PAR for the production of chiral alcohols from acetophenone derivatives, β-keto-esters and *N*-Boc-3-pyrrolidinone with a high enantioselectivity and yield. The process could function without the additional coenzyme regeneration system and produced a large amount of product, because PAR itself was able to regenerate NADH in the presence of 2-propanol.

MATERIALS AND METHODS

Chemicals

2′,4′-Dichloroacetophenone (Table 1, **1**), *m*-chlorophenacyl chloride (**2**), 3′-methoxyacetophenone (**3**), 3,4-dimethoxyphenylacetone (**4**), *N*-Boc-3-pyrrolidinone (**5**), methyl 4-bromo-3-oxobutanoate (**14**), ethyl 4-bromo-3-oxobutanoate (**15**), isopropyl 4-bromo-3-oxobutanoate (**16**), octyl 4-bromo-3-oxobutanoate (**17**), isopropyl 4-cyano-3-oxobutanoate (**18**), (*R,S*)-2-chloro-1-(3-chlorophenyl)ethanol, (*R*)-2-chloro-1-(3-chloro-phenyl)ethanol (**19**), (*R,S*)-1-(2,4-dichlorophenyl)ethanol, (*R,S*)-1-(3-methoxyphenyl)ethanol (*R,S*)-1-(2,4-di-methoxyphenyl)ethanol, methyl (*R,S*)-4-bromo-3-hydroxybutanoate), methyl (*S*)-4-bromo-3-hydroxybutanoate, (*R,S*)-*N*-Boc-3-pyrrolidinol and (*R*)-*N*-Boc-3-pyrrolidinol were kindly supplied by Sumitomo Chemical Industries, Osaka, Japan. Chloroacetone (**6**), methyl 4-oxobutanoate (**9**), *tert*-butyl 3-oxobutanoate (**11**), methyl 3-oxopentanoate (**12**), and ethyl 4-chloro-3-oxobutanoate (**13**) were purchased from Kanto Chemical Co., Inc., Tokyo, Japan. Ethyl 3-oxobutanoate (**10**) and 1,1-dichloroacetone (**7**) were from Tokyo Kasei Co., Ltd, Tokyo, Japan. (*S*)-2-Phenyl-1-propanol was from Fluka Chemie AG, Buchs, Switzerland, and (*R,S*)-1-phenyl-2-propanol, 3-chloro-2-butanone (**8**), ethyl (*R*)- and (*S*)-3-hydroxybutanoates (**23**), ethyl (*R*)- (**24**) and (*S*)-4-chloro-3-hydroxybutanoates were from Aldrich Chemical Co., USA. Other reagents used were of analytical grade.

Bacterial strain, vectors and culture conditions

The construction of the expression vector pUAR was described previously [19]. The entire *par* gene was ligated downstream of the *lac* promoter of *Bam*HI/*Pst*I-treated pUC118. *E. coli* JM109 was used as a host strain. The other expression vector, pKAR, was constructed from pUAR and pKK223-3 (Amersham Pharmacia Biotech). The *par* gene which had been cut out from pUAR with *Pst*I and *Sma*I was ligated downstream of the *tac* promoter of *Pst*I/*Sma*I-treated pKK223-3 and used to transform *E. coli* JM109. *E. coli* cells transformed with pUAR and pKAR were cultured at 37 °C and 30 °C, respectively, in Luria-Bertani medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.0) containing 0.1 $mg{\cdot}mL^{-1}$ of ampicillin and 0.01% $ZnCl_2$, unless otherwise stated. The addition of $ZnCl_2$ was necessary to obtain a high level of PAR activity. For induction of the gene under the control of the *lac* or *tac* promoter, 0.4 mM isopropyl thio-β-D-galactoside (IPTG) was added to Luria–Bertani medium.

Purification of recombinant PAR

Recombinant PAR was purified from *E. coli* (pUAR) cells grown on Luria–Bertani medium to which was added 0.01% $ZnCl_2$, 0.1 $mg{\cdot}mL^{-1}$ of ampicillin and 0.4 mM IPTG, as described previously [19].

Enzyme assay and determination of substrate specificity

PAR activity was assayed at 25 °C by measuring the decrease in absorption at 340 nm of NADH ($\varepsilon = 6220\ M^{-1}{\cdot}cm^{-1}$). The reaction mixture consisted of 3.0 μmol of phenylacetaldehyde, 0.4 μmol of NADH, 75 μmol of KPB (pH 7.0) and 5 μL of enzyme solution in a total volume of 1.5 mL, as described previously [19]. One unit of enzyme was defined as the amount that converted 1 μmol NADH and phenylacetaldehyde in 1 min at 25 °C. When the activity was measured using acetophenone as a substrate, it was found to have decreased to 35% of that for phenylacetaldehyde.

The reaction mixture for the assay of the oxidative reaction of PAR consisted of 75 μmol Tris/HCl buffer (pH 8.0), 4.5 μmol NAD^+, 15 μmol of each substrate and an appropriate amount of PAR in a total volume of 1.5 mL. The reaction was monitored by a spectrophotometer on the basis of the increase of NADH at 340 nm and 25 °C.

The substrate specificity of PAR was determined spectrophotometrically by measuring the decrease in the absorption of NADH at 340 nm using 3 mM of each substrate and the purified enzyme. The reaction conditions were the same as those for the PAR assay.

