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Biocatalytic reduction system for the production of chiral methyl (*R*)/(*S*)-4-bromo-3-hydroxybutyrate

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ABSTRACT

An effective method for producing methyl 4-bromo-3-hydroxybutyrate enantiomers was developed using an engineered protein. *Escherichia coli* transformant cells containing a mutant β-keto ester reductase (KER-L54Q) from *Penicillium citrinum* and a cofactor-regeneration enzyme such as glucose dehydrogenase (GDH) or *Leifsonia* sp. alcohol dehydrogenase (LSADH) were used to produce methyl (*S*)-4-bromo-3-hydroxybutyrate from methyl 4-bromo-3-oxobutyrate. On the other hand, the production of methyl (*R*)-4-bromo-3-hydroxybutyrate was achieved by asymmetric reduction of methyl 4-bromo-3-oxobutyrate with a mutant phenylacetaldehyde reductase (PAR-HAR1) from *Rhodococcus* sp. ST-10.

Chiral 4-bromo-3-hydroxybutyrate is an important synthetic intermediate. (*R*)-4-bromo-3-hydroxybutyrate is used for the synthesis of (*R*)-carnitine, which plays an important role in human metabolism and the transport of long chain fatty acids across the mitochondrial membrane.[1] On the other hand, (*S*)-4-bromo-3-hydroxybutyrate is used as a key structural synthon for the synthesis of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in cholesterol biosynthesis.[2] In addition, optically active 4-amino-3-hydroxybutyric acid (GABOB) and 4-hydroxypyrrolidone can be easily prepared from optically active 4-bromo-3-hydroxybutyrate. However, enzymatic asymmetric reduction of 4-bromo-3-oxobutyrate has rarely been investigated as a means of producing chiral 4-bromo-3-hydroxybutyrate. One of the major reasons for this is the low reactivity and enantioselectivity of enzymes because many of them are sensitive to brominated compounds including 4-bromo-3-oxobutyrate. Recently, we reported the isolation of cDNA encoding *Penicillium citrinum* β-keto ester reductase (KER)[3,4] and the gene that encodes *Leifsonia* sp. alcohol dehydrogenase (LSADH),[5–7] which are able to catalyze the reduction of methyl 4-bromo-3-oxobutyrate (BAM) to methyl (*S*)-4-bromo-3-hydroxybutyrate ((*S*)-BHBM) with high optical purity. We succeeded at expressing them efficiently in *Escherichia coli*, and the *E. coli* cells obtained showed high productivity as biocatalysts. Moreover, protein engineering was performed using error-prone PCR-based random mutagenesis to improve the thermostability and enantioselectivity of KER, and KER-L54Q was selected.[8]

Itoh et al. reported that phenylacetaldehyde reductase (PAR) from the styrene-assimilating bacteria *Rhodococcus* sp. ST-10, which has a broad substrate range, reduced isopropyl 4-bromo-3-oxobutyrate to yield (*R*)-isopropyl 4-bromo-3-hydroxybutyrate with an enantiomeric excess (ee) of 98.4%.[9] Recently, we obtained an engineered PAR mutant (HAR1) that can operate in relatively high concentrations of 2-propanol.[10,11] Here, we report the effective biocatalytic production of both enantiomers of methyl 4-bromo-3-hydroxybutyrate from its ketone.

KER is as an NADPH-dependent reductase, and the $NADP^+$ produced can be recycled to NADPH by the glucose dehydrogenase (GDH) reaction as shown in Scheme 1. In this system, the formation of gluconic acid via gluconolactone leads to a decrease in pH. Therefore, this system requires control of pH of the reaction mixture. On the other hand, we have developed an alternative hydrogen-transfer bioreduction process involving NADH-dependent LSADH or PAR that does not require an additional coenzyme regeneration system. LSADH and PAR can effectively regenerate NADH by transferring hydrogen from 2-propanol (Scheme 1). 2-Propanol is a suitable hydrogen donor for bioreduction because of its chemical properties and low cost. Furthermore, acetone, which is a by-product of the cofactor-regeneration reaction, is

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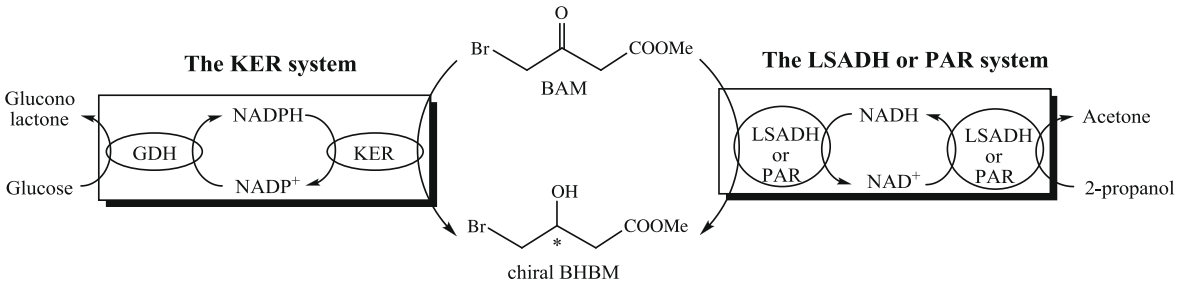

Scheme 1. Asymmetric reduction of BAM and recycling of the coenzyme.

easily removed from the reaction mixture by aeration and does not generate a pH change during the enzymatic reaction.

