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Bioreactor Systems for the Production of Optically Active Amino Acids and Alcohols

Hirokazu Nanba,* Yoshihiko Yasohara, Junzo Hasegawa, and Satomi Takahashi

Kaneka Corporation, Takasago, Hyogo 676-8688, Japan

Abstract:

Industrial enzyme catalytic reactions for the production of useful optically active compounds have been developed. The first example is a unique process for the production of D-*p*-hydroxyphenylglycine (D-HPG) from D,L-5-*p*-hydroxyphenylhydantoin. This process involves two immobilized enzyme processes, hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase (DCase). A thermophilic bacterium producing thermostable hydantoinase and a mesophilic bacterium producing DCase were isolated, and the thermostability of the latter enzyme was improved by a protein-engineering technique. Both enzymes could be reused in long-term repeated batch reactions as immobilized enzymes for industrial production. The second example is an enzymatic reduction system for the production of optically active alcohols from the corresponding carbonyl compounds. Effective production of ethyl (*S*)-4-chloro-3-hydroxybutyrate was achieved with high productivity (350 g/L, >99% ee) by constructing the recombinant *Escherichia coli* overproducing both carbonyl reductase (S1) and the cofactor-regenerating enzyme, glucose dehydrogenase. We also found various types of reducing enzyme that are useful for the production of various compounds.

1. Introduction

Optically active compounds such as chiral amino acids and alcohols have been widely recognized as important synthetic intermediates for pharmaceuticals.[1] For the preparation of these chemicals, biocatalysts have proven to be a useful tool. Enzymatic reactions can typically be carried out in aqueous medium at ambient temperature under atmospheric pressure, and high selectivity has been observed in the reactions, especially enantio- and regioselectivities. Further, the introduction of new biotechnologies such as protein engineering and recombinant DNA techniques have led us to develop a "super enzyme" reaction system, which enables highly efficient enzymatic reactions applicable to the industrial production of useful chemicals.

We have succeeded in establishing several enzyme technologies,[2] which have been used for the industrial production of intermediates of optically active pharmaceuticals. Among the technologies, we describe here the establishment of a bioreactor system for D-amino acid production, and an enzymatic reduction system for optically active alcohols.

2. Application of a Bioreactor for D-Amino Acid Production

D-Amino acids have become increasingly important in the pharmaceutical field as intermediates or chiral synthons for the preparation of β-lactam antibiotics, physiologically active peptides, pyrethroids, and the like. We have established an economical and efficient production process for D-amino acids using microbial hydantoinase (Scheme 1).[3] In this process, D,L-5-substituted hydantoin can be quantitatively transformed into the corresponding optically active *N*-carbamoyl-D-amino acids by dynamic kinetic resolution. D-*p*-Hydroxyphenylglycine (D-HPG), which is used as the side chain of semisynthetic penicillins and cephalosporins, has been produced by this process. Among D-amino acids, the highest demand is for D-HPG, and several thousand metric tons of it have been produced per year. First, we developed a hydantoinase process involving hydrolysis of the corresponding hydantoin with microbial cells and chemical decarbamoylation. At present, this process has been improved and has become a bioreactor system involving two-step reactions with immobilized enzymes, i.e., hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase (DCase).[4] This unique process has the advantage of low production costs and reduced waste.

In this section, we describe the development of the bioreactor system, especially noting the improvement of DCase stability during long-term usage as an immobilized enzyme by means of a molecular engineering technique using a directed evolution method.[5−7]

As shown in Scheme 1, the hydantoinase reaction, which quantitatively converts D,L-5-substituted hydantoin to *N*-carbamoyl-D-*p*-hydroxyphenylglycine, is the first step in the newly established bioreactor system for the production of

* Corresponding author. E-mail: Hirokazu.Nanba@kaneka.co.jp.

(1) Crosby, J. Chirality in Industry: An Overview. In *Chirality in Industry*; Collins, A. N., Sheldrake, G. N., Crosby, J., Eds.; John Wiley & Sons: Chichester, 1992; pp 1−66.