Enzymatic reaction and reaction mixture analysis

The reaction mixture for measuring the products from ketones consisted of 1–8% (w/v) of each substrate, 1 μmol NAD^+, 50 μmol potassium phosphate buffer (KPB) (pH 7.0), 5% (v/v) 2-propanol, and ≈ 60 mg (wet weight) of recombinant *E. coli* cells from a 5-mL of culture broth containing ≈ 2.5 U of PAR in a total volume of 1 mL, and was suspended in a 2-mL polypropylene tube in a Mini-incubator M-36 (Taitec, Saitama, Japan) with shaking (2500 r.p.m.). The insoluble substrate was suspended in the reaction mixture, and the reaction was allowed to proceed for 24 h at 25 °C. The product was extracted with the same volume of ethyl acetate and analyzed by GC or HPLC. GC was performed using a Shimadzu GC-14 A system equipped with a coiled column (3 mm × 2 m) packed with Thermon 1000 (5% on Chromosorb W) or a GC-18 A system equipped with a capillary column (DB-1, 0.25 mm × 30 m, J & W Scientific, CA, USA) with an FID (flame ionization detector). GC was carried out under the following conditions: a column temperature of 150 °C, injection and detection temperatures of 215 and 240 °C, and a flow rate of 50 $mL{\cdot}min^{-1}$ of N_2 for *N*-Boc-3-pyrrolidinone (Boc-PN) (GC-14 A system); column temperature of 100 °C for 5 min then raised by 5 $°C{\cdot}min^{-1}$ for 8 min and 20 $°C{\cdot}min^{-1}$ for 2 min to 180 °C and held for 1 min, injection and detection temperatures of 200 and 250 °C, and a flow rate of

1 mL·min^{-1} of He for ethyl 4-chloro-3-oxobutanoate (ECOB) (GC-18 A system). The products and substrates showed the following retention times (min): Boc-PN, 3.4; *N*-Boc-3-pyrrolidinol (Boc-PL), 11.0; ECOB, 7.6; ethyl 4-chloro-3-hydroxybutanoate (ECHB), 8.6. HPLC proceeded using the Shimadzu LC-10AT system with a TSK gel ODS-80T column (4.6 × 150 mm, Tosoh, Tokyo, Japan) for *m*-chlorophenacyl chloride (*m*-CPC). The mobile phase was H_2O/acetnitrile (3 : 2) flowing at a rate of 1 mL·min^{-1} at 35 °C. The compounds were spectrophotometrically detected at 273 nm, and the retention times of *m*-CPC and 2-chloro-1-(3-chlorophenyl)ethanol (CCE) were 12.3 and 7.3 min, respectively.

The NAD^+/NADH ratio in the reaction mixture was also determined by HPLC using the above system. The reaction mixture was centrifuged to remove cells and insoluble substrate, and the supernatant was used as a sample. The mobile phase was 50 mM KPB (pH 6.0)/acetonitrile (97 : 3) flowing at a rate of 1 mL·min^{-1} at 35 °C. The compounds were spectrophotometrically detected at 260 nm, and the retention times of NAD^+ and NADH were 3.1 and 4.7 min, respectively.

Determination of absolute configuration of products

To determine the absolute configuration of the alcohol produced from ketone, the product extracted with ethyl acetate from the reaction mixture was analyzed using GC equipped with a Chirasil-DEX CB chiral column (0.25 mm × 25 m, 0.25-μm film, Chrompack, the Netherlands) and FID. Helium gas was used as a carrier at 20 p.s.i. The split ratio was 100, the injection and detection temperatures were 220 °C, and the column temperature was 80 °C for 10 min then raised up to 150 °C at 7 °C·min^{-1} and maintained at 150 °C for 3 min. Retention times were 6.3 min for (*R*)- and 6.5 min for ethyl (*S*)-3-hydroxybutanoate (**23**).

To determine the absolute configuration of the other alcohols, they were purified by thin layer chromatography if necessary, and analyzed by HPLC with a chiral column. Analytical HPLC was performed with a Shimadzu LC-10AT system using a Chiralcel OB-H, OD-H or OF (4.6 × 250 mm, Daicel Chemical Industries, Ltd, Tokyo, Japan) under the following conditions at room temperature, unless otherwise indicated: mobile phase of hexane/2-pronanol (9 : 1), flow rate of 1.0 mL·min^{-1}, detection at 254 nm, retention time of 12.4 min for (*S*)- (**20**) and 17.2 min for (*R*)-1-(3-methoxyphenyl)ethanol (Chiralcel OB-H); retention time of 17.3 min for (*R*)- and 19.4 min for (*S*)-1-(3,4-dimethoxyphenyl)-2-propanol (**21**)(Chiralcel OB-H); flow rate of 0.5 mL·min^{-1} (30 °C), retention time of 8.2 min for (*S*)- (**22**) and 11.2 min for (*R*)-1-(2,4-dichlorophenyl)ethanol (Chiralcel OB-H); flow rate of 0.5 mL·min^{-1} (35 °C), retention times were 9.2 min for (*R*)- (**19**) and 10.0 min for (*S*)-CCE (Chiralcel OB-H); flow rate of 1.0 mL·min^{-1} (30 °C), detection at 220 nm, retention time of 7.8 min for (*R*)- (**24**) and 8.3 min for (*S*)-ECHB (Chiralcel OB-H); mobile phase was a hexane/2-pronanol (4 : 1), flow rate of 0.5 mL·min^{-1} (40 °C), detection at 220 nm, retention time of 14.1 min for (*S*)- (**25**) and 16.1 min for (*R*)-Boc-PL (Chiralcel OF); mobile phase of hexane/2-pronanol (49 : 1) plus 0.1% trifluoroacetic acid (40 °C), flow rate of 0.5 mL·min^{-1}, detection at 220 nm, retention time of 17.2 min for (*R*)- (**26**) and 18.8 min for isopropyl (*S*)-4-bromo-3-hydroxybutanoate (Chiralcel OD-H). The enantiomeric purity (% e.e.) of the product was determined by GC or HPLC.