BAM and BHBM are reactive and unstable compounds, especially in aqueous solution. Therefore, an organic solvent/water two-phase system is required for the asymmetric biocatalytic reduction of BAM. In the KER system, *E. coli* cells that overproduce mutated KER (KER-L54Q) along with GDH were applied to the conversion of BAM. In the LSADH and PAR system, *E. coli* cells harboring pKELA or pHAR1 and expression plasmids that overproduce LSADH and mutant PAR (HAR1: A3S, I4L, E12G, D42L, K67R, L125 M, S173P, and A327 V), respectively, were applied to the conversion of BAM. The *E. coli* cells were cultivated in Luria–Bertani (LB) medium containing an appropriate amount of antibiotics and inducer in a flask, before being collected by centrifugation and used as biocatalysts as previously reported.[7,8,11] The *E. coli* cells transformed with pHAR1 were cultured in LB medium containing 0.01% (w/v) $ZnCl_2$, 100 μg/ml ampicillin, and 0.4 mM isopropyl-β-D-thiogalactopyranoside to obtain a high level of PAR activity.[11] The bioreduction process was monitored by analytical gas chromatography (GC). The results are summarized in Table 1.

In the KER system, the BAM had disappeared after only 4 h, and (*S*)-BHBM was produced with a molar yield of 86% (589 mM, 116 mg/ml in butyl acetate) and an optical purity of >99% ee.[12] In the LSADH system, the conversion of BAM stopped after 53 h, the molar yield was 97% (413 mM, 81 mg/ml in butyl acetate), and the optical purity was >99% ee.[13] In the PAR system, the conversion of BAM was almost 100% after 10 h, (*R*)-BHBM was produced with a molar yield of 80% (176 mM, 35 mg/ml in butyl acetate), and the optical purity was >99% ee.[14]

Figure 1 shows the time-course of the reduction of BAM to (*S*)-BHBM by recombinant *E. coli* cells producing KER-L54Q. The yield increased with time, which proved that KER-L54Q and GDH maintained their catalytic activity in concentrated BAM solution containing 50% (v/v) *n*-butyl acetate.

Although the enantioselectivity of the three enzymes catalyzing the reduction of BAM was satisfactory (>99% ee), their reactivity and productivity varied among the three systems under the optimal conditions for each system. When the KER system was used, the final accumulated (*S*)-BHBM concentration was the highest among the three systems, and it also had the shortest reaction time and used a small amount of biocatalysts. In the LSADH system, it took 53 h or more to complete the BAM reduction. In contrast,

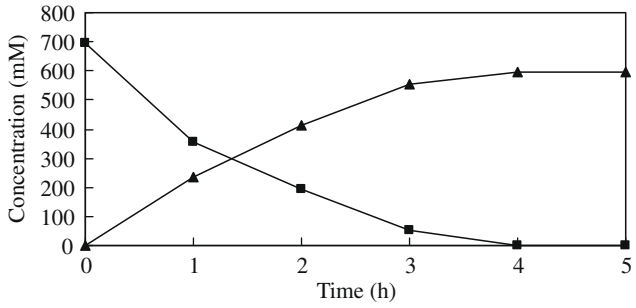

Figure 1. Time course of the reduction of BAM to (*S*)-BHBM by recombinant *E. coli* cells expressing KER-L54Q. (■), BAM; (▲), (*S*)-BHBM.

when the PAR system was used, the complete conversion of BAM was attained after 10 h, but the resultant concentration of (*R*)-BHBM was low, and a large amount of biocatalysts was required. The performance of biocatalysts is dependent on the specific activity of the enzyme for the substrate, the amount of enzyme expressed in *E. coli* cells, and the stability of the enzyme during the reaction. Table 2 displays the properties of the three biocatalysts tested. The results showed that the high performance of the KER system was due to its high specific activity for BAM and its stability in the reaction mixture. KER from various microorganisms was screened for its suitability for converting BAM to (*S*)-BHBM in a two-phase system of water-*n*-butyl acetate, and as a result, KER was engineered to increase its thermostability, giving KER-L54Q. Therefore, KER-L54Q was considered to be adapted to the conversion of BAM. On the other hand, LSADH and PAR-HAR1 showed less reactivity and tolerance toward *n*-butyl acetate compared with KER-L54Q. Although the BAM reduction performance of these systems is not very high, they are easily controlled and can be inexpensively industrialized, since they do not require additional coenzyme regeneration systems or cause a pH change during the reaction. Furthermore, they prefer NADH as a cofactor, which is more stable and inexpensive than NADPH. It was already noted that *n*-butyl acetate has negative effects on the LSADH and PAR-HAR1 systems.[9] Currently, we are investigating an alternative organic solvent for these systems.