(2) (a) Takahashi, S. In *Biotechnology of Amino Acid Production*; Aida, K., Ed.; Kodansha and Elsevier: Tokyo, 1986; p 269. (b) Hasegawa, J. *J. Synth. Org. Chem.* **1987**, 33. (c) Takahashi, S. In *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation*; Flickinger, M. C., Drew, S. W., Eds.; John Wiley & Sons: New York, 1999; p 1963. (d) Ikenaka, Y.; Takahashi, S. In *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation*; Flickinger, M. C., Drew, S. W., Eds.; John Wiley & Sons: New York, 1999; p 100. (e) Ohashi, T.; Hasegawa, J. In *Chirality in Industry*; Collins, N., Ed.; John Wiley & Sons: New York, 1992; pp 249, 269. (f) Hasegawa, J.; Nagashima, N. In *Stereoselective Biocatalysis*; Patel, R. N., Eds.; Marcel Dekker, Inc: New York, 2000; p 343. (g) Nanba, H.; Nakai, T; Yasohara, Y; Morikawa, S; Hasegawa, J. *J. Synth. Org. Chem. Jpn.* **2004**, *62*, 1102.

(3) (a) Yamada, H.; Takahashi, S; Kii, Y.; Kumagai, H. *J. Ferment. Technol.* **1978**, *56*, 484. (b) Takahashi, S.; Kii, Y.; Kumagai, H.; Yamada, H. *J. Ferment. Technol.* **1978**, *56*, 492. (c) Takahashi, S.; Ohashi, T.; Kii, Y.; Kumagai, H.; Yamada, H. *J. Ferment. Technol.* **1979**, *57*, 328.

(4) (a) Nanba, H.; Ikenaka, Y.; Yamada, Y.; Yajima, K.; Takano, M.; Takahashi, S. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 875. (b) Ikenaka, Y.; Nanba, H.; Yamada, Y.; Yajima, K.; Takano, M.; Takahashi, S. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 882.

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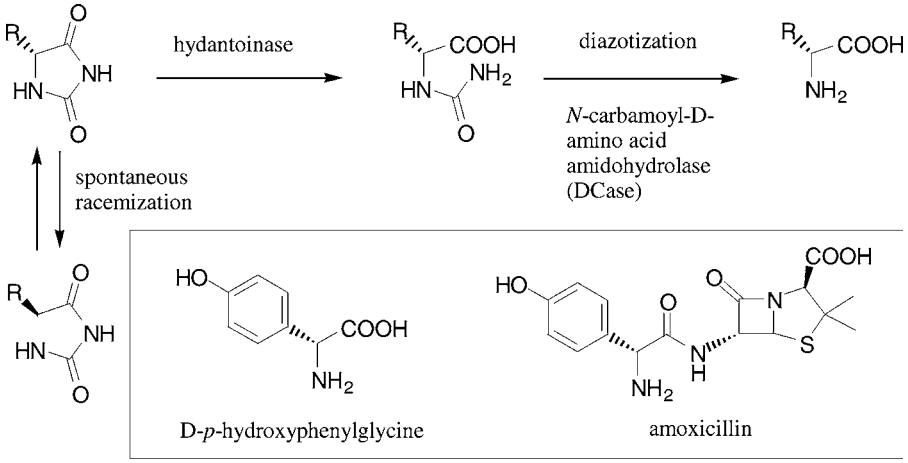
Scheme 1. **D-Amino acid production process**



D-HPG. The hydantoinase of thermophilic microorganisms that had been isolated from a soil sample and had been used in the cell reaction process for the production of D-HPG has high stability and activity. Because of the high stability of the enzyme, it can be reused in long-term repeated batch reactions as an immobilized enzyme for the bioreactor process.

The second step in the bioreactor is the DCase reaction, which involves decarbamoylation of *N*-carbamoyl-D-*p*-hydroxyphenylglycine. We first tried to obtain DCase-producing microorganisms from natural sources for the substitution of chemical decarbamoylation to the enzymatic reaction. *Agrobacterium* sp. strain KNK712, which effectively produces DCase, was then isolated from a soil sample.[4a] The DCase from *Agrobacterium* was found to have high specific activity and broad substrate specificity toward *N*-carbamoyl-D-amino acids, including *N*-carbamoyl-D-HPG. The thermostability of the enzyme, however, was low, with its remaining activity after treatment at 60 °C for 20 min being only 5%.[4a]