RESULTS

Expression of PAR

The PAR activity in the cell-free extracts of *E. coli* transformants is shown in Table 1. Culture temperature affected the enzyme expression level. As the highest activity was obtained for *E. coli* JM109 (pUAR) cultured at 37 °C for 22 h, these cells were used for the resting-cells reaction. However, the PAR activity in recombinant *E. coli* JM109 (pUAR) cells often decreased by sequential inoculation. Therefore, cells freshly transformed with pUAR were used for the reaction.

Substrate and stereospecificity of PAR

PAR has a quite broad substrate specificity for many ketones including 2-alkanones and arylketones [17,18]. Table 2 summarizes the activities for some ketones tested to identify alcohols potentially useful as pharmaceutical intermediates using the purified PAR. PAR showed strong activity toward the acetophenones substituted with a methoxy group or chlorine, especially 3′- and/or 4′-positions (**1**–**4**). The preference of PAR for these compounds has been reported previously [17]. It was also found that PAR acted on some β-ketoesters (**9**–**18**) including 4-chloro- and 4-bromo-3-oxobutanoates. The activity for β-ketoesters increased fivefold to 50-fold when the methyl ester moiety was substituted by an ethyl, isopropyl or *tert*-butyl group. However, the activity level for the 4-cyano substituent of 3-oxobutanoate (**18**) was quite low. Despite of its low activity for acetone (relative activity at 3 mM in pH 6.0, 2.5% for that of acetophenone, 0.8% in pH 7.0), PAR showed strong activity towards chloroacetone derivatives (**6**–**8**). PAR also showed sufficient activity for *N*-Boc-pyrolidinone (Boc-PN) (**5**).

PAR transfers the pro-*R* hydrogen of NADH to the *re* face of the carbonyl group of acetophenone derivatives [17]. As exemplified in Table 3, the recombinant *E. coli* system expressing PAR gave the (*S*)-form chiral alcohols from the corresponding ketones with an enantiomeric purity of 98.4 to ≫ 99% e.e. The absolute configuration of the purified 1-(3,4-dimethoxyphenyl)-2-propanol (**21**) product $\{[\alpha]_D^{25} = +16.4$ (c = 1, $CH_3OH)\}$ was determined to be the (*S*)-form by a comparison with standard (*S*)-1-phenyl-2-propanol $\{[\alpha]_D^{25} = +22.8$ (c = 1, $CH_3OH)\}$. *m*-CCP (**2**), ECBO (**13**) and isopropyl 4-bromo-3-oxobutanoate (**16**)

Table 1. Effect of cultivation temperature on recombinant PAR.

	Activity (U·L^{-1} of culture)		
	Temperature (°C)		
Transformant	30	37	40
E. coli (pKAR)	390	280	177
E. coli (pUAR)	30	476	157

Table 2. Substrate specificity of recombinant PAR.

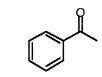
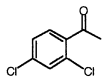
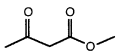
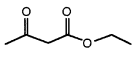
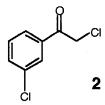
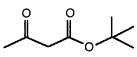
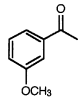
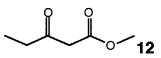
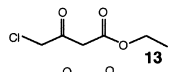
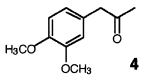
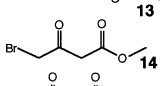
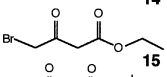
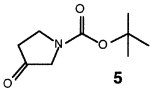
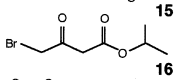
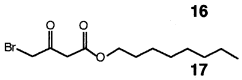
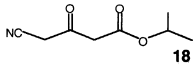
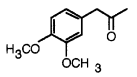
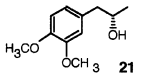
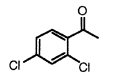
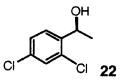
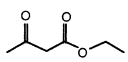
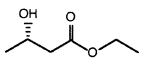
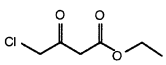
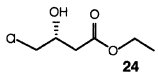
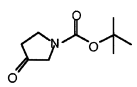
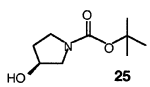
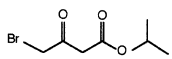
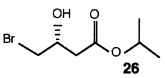
substrate	relative activity (%)	substrate	relative activity (%)
	100	 **8**	122
 **1**	546	 **9**	154
		 **10**	868
 **2**	258	 **11**	2247
 **3**	744	 **12**	5
		 **13**	187
 **4**	70	 **14**	18
		 **15**	274
 **5**	200	 **16**	994
 **6**	188	 **17**	545
 **7**	449	 **18**	7