In conclusion, an effective method for producing chiral BHBM was developed using biocatalytic reduction. The KER (KER-L54Q),

Table 1
Biocatalytic reduction of BAM

Enzyme	Coenzyme	Wet cells (w/w-BAM)	Reaction time (h)	Final concn of BHBM (mM)	Yield (%)	ee[a] (%)
KER-L54Q	NADPH	0.23	4	589	86	(*S*) >99
LSADH	NADH	0.55	53	413	97	(*S*) >99
PAR-HAR1	NADH	6.6	10	176	80	(*R*) >99

[a] The stereoisomers of BHBM were analyzed by GC using a Shimadzu GC-17A system equipped with a Chirasil-DEX CB column (0.53 mm × 25 m, 0.25 μm film, CHROMPACK, Ltd, USA) and an FID. The samples were treated with trifluoroacetic acid anhydride in chloroform to convert them to a trifluoroacetyl derivative of BHBM.

Table 2
Properties of BAM reduction enzymes

Enzyme	Specific activity for BAM[a] (U/mg protein)	Activity in *E. coli* cells[b] (U/g wet cells)	Half-life in the two-phase system[c] (h)
KER-L54Q	144	179	17
LSADH	9.1	13	3
PAR-HAR1	0.5	0.4	0.5

[a] Each purified enzyme activity for BAM was assayed spectrophotometrically at 30 °C by measuring the decrease in the absorption of NADPH or NADH at 340 nm. The reaction mixture consisted of 2.0 μmol of BAM, 0.1 μmol of NADPH or NADH, 12 or 7.5 μmol of KPB (pH 7.0), and 5 or 50 μl of enzyme solution in a total volume of 0.2 ml. One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of NADPH or NADH within 1 min under these conditions.

[b] The reaction mixtures for the conversion of BAM contained 0.26 mmol of BAM; 2.9 μmol $NADP^+$ or 3.0 μmol NAD^+; 0.56 mmol glucose or 0.56 mmol of 2-propanol; and 16, 30, or 52.8 mg wet weight of *E. coli* transformant cells overproducing KER-L54Q, LSADH, or PAR-HAR1, respectively, in 2 ml of 0.1 M KPB (pH 7.0), to which 2 ml of *n*-butyl acetate was added. The mixture was then stirred vigorously at 30 °C for 30 min. The molar conversion yield was calculated from the concentrations of BAM and BHBM in the *n*-butyl acetate layer. One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of BAM within 1 min under these conditions.

[c] *E. coli* transformant cells overproducing KER-L54Q, LSADH, or PAR-HAR1 were cultivated in Luria–Bertani medium containing an appropriate amount of antibiotics and inducer in a flask, and the cells were later collected by centrifugation and used as biocatalysts as previously reported.[7,8,11] The harvested cells were suspended in 0.1 M KPB (pH 7.0), and the cell concentration was adjusted to 200 mg wet cells per milliliter. The cells were then disrupted with a Multibeads shocker (Yasui Kikai). After centrifugation, the supernatant was collected. One ml of *n*-butyl acetate was added to 1 ml of the supernatant, and each mixture was stirred at 30 °C. The resultant enzyme solution was collected, and each enzyme activity was assayed spectrophotometrically at 30 °C as described above. Half-life corresponds to the time required for the activity of the enzyme to be reduced to half of its initial value.

LSADH, and PAR (PAR-HAR1) systems showed high enantioselectivity and productivity in severe reaction media containing a high concentration of BAM/BHBM and *n*-butyl acetate. Our biocatalytic systems were found to be useful and complementary tools for synthetic organic chemistry. These systems could be used in the preparation of optically pure alcohols for industrial applications.

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12. The reaction mixture (40 ml reaction volume) contained recombinant *E. coli* cells collected from a 100 ml culture and 20 ml of *n*-butyl acetate. Reaction conditions: 30 °C with stirring (650 rpm), BAM: 13.9 mmol, wet cells: 0.6 g, glucose: 4.0 g, $NADP^+$: 15 mg, potassium phosphate buffer (0.1 M, pH 6.5): 20 g, and *n*-butyl acetate: 17.6 g.
13. The reaction mixture (30 ml reaction volume) contained recombinant *E. coli* cells collected from a 200 ml culture and 15 ml of *n*-butyl acetate. Reaction conditions: 30 °C with stirring (650 rpm), BAM: 6.4 mmol, wet cells: 0.7 g, 2-propanol: 1.2 g, NAD^+: 30 mg, potassium phosphate buffer (0.1 M, pH 7.0): 15 g, and *n*-butyl acetate: 13.2 g.
14. The reaction mixture (10 ml reaction volume) contained recombinant *E. coli* cells collected from a 400 ml culture and 5 ml of *n*-butyl acetate. Reaction conditions: 30 °C with stirring (650 rpm), BAM: 1.1 mmol, wet cells: 1.4 g, 2-propanol: 0.5 g, NAD^+: 7.5 mg, 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (0.05 M, pH 7.0): 5 g, and *n*-butyl acetate: 4.4 g.