For the practical use of the DCase as an immobilized enzyme, both high reactivity toward *N*-carbamoyl-D-HPG and high stability are required. We therefore tried to improve the thermostability of *Agrobacterium* DCase by means of directed evolution.[5−7] The DCase gene from the *Agrobacterium* sp. strain KNK712 was cloned and was overexpressed in *Escherichia coli* cells;[4a] i.e., the DCase accounted for 50% of the soluble protein in the cells. Next, the DCase gene was randomly mutagenized in vitro; then selection of the DCase was performed, which improved its thermostability.[5,6] The screening was carried out by means of a high-throughput method, i.e., a colorimetric colony assay method, by which colonies of the remaining DCase activity after heat treatment (65 °C, 5 min) could be detected on the basis of the generation of coloring matter by the enzymatic coupling reaction. From approximately 34,000 colonies, we found 16 clones that had high thermostability and showed no reduction of activity in crude extract. From the sequence analysis of the DCase genes, it became apparent that the increase in thermostability was caused by an amino acid change of the 57th amino acid from His to Tyr, the 203rd amino acid from Pro to Ser or Leu, or the 236th amino acid from Val to Ala. The thermostable temperature, which was a parameter of thermostability and was defined as the temperature that caused a 50% decrease in DCase activity following heat treatment for 10 min, was 61.8 °C for the wild-type DCase. As a result of the mutation, the thermostable temperature of mutant DCases His57Tyr, Pro203Leu, Pro203Ser, and Val236Ala increased to 67.3, 68.0, 66.5, and 71.4 °C, respectively (Table 1).[5]

Table 1. **Thermostability analysis of mutant DCases[a]**

location of mutation	substituted amino acid	thermostable temperature (°C)[b]
57 His	Tyr[c]	67.3
	Leu[d]	67.5
203 Pro	Leu[c]	68.0
	Ser[c]	66.5
	Ala[d]	67.7
	Asn[d]	67.0
	Glu[d]	70.0
	His[d]	65.2
	Ile[d]	67.2
	Thr[d]	67.5
236 Val	Ala[c]	71.4
	Ser[d]	72.0
	Thr[d]	69.5
wild-type	−	61.8

[a] The mutagenized DCase genes were analyzed, and the amino acid substitutions resulting from the mutations were deduced. [b] Defined as the temperature of heat treatment for 10 min that caused a decrease in DCase activity of 50%. [c] Obtained by random mutagenesis using $NH_2OH{\cdot}HCl$ or $NaNO_2$. [d] Obtained by site-directed substitutions using saturation mutagenesis.

Next, to obtain more thermostable mutant DCases, we carried out saturation mutagenesis. In addition to the known amino acid changes mentioned above, the following changes at each site were found to increase the thermostability: the 57th His was changed to Leu; the 203rd Pro to Ala, Asn, Glu, His, Ile, or Thr; and the 236th Val to Ser or Thr (Table 1).[5b] Further, we constructed multiple mutants with combina-

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(7) (a) Nanba, H.; Takahashi, S. *Seibutsu-kogaku kaishi* **1999**, *77*, 433. (b) Takahashi, S.; Nanba, H.; Ikenaka, Y.; Yajima, K. *Nippon Nogeikagaku Kaishi* **2000**, *74*, 961.

Table 2. **Comparison of the properties of the wild-type and mutant DCases**

	wild-type DCase	mutant DCase (His57Tyr/Pro203Glu/Val236Ala)
V_{max} (μmol·min^{-1}·mg^{-1}. C-D-HPG[a])	9.0	7.6
K_m (mM·pH7·C-D-HPG[a])	0.89	1.3
optimum pH	7.0	6.4
pH stability	6.5−7.5	6.0−8.0
optimum temperature (°C)	65	75
thermostable temperature[b] (°C)	61.8	80.8
substrate specificity	C-D-AA[c]	C-D-AA[c]

[a] C-D-HPG represents *N*-carbamoyl-D-*p*-hydroxyphenylglycine. [b] Defined as the temperature of heat treatment for 10 min which caused a decrease in the DCase activity of 50%. [c] C-D-AA represents *N*-carbamoyl-D-amino acid, and shows that these enzymes react only with *N*-carbamoyl-D-amino acids as substrates, and do not react with L-form substrates and D-form substrates with other *N*-substituted groups, such as *N*-acetyl- or *N*-formyl-D-amino acids.