Table 3. Enantioselectivity of PAR-catalyzed reduction of ketones.

substrate	product	enantiomeric excess (%)
	 **19**	>>99 (*R*)-form
	 **20**	>>99 (*S*)-form
	 **21**	>>99 (*S*)-form
	 **22**	>>99 (*S*)-form
	 **23**	>>99 (*S*)-form
	 **24**	99 (*R*)-form
	 **25**	>>99 (*S*)-form
	 **26**	98.4 (*R*)-form

were reduced to the (*R*)-form alcohols (**19**), (**24**) and (**26**) according to the reduction rule of PAR. Among the ketones tested, the e.e. values of the produced alcohols (**19**–**23**, **25**) were ≫ 99%, except those of ECHB (**24**) and isopropyl 4-bromo-3-hydroxybutanoate (**26**), which were 98 and 98.4%, respectively.

Kinetic parameters of the PAR reaction

To evaluate the PAR reaction, several kinetic constants were determined from Lineweaver–Burk plots using the purified PAR (Table 4). Although the K_m value for 2-propanol reached 270 mM at pH 7.0 and 440 mM at pH 8.0, the k_{cat} value was also quite high, indicating that the oxidative reaction was sufficient to reduce NAD^+ at a high concentration of 2-propanol as indicated in the next section.

Figure 1 shows the pH dependency of PAR in the reductive and oxidative reactions. The optimum pH for the oxidation of 2-propanol by PAR was around 10.5. The data suggested that pH could control the reaction rates of PAR for the desired reductive reaction and the regeneration of NADH depending on the oxidation of 2-propanol.

Optimized conditions for PAR-catalyzed production of (*R*)-CCE

To evaluate the PAR asymmetric reduction system, we used the recombinant *E. coli* cells that express PAR and are at rest, because they were easy to handle and effective. As the model substrate for the reaction, we chose *m*-CPC (**2**), which was insoluble in water and a moderately good substrate of PAR.

Table 4. Kinetic constants of the PAR. The reaction mixture for the assay of the reductive reaction consisted of varying amounts of phenylacetaldehyde or acetophenone, 0.4 μmol of NADH, 75 μmol of KPB (pH 7.0) and 5 μL of enzyme solution in a total volume of 1.5 mL, and that of the oxidative reaction consisted of 75 μmol KPB (pH 7.0) or Tris/HCl buffer (pH 8.0), 4.5 μmol NAD^+, varying amounts of 2-propanol and 5 μL of enzyme solution in a total volume of 1.5 mL. The reaction was performed at 25 °C, and the increase or decrease of NADH was spectrophotometrically measured at 340 nm.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Phenylacetaldehyde	2.0	254	127.0
Acetophenone	1.54	89	57.8
NADH[a]	0.01	–	–
2-Propanol	440 (pH 8)	2387	5.4
	270 (pH 7)	664	2.5
NAD^+	0.14	–	–

[a] Data from [18] using phenylacetaldehyde as a substrate.

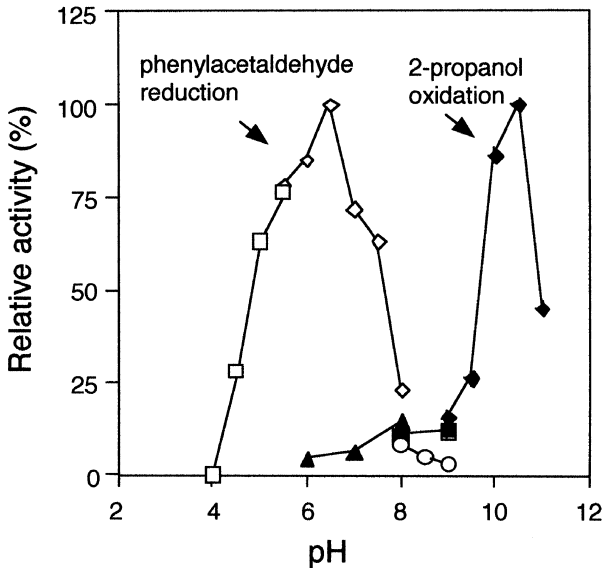


Fig. 1. PAR activity as a function of pH in the reduction of phenylacetaldehyde [18] and oxidation of 2-propanol. The activity of purified PAR was measured in the following 0.1 M buffers: citrate-K_2HPO_4 (pH 4.0–5.5) (□), KPB (pH 5.5–8.0) (◇), Tris/glycine (pH 8.0–9.0) (○) in the reductive reaction, KPB (pH 6.0–8.0) (▲), Tris/HCl (pH 8.0–9.0) (■), glycine/NaOH (pH 9.0–11) (◆) in the oxidative reaction. The reaction mixture for the assay of the reductive reaction consisted of 4.5 μmol of phenylacetaldehyde, 0.4 μmol of NADH, and the enzyme solution in a total volume of 1.5 mL, and that of the oxidative reaction consisted of 4.5 μmol NAD^+, 15, mol of 2-propanol and the enzyme solution in a total volume of 1.5 mL.

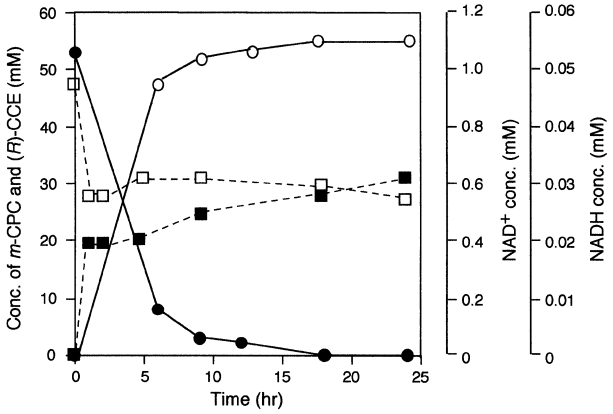


Fig. 2. Time course of the production of (*R*)-CCE and the NAD^+/NADH concentrations. The reaction mixture consisted of 1% *m*-CCP (w/v), 1 μmol NAD^+, 50 μmol potassium phosphate buffer (KPB)(pH 7.0), 5% (v/v) 2-propanol, and ≈ 60 mg (wet weight) of recombinant *E. coli* cells from 5 mL of culture broth containing ≈2.5 U of PAR in a total volume of 1 mL. Symbols: ●, (*R*)-CCE produced; ○, *m*-CCP remaining [initial conc. of 1% (53 mM)]; ma, NAD^+ (initial conc. of 1 mM), NADH. The data are the average of three measurements.

Using the reaction mixture described in Materials and methods with vigorous shaking, 1% (53 mM) *m*-CPC was completely converted into (*R*)-CCE (**19**) within 20 h (Fig. 2). The reaction conditions were varied to attain the highest productivity. The optimal pH of the reaction mixture was around 7.0, and a similar conversion was obtained between pH 6.5 and 7.5.

To regenerate NADH *in situ*, several secondary alcohols (5% v/v) were tested, including 2-propanol (relative activity at 3 mM: 6%), 2-butanol (15%), 2-pentanol (100%), 2-hexanol (240%), 2-heptanol (410%) and 2-octanol (422%), which were found to serve as a substrate for the oxidative reactions of PAR. 2-Propanol gave the best result followed by 2-heptanol, 2-octanol and 2-butanol (Fig. 3). Without the addition of 2-propanol, no conversion was observed. The optimal concentration for 2-propanol in the reaction mixture was between 3% (392 mM) and 7% (914 mM), and (*R*)-CCE production was inhibited above 10% (Fig. 4). This suggested that more than 10% (v/v) 2-propanol in the reaction mixture denatured the PAR.

The NAD^+ concentration in the reaction mixture was optimized. As shown in Fig. 5, a sufficient conversion was observed at more than 0.5 mM after 24 h. Therefore, we generally used 1 mM NAD^+ in the reaction mixture. The results also indicated that the endogenous NAD^+/NADH in the *E. coli* cells was insufficient for a smooth reaction. During the reaction, a part of NAD^+ was rapidly reduced into NADH, then NAD^+ and NADH concentrations were maintained to be 0.6 ± 0.05 mM and 0.025 ± 0.005 mM, respectively (Fig. 2). These concentrations were approximately 2.5–4 times higher than the K_m values of PAR (0.14 mM for NAD^+, 0.01 mM for NADH), suggesting that the most effective redox reaction proceeds under such a condition.

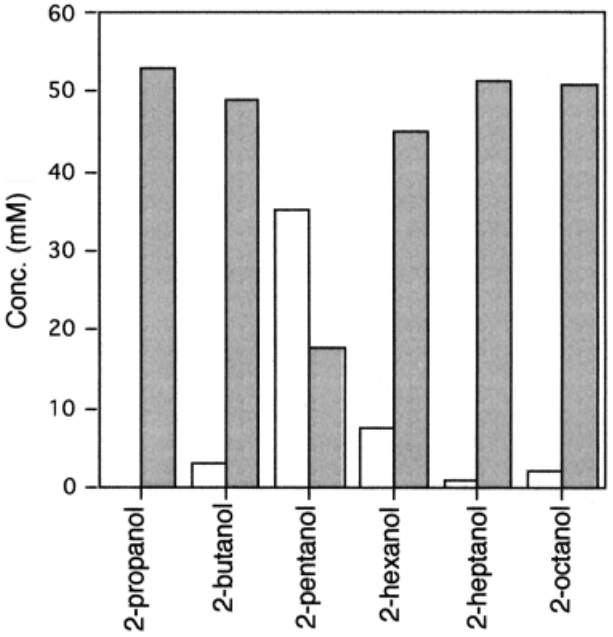


Fig. 3. Effect of secondary alcohols on (*R*)-CCE production. Each secondary alcohol was added to the reaction mixture at 5% (v/v) instead of 2-propanol as described in Fig. 2. The amount after 24 h of (*R*)-CCE produced from 1% (53 mM) *m*-CCP was drawn as a grey bar, and *m*-CCP remaining as a white bar. Complete conversion was observed for 2-propanol. The data are the average of three measurements.