Table 3. **Properities of COBE-reducing enzymes in *Candida magnoliae***

	S1	S3	S4	R
native M_r[a]	77,000	67,000	86,000	33,000
subunit M_r	32,000	30,000	29,000	35,000
number of subunits	2	2	2	1
family	SDR[b]	SDR	SDR	aldo-keto reductase
cofactor	NADPH	NADPH	NADPH	NADPH
optimum pH	5.5		6.0	7.0
optimum temperature	55 °C	50 °C	50 °C	40 °C
stereoselectivity for COBE	>99% ee for (*S*)	52% ee for (*S*)	51% ee for (*S*)	> 99% ee for (*R*)

[a] Molecular weight. [b] Short-chain alcohol dehydrogenase/reductase.

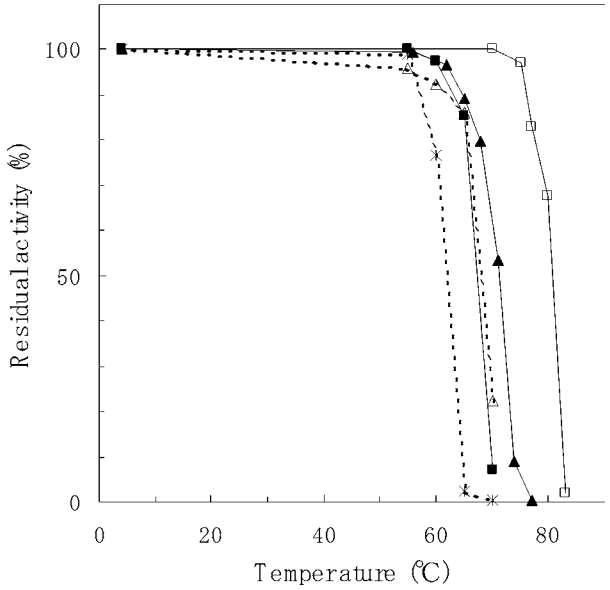


Figure 1. **Effects of temperature on the stability of the mutant enzymes. The remaining activities of the wild-type DCase (···×···), His57Tyr (—■—), Pro203Leu (···△···), Val236Ala (—▲—), and His57Tyr/Pro203Glu/Val236Ala (—□—) are expressed as a percentage of the activity of each nonheated enzyme.**

tions of two or three thermostability-related amino acid substitutions. The resulting mutant DCases showed a cumulative increase in thermostability by the accumulation of the individual mutations. The most thermostable enzyme, His57Tyr/Pro203Glu/Val236Ala, which had three amino acid substitutions, showed an increase of approximately 19 °C in the thermostable temperature (80.8 °C, mutant 455M in Figure 1). It also showed increased stability in the lower and

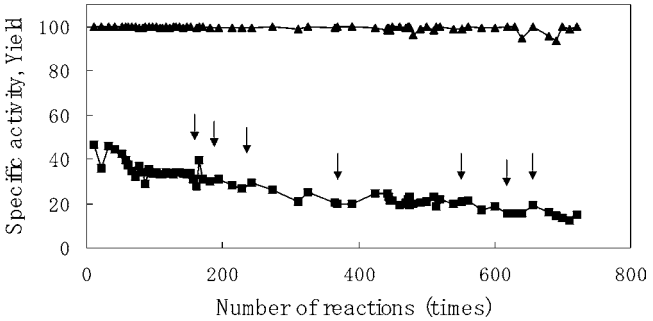


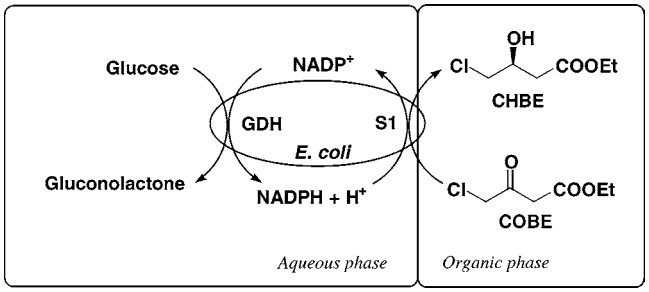
Figure 2. **Operation of immobilized DCase (mutant 455M) in the plant. The yield (%) of the reaction (▲) and the specific activity (units/g) of immobilized DCase (■) are expressed. Addition of immobilized DCase is expressed by arrows.**

higher pH regions, and maintained sufficient activity for practical use[6] (Table 2). On the basis of the determination of the crystal structure of the wild-type DCase at a 1.7 Å resolution,[8] we were able to explain the thermostable properties such as hydrophobic interactions, relaxation of the strain energy of the peptide backbone, and the release in the strain of the side-chain conformation.