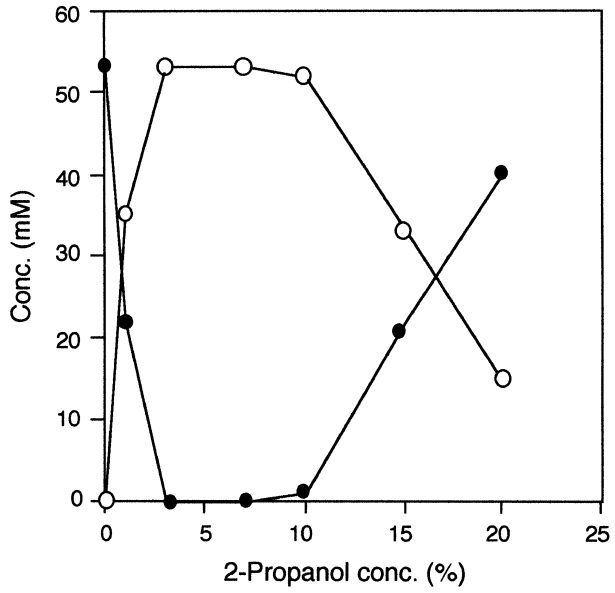


Fig. 4. Optimal 2-propanol concentration for (R)-CCE production. The reaction was carried out with varying the initial 2-propanol concentration as described in Fig. 2. Symbols: ○, (*R*)-CCE produced; ●, *m*-CCP remaining [initial conc. of 1% (53 mM)] after 24 h. The data are the average of three measurements.

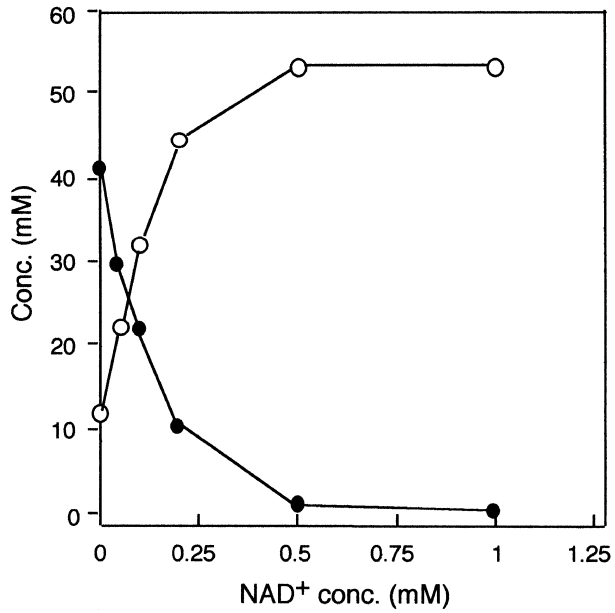


Fig. 5. Effect of NAD^+ concentration in the reaction mixture on (R)-CCE production. The reaction was carried out with varying the initial NAD^+ concentration as described in Fig. 2. Symbols: ○, (*R*)-CCE produced; ●, *m*-CCP remaining [initial conc. of 1% (53 mM)] after 24 h. The data are the average of three measurements.

When the number of *E. coli* cells in the reaction mixture was increased threefold (180 mg wet cells·mL^{-1}), 3% (159 mM) *m*-CPC was converted to (*R*)-CCE with a molar yield of 93.2% and optical purity of 99.5% e.e. after 24 h (Fig. 6). The increase of *E. coli* cells apparently raised the reaction rate and analytical yield of (*R*)-CCE.

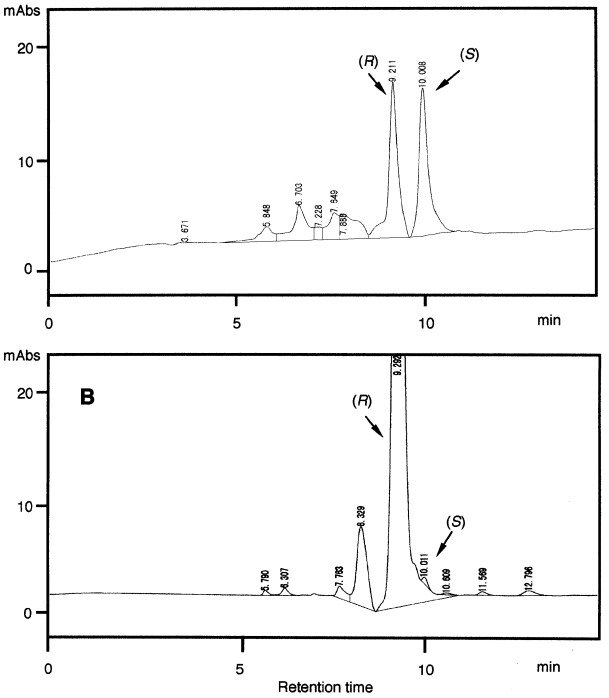


Fig. 6. HPLC analysis of CEE enantiomers. (A) Racemic CEE standard. (B) Enzymatically produced (*R*)-CEE (**19**). Each sample was loaded on a column of Chiralcel OB-H (4.6 × 250 mm), which was attached to a Shimadzu LC-10AT HPLC system, and analyzed under the conditions as described in Materials and methods.