Immobilization of the DCase mutant 455M, which is highly produced by the recombinant *E. coli*,[9] was carried out by adsorbing a macroporous phenol formaldehyde resin with tertiary amine as a functional group, followed by cross-linking with glutaraldehyde.[10] When immobilized enzyme was repeatedly used in batch reactions, the thermostable DCase showed improved stability and was practically ap-

(8) Nakai, T.; Hasegawa, T.; Yamashita, E.; Yamamoto, M.; Kumasaka, T.; Ueki, T.; Nanba, H.; Ikenaka, Y.; Takahashi, S.; Sato, M.; Tsukihara, T. *Structure* **2000**, *8*, 729.
(9) Nanba, H.; Ikenaka, Y.; Yamada, Y; Yajima, K; Takano, M.; Takahashi, S. *J. Biosci. Bioeng.* **1999**, *87*, 149.

Scheme 2. **Enzymatic reduction system for (*S*)-CHBE production by recombinant *E. coli* coproducing carbonyl reductase and glucose dehydrogenase**



plicable to the production of D-amino acids.[7a] Immobilized DCase has been used as a bioreactor system together with immobilized hydantoinase for the commercial production of D-HPG, and more than 700 long-term repeated batch reactions have been realized (Figure 2).[7a] This bioreactor process has enabled an improvement of the reaction and purification yields, and a reduction in byproducts and waste. As a result, both production costs and the environmental burden have been reduced.

3. Enzymatic Reduction System

The asymmetric reduction of prochiral carbonyl compounds is an attractive methodology for synthesizing optically active alcohols. Many trials have been carried out to develop effective methodologies in the fields of both organic chemistry and biological chemistry. Regarding biological reduction, there have been numerous reports of using Baker's yeast as a catalyst,[11] but whole microbial cells such as Baker's yeast are used primarily in the laboratory. There are two main obstacles to achieving effective enzymatic reduction. The first is insufficient stereoselectivity, mainly because the microorganisms have some reducing enzymes that have different levels of stereoselectivity. The other is the supply of the reduced form of the coenzyme. Many reducing enzymes require NADH (reduced nicotinamide adenine dinucleotide) or NADPH (reduced nicotinamide adenine dinucleotide phosphate) as hydrogen donors. These are expensive and not industrially available. The oxidized forms of the coenzymes, NAD^+ and $NADP^+$, are industrially available, but still expensive for practical use. Therefore, the enzymatic regeneration of the reduced coenzyme from a catalytic amount of oxidized coenzyme is effective. However, in the whole cell reaction, it is difficult to control a coenzyme supplement system because the cell is a "black box."

(10) (a) Nanba, H.; Ikenaka, Y.; Yamada, Y.; Yajima, K.; Takano, M.; Ohkubo, K; Hiraishi, Y.; Yamada, K.; Takahashi, S. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1839. (b) Nanba, H.; Ikenaka, Y.; Yamada, Y.; Yajima, K.; Takano, M.; Ohkubo, K; Hiraishi, Y.; Yamada, K.; Takahashi, S. *J. Mol. Catal., B: Enzym.* **1999**, *6*, 257.

(11) (a) Chin-Joe, I.; Straathof, A. J. J.; Pronk, J. K.; Jongejan, J. A.; Heijnen, J. J. *Biotechnol. Bioeng.* **2001**, *75*, 29. (b) D'Arrigo, P.; Pedrocchi-Fantoni, G.; Servi, S. *Adv. Appl. Microbiol.* **1997**, *44*, 81. (c) Kometani, T.; Yoshii, H.; Kitatsuji, E.; Nishimura, H.; Matsuno, R. *J. Ferment. Technol.* **1993**, *76*, 33. (d) Csuk, R.; Glanzer, B. I. *Chem. Rev.* **1991**, *91*, 49. (e) Nakamura, K.; Kawai, Y.; Nakajima, N.; Ohno, A. *J. Org. Chem.* **1991**, *56*, 4778. (f) Ward, O. P.; Young, C. S. *Enzyme Microb. Technol.* **1990**, *12*, 482. (g) Shieh, W. R.; Gopalan, A. S.; Sih, C. J. *J. Am. Chem. Soc.* **1985**, *107*, 2993. (h) Zhou, B.; Gopalan, A. S.; VanMiddlesworth, F.; Shieh, W. R.; Sih, C. J. *J. Am. Chem. Soc.* **1983**, *105*, 5925.