Production of (*R*)-ECHB

We applied the optimized conditions obtained with (*R*)-CCE to the conversion of ECOB (**13**). An organic solvent and water two-phase system was used for the reaction, because ECOB was unstable in aqueous solution. The same volume of water immiscible organic solvent was added to the reaction mixture as shown in Fig. 7, and the reaction was performed in a total volume of 1 mL. The whole reaction mixture consisted of 50% (v/v) organic solvent, 1% (61 mM) ECOB and 5% (v/v) 2-propanol, 25 μmol KPB, 0.5 μmol NAD^+, and 60 mg of *E coli* cells. The conversion ratio changed markedly when the organic solvent was added (Fig. 7). *n*-Decane, *n*-octane and cyclohexane gave almost 100% conversion. But only a slight or no reaction was observed with methyl acetate and ethyl acetate. The effect of these compounds on PAR reaction was examined by adding 50 mM each compound to the reaction mixture using the purified PAR and acetophenone as a substrate. Strong inhibition of PAR were observed by these compounds: methyl acetate, 31%; ethyl acetate, 52%; butyl acetate, 85%; ethyl propionate 74%. Therefore, we used *n*-octane as an organic phase solvent in subsequent experiments. Under optimized conditions in the water/*n*-octane two-phase system, 2% (122 mM) ECOB was reduced to almost 100% ECHB after 15 h (data not shown). The efficiency of the conversion was investigated with an initial substrate concentration of between 1 and 5% (310 mM). The molar conversion yield of ECHB (**24**) and the optical purity of the (*R*)-isomer from 3% (163 mM) ECOB was 94 and 99% e.e., respectively, after 24 h. However, the analytical conversion

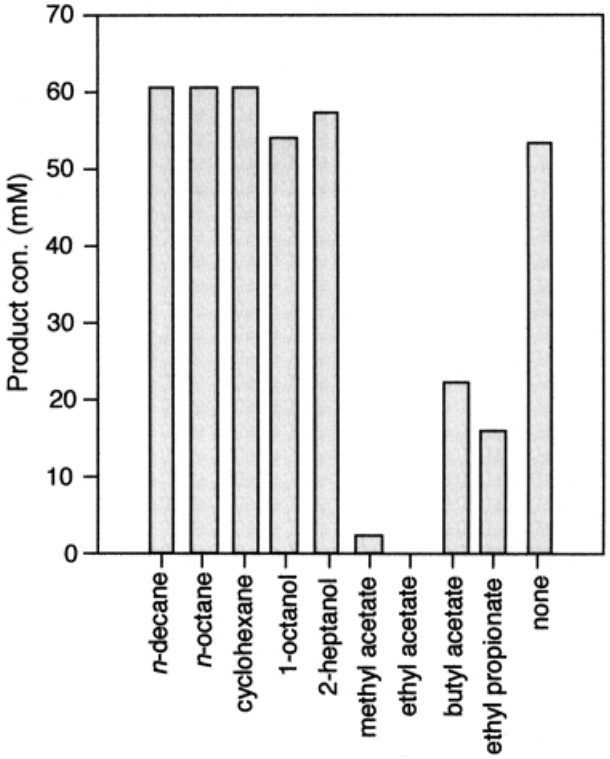


Fig. 7. Effect of organic solvent on the production of (*R*)-ECHB with a two-phase system. The whole reaction mixture consisted of 50% (v/v) organic solvent, 1% (61 mM) ECOB and 5% (v/v) 2-propanol, 25 μmol KPB, 0.5 μmol NAD^+, and 60 mg of *E. coli* cells in a total volume of 1 mL. The bar indicates the (*R*)-ECHB produced from 1% ECOB after 24 h. Complete conversion was observed for *n*-decane, *n*-octane and cyclohexane.

yield rapidly decreased when more than 4% ECOB was added to the reaction mixture even with the increased dose of *E. coli* cells. This suggested that the high concentration of ECOB inhibited the PAR reaction.

Production of (*S*)-Boc-PL

Accumulation of (*S*)-Boc-PL (**25**) from Boc-PN (**5**) was tested next, because (*S*)-pyrrolidinol is a useful intermediate for pharmaceuticals. Boc-PN with the relatively high solubility in water (15 $mg{\cdot}mL^{-1}$) was used to represent such compounds. PAR showed efficient activity for *N*-Boc-derivative of 3-pyrrolidinone, suggesting that other heterocyclic ketones would be reduced by PAR after an appropriate modification.

Under the optimized conditions obtained with (*R*)-CCE 2% (108 mM) Boc-PN was reduced to almost 100% Boc-PL after 12 h (data not shown). The efficiency of the conversion was investigated with an initial substrate concentration of between 1 and 8%. More than 90% conversion was observed at a substrate concentration of less than 4%. The molar conversion yield and the optical purity of the (*S*)-isomer from 5% (270 mM) Boc-PN was 85 and $\gg$ 99% e.e., respectively, after 24 h. However, the analytical conversion yield decreased with > 4% Boc-PN, even with increased doses of *E. coli* and 2-propanol (Fig. 8).