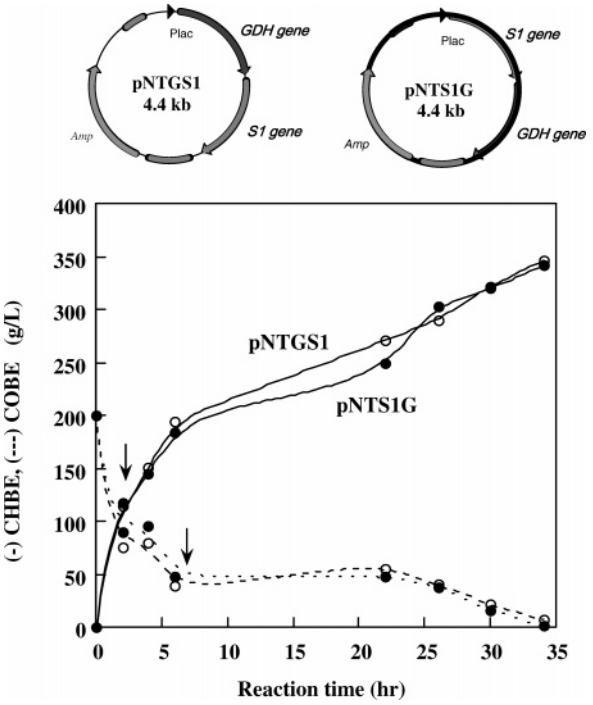
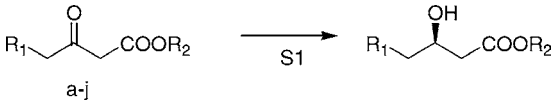


Figure 3. **Asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant in a water/solvent, two-phase system. The upper figures show the structures of two plasmid vectors for the construction of the recombinant *E. coli* coproducing carbonyl reductase S1 and glucose dehydrogenase. The lower figure shows the time course of the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by using the recombinant cells harboring each plasmid vector. The experimental conditions were previously described.[18] Dashed lines indicate the concentration of COBE, and full lines indicate the concentration of CHBE. Symbols: (○) *E. coli* HB101/pNTGS1; (●) *E. coli* HB101/pNTS1G.**

Table 4. **Enantioselective reduction of alkyl 3-oxobutanoate by carbonyl reductase S1**

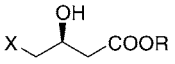
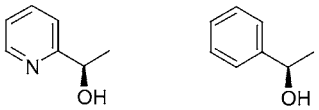
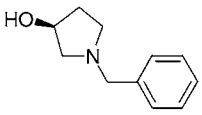
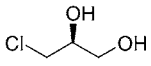
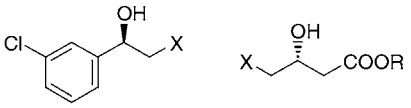
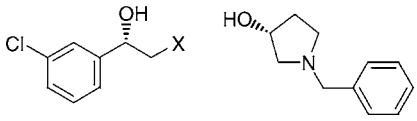


R_1	R_2	relative activity (%)[a]	% ee[b]
Cl	CH_2CH_3	100	>99
Br	CH_2CH_3	72	>99 ***S***
I	CH_2CH_3	16	>99 ***S***
Cl	$(CH_2)_7CH_3$	4	>99 ***S***
N_3	CH_2CH_3	0	N.T.[c]
$C_6H_5CH_2O$	CH_2CH_3	21	21 ***S***
HO	CH_2CH_3	80	>99 ***S***
H	CH_2CH_3	7	>99 ***R***
H	$C(CH_3)_3$	0	N.T.
CH_3CH_2	CH_2CH_3	0.5	N.T.

[a] The substrate specificity of carbonyl reductase S1 was measured as the enzyme activity for each substrate. The assay condition was previously described.[19] [b] The reaction mixture, comprising 25 mL of broth of *Escherichia coli* HB101/pNTS1G, 10 mmol of substrate, 10 mmol of glucose, 3.2 mg of $NADP^+$, 25 mg of Triton X-100, and 25 mL of *n*-butyl acetate, was stirred at 30 °C for 20 h. [c] Not tested.

We therefore tried to construct an "enzymatic reduction system" for the industrial production of various kinds of optically active alcohols. In this section, we describe the establishment of the enzymatic reduction system and its

Table 5. **Reductase library for asymmetric reductions**

Enzyme	Source	Cofactor	Product
Carbonyl reductase	*Candida magnoliae*	NADPH	
Alcohol dehydrogenase	*Candida maris*	NADH	
Carbonyl reductase	*Micrococcus luteus*	NADPH	
Glycerol dehydrogenase	*Serratia marcescens*	NADH	
Carbonyl reductase	*Rhodotorula glutinis* var. *dairenensis*	NADPH	
Carbonyl reductase	*Devosia riboflavina*	NADH	

applications, i.e., finding tough and highly efficient reductases and overproducing two enzymes, a carbonyl reductase and a cofactor regeneration enzyme, in the same *E. coli* cells by use of recombinant DNA techniques.

Optically active ethyl 4-chloro-3-hydroxybutyrate (CHBE) is a useful building block. An asymmetric reduction of ethyl 4-chloro-3-oxobutyrate (COBE) is the most economical way of synthesizing CHBE. Shimizu et al. have reported that the combination of an aldehyde reductase from *Sporobolomyces salmonicolor* and a coenzyme-regenerating system containing glucose dehydrogenase allows the accumulation of 300 g/L of (*R*)-CHBE.[12] On the other hand, some whole cells of microorganisms, *Geotrichum candidum*[13] and *Zygosaccharomyces rouxii*,[14] were found to produce 5 g/L and 13 g/L of (*S*)-CHBE, respectively. The whole cells of *Candida magnoliae*, which we discovered from the screening experiment, produced 90 g/L of (*S*)-CHBE with 96.6% ee.[15] *Candida magnoliae* has at least four COBE-reducing enzymes, S1, S3, S4, and R (Table 3).[16] NADPH-dependent carbonyl reductases (S1, S3, and S4) reduce COBE to (*S*)-CHBE (>99% ee, 52% ee, and 51% ee, respectively), and an NADPH-dependent aldehyde reductase (R) reduces COBE to optically pure (*R*)-CHBE. We focused on the reductase S1 because of its high stereoselectivity. For the regeneration of coenzyme, two dehydrogenases, formate dehydrogenase and glucose dehydrogenase, are well-known.[17] Formate dehydrogenase oxidizes formate to carbon dioxide with NAD^+ reduction, and glucose dehydrogenase oxidizes glucose to gluconolactone, which is spontaneously converted to gluconic acid with $NAD(P)^+$ reduction. For the effective regeneration of coenzyme, we constructed a recombinant *E. coli* that overproduced both carbonyl reductase S1 from *Candida magnoliae* and glucose dehydrogenase from *Bacillus* sp. (Scheme 2). In the water/organic solvent system, 350 g/L of (*S*)-CHBE accumulated in the organic phase, with 85% molar yield (Figure 3). In the aqueous mono-phase system, 208 g/L of (*S*)-CHBE was accumulated by continuous feeding of COBE, which is unstable in water. The calculated turnover number of $NADP^+$ to CHBE was 21,600 mol/mol in this case. The optical purity of the formed (*S*)-CHBE was >99% ee.[18] The product can be easily extracted with organic solvent from the reaction mixture. The recombinant *E. coli* was also a useful catalyst for the synthesis of various optically active 3-hydroxyacid ester derivatives (Table 4).[19]

Further, we obtained the mutant reductase S1, which modified the cofactor specificity and which was applied to the asymmetric reduction of COBE to (*S*)-CHBE in cooperating with the NADH regeneration system of formate