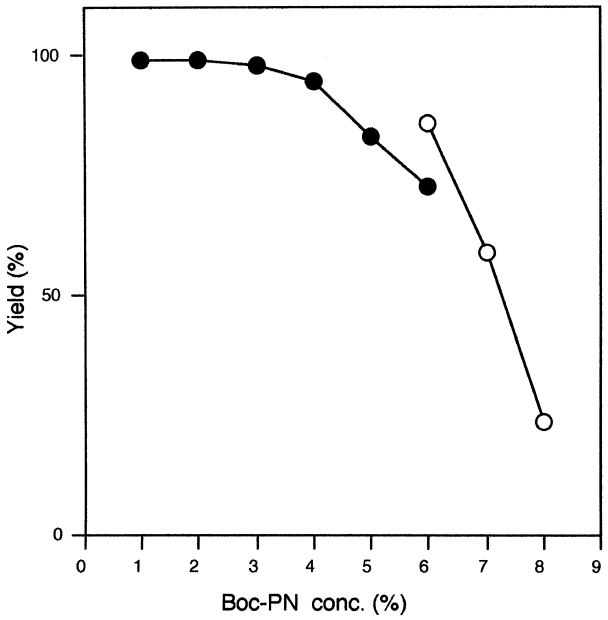


Fig. 8. Molar conversion yield of (*S*)-Boc-PL depending on the initial substrate concentration. The reaction mixture consisted of 1–8% (54–432 mM) Boc-PN, 1 μmol NAD^+, 50 μmol potassium phosphate buffer (KPB) (pH 7.0), 5% (v/v) 2-propanol, and $\approx$ 60 mg (wet weight) of recombinant *E. coli* cells in a total volume of 1 mL. At an initial Boc-PN concentration of more than 5%, 2-propanol was added to 1% excess over the indicated substrate concentration. Symbols: ●, $\approx$60 mg of wet cells were used; ○, $\approx$120 mg wet of cells were used.

DISCUSSION

PAR showed quite a broad substrate specificity for various ketones including substituted acetophenone derivatives, β-ketoesters, chloroacetones, etc. (Table 2). It became clear that PAR was able to catalyze the asymmetric reduction of more than 30 kinds of ketones [17,18]. PAR, however, had quite a strict stereospecificity for the reduction reaction. As shown in Fig. 9, the absolute configuration of the product is clear from the PAR reaction. This reaction mechanism gave definite chiral alcohols. Properties such as a broad substrate spectrum, strict stereospecificity and NAD^+/NADH-dependency are desirable features for a biocatalytic asymmetric reduction process.

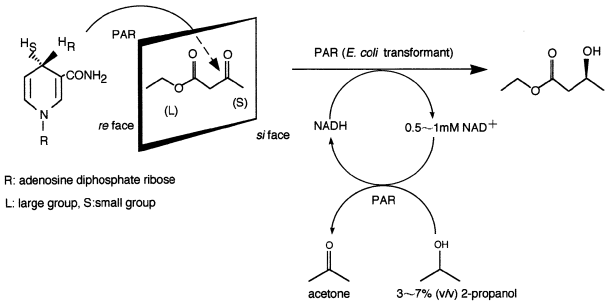


Fig. 9. Stereochemistry of PAR [17] and a schematic representation of the PAR reduction process.

For the enzymatic reduction to proceed efficiently, however, it is essential to regenerate NADH *in situ*. We revealed that PAR itself could reproduce NADH when 2-propanol was present in the reaction system. It was found that to regenerate NADH, PAR can utilize not only 2-propanol but also other secondary alcohols such as 2-butanol and 2-heptanol. Although 2-propanol was not a good substrate for PAR judging from its K_m value (Table 4), there was enough activity to reproduce NADH when it was added to the reaction mixture at high concentration (400–1000 mM). As PAR hardly catalyze the reduction of acetone under the conditions tested, the PAR/2-propanol system could supply NADH without loss. High molar conversion yields and short reaction times observed for three substances reflected the efficiency of the regeneration of NADH by the PAR/2-propanol system. 2-Propanol is superior for the biocatalytic reaction because (a) it is cheap; (b) it can elevate the solubility of water-insoluble substances in the reaction mixture; (c) there is no need to adjust pH during the reaction compared with the glucose/glucose dehydrogenase system to give gluconic acid; and (d) the acetone produced has a less inhibitory effect on the enzyme compared with the aldehydes produced from normal alcohols. Although a one-enzyme-catalyzed reaction, where a single enzyme is responsible for a desired reaction and the regeneration of cofactor was demonstrated by alcohol dehydrogenases from *Pseudomonas* [8] and *Thermoanaerobium* [9] with 2-propanol, accumulations of the products were insufficient. Our process provided a very efficient enzymatic asymmetric reduction of practical benefit.

We showed the potential applications of this process using *E. coli* recombinant PAR and three different substrates. The reduction of *m*-CPC showed that the process is suitable for many water-insoluble compounds suspended in solution. For the conversion of ECOB, which is unstable in water, an *n*-octane, *n*-decane or cyclohexane-water two-phase system was effective. Many compounds are probably suited to such a two-phase system. Greater productivity was expected for the conversions of *m*-CCP and Boc-PN by using the two-phase system. We presumed that the fall in conversion efficiency of ECOB and Boc-PN at high initial concentrations is due to the lack of tolerance of PAR for water-soluble organic compounds. Making the PAR more tolerant to organic compounds through protein engineering, and optimizing the conditions for each substance used, would improve the practical productivity of our reduction system.

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