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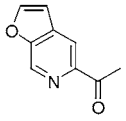
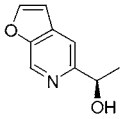
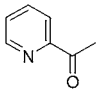
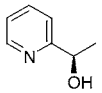
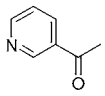
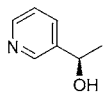
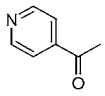
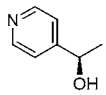
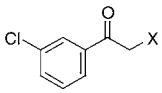
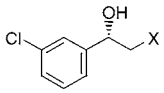
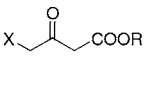
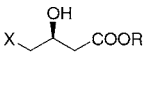
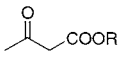
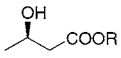
Table 6. **Stereoselectivity of AFPDH for various carbonyl compounds**

Substrate	Product	Optical purity of the product (% ee)[a]
		>99.9 (*R*)
		99.7 (*R*)
		>99.9 (*R*)
		>99.9 (*R*)
		99.8 (*S*)
		>99.9 (*S*)
		>99.9 (*R*)

[a] The reaction mixture comprising 0.5 unit of AFPDH, 0.05 mmol of substrate, 20 mg of glucose, 0.5 mg of NAD^+, and 4 units of commercial glucose dehydrogenase in a total volume of 1 mL, 100 mM of potassium phosphate buffer (pH 6.5), was stirred at 30 °C for 17 h.

dehydrogenase[20] and formate, by a rational design method based on the three-dimensional structure of the protein.[21,22] The formate dehydrogenase system utilizes a more benign reaction because its product is cleaner than that of glucose dehydrogenase.

Regarding the enzymatic reduction system, an effective recombinant biocatalyst can be prepared by replacing the carbonyl reductase S1 gene with the other desired carbonyl reductase gene. We have an enzyme library of some original reductases that can be used for the production of various kinds of important chiral alcohols (Table 5). For example, a novel NADH-dependent alcohol dehydrogenase from *Candida maris* (AFPDH) has broad substrate specificity and excellent stereoselectivity toward various carbonyl compounds (Table 6).[23] In addition to the asymmetric reduction of ketones, this alcohol dehydrogenase can also catalyze the stereoselective oxidation of alcohols. Therefore, the optical resolution of cheap racemic alcohol can be carried out by using this enzyme. Carbonyl reductase from *Rhodotorula glutinis* can reduce the phenacyl halide derivatives to the corresponding chiral halohydrin derivatives, which can be easily converted to chiral styrene oxide derivatives.[24] Both isomers of 3-pyrrolodinol derivatives can be prepared by choosing the enzyme between the reductase from *Micrococcus luteus* and the reductase from *Devosia riboflavina*.

The enzymatic reduction system is a powerful tool for the synthesis of optically active hydroxyl compounds. But, in general, the substrate specificity of a reducing enzyme is not very broad, which limits the application. We could solve this problem by making various types of useful enzymes and constructing the recombinant *E. coli* that coproduces the reductase and the coenzyme-regenerating enzyme as a biocatalyst for industrial preparation of various kinds of useful chiral alcohols.

4. Conclusion

Historically, industrial production of D-HPG has been accomplished by the use of the classical optical resolution method. In 1979, we established the hydantoinase process, which involves microbial hydrolysis of 5-substituted hydantoins and chemical decarbamoylation, in collaboration with H. Yamada and his colleagues. Since then, we have made continuous efforts to improve the process and have created an efficient bioreactor system. A thermostable hydantoinase from a thermophilic bacterium and a DCase from a mesophilic bacterium were obtained from natural sources, and the thermostability of the DCase was highly improved by a molecular engineering technique. Both enzymes could be reused in long-term repeated batch reactions as immobilized enzymes. We consider the bioreactor system to be the most economical method for producing D-HPG; it is also more environmentally friendly and saves resources in comparison with the conventional method.

Our second goal was to establish an enzymatic reduction system, i.e., a general methodology for the production of optically active alcohols. We developed a process for (*S*)-CHBE coupled with COBE reductase and GDH as the regenerating enzyme of NADPH in collaboration with Shimizu and his colleagues. One of the points of this study was to find an enzyme with high tolerance to a substrate and product, and with high stereoselectivity, and another was to construct an effective system with a reductase and an effective cofactor regeneration enzyme. As a result, we achieved high productivity of (*S*)-CHBE (350 g/L, >99% ee). Since the industrial production of (*S*)-CHBE began, we have searched for and found various types of reducing enzymes for industrial application to produce a variety of useful compounds.

We have described herein some of the remarkable abilities of enzymes and noted examples of the application of the "super enzymes" in the chemical industry.

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