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Efficient Synthesis of a Chiral Precursor for Angiotensin-Converting Enzyme (ACE) Inhibitors in High Space-Time Yield by a New Reductase without External Cofactors

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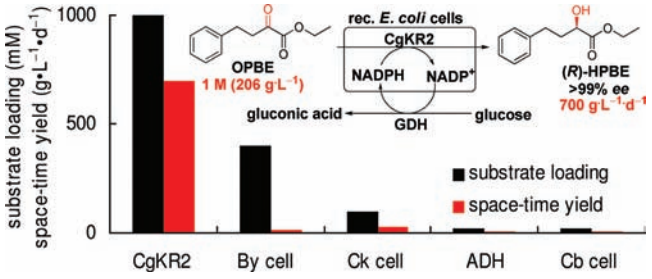
ABSTRACT



A new reductase, CgKR2, with the ability to reduce ethyl 2-oxo-4-phenylbutyrate (OPBE) to ethyl (*R*)-2-hydroxy-4-phenylbutyrate ((*R*)-HPBE), an important chiral precursor for angiotensin-converting enzyme (ACE) inhibitors, was discovered. For the first time, (*R*)-HPBE with >99% *ee* was produced via bioreduction of OPBE at 1 M without external addition of cofactors. The space-time yield (700 g·L^{-1}·d^{-1}) was 27 times higher than the highest record.

Chiral alcohols have wide applications in the synthesis of numerous pharmaceuticals.[1] Ethyl (*R*)-2-hydroxy-4-phenylbutyrate ((*R*)-HPBE), for instance, is an important building block for the production of various angiotensin-converting enzyme (ACE) inhibitors, which are used to treat hypertension and congestive heart failure, such as benazepril, cilazapril, enalapril, and ramipril.[2] A series of technical processes for the synthesis of (*R*)-HPBE have been explored, including resolution of the corresponding racemate,[3] reduction of 2-oxo-4-phenylbutanoic acid or its derivates,[4] and a chemical multistep asymmetric synthesis.[5] Among these routes, the asymmetric synthesis of (*R*)-HPBE with reductases has attracted much attention due to its significant advantages such as high conversion, eco-friendliness (neutral pH and room temperature), and remarkable stereoselectivity making it preferable to other

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methods.[6] Chen et al. employed *Candida boidinii* CIOC21 for the production of (*R*)-HPBE, yielding 99% *ee* in aqueous medium with 20 mM substrate under the optimal conditions.[2b] Zhang et al. reported the production of (*R*)-HPBE by whole cells of *Candida krusei* SW2026 in 99.7% *ee* and 95.1% yield by reducing 2.5 g/L of OPBE.[2c] However, the *ee* of the product decreased to 97.4% in the presence of 20 g/L of OPBE. Despite the reported enzyme-mediated asymmetric reduction to yield (*R*)-HPBE with excellent stereoselectivity, there were still drawbacks including low substrate loading, low space-time yield, and external addition of expensive cofactors, which restricted the industrial applications. Interestingly, in our recent work, the biosynthesis of (*S*)-HPBE was observed at very high substrate loading (620 g/L) with >99% *ee* in the absence of external cofactors.[7] Although (*S*)-HPBE is not the normally employed enantiomer used as an intermediate of ACE inhibitors, we still believed that the production of (*R*)-HPBE by the same pathway might also be achieved by developing new reductases.

To discover and develop promising and versatile reductases, many new routes have been adopted.[8] Among them, a genome mining method is an effective strategy employed for the discovery of novel biocatalysts. For example, a novel NADH-dependent reductase newly discovered by genome mining from *Streptomyces coelicolor* shows notable catalytic performance in the reduction of prochiral ketones.[8c]

Herein, we also employed a genome mining method to discover enzymes for stereoselective synthesis of (*R*)-HPBE. Three reductases from *Saccharomyces cerevisiae* (YDL124w, YDR368w, YGL185c) are known to possess excellent enantioselectivity toward OPBE.[9] A series of predicted putative carbonyl reductases were selected as the candidates on the basis of pBLAST searching with the three reported reductases from *Saccharomyces cerevisiae* as query sequences. Five genes (Genbank accession numbers: CAG61069.1, ABN67667.2, CAG57781.1, CAG62011.1, and ABN65769.2) bearing 29−67% amino acid identities with the three probe reductases were cloned and expressed in *Escherichia coli* BL21 (DE3). After one round of screening, CgKR2 from *Candida glabrata* (Genbank accession: CAG61069.1) was identified as the one with the highest activity and enantioselectivity toward OPBE, giving optically pure (*R*)-HPBE from the bioconversion of 10 mM substrate. Then a second round of mining based upon pBLAST searching was carried out with CgKR2 as the query sequence. As a result, 8 genes (Genbank accession numbers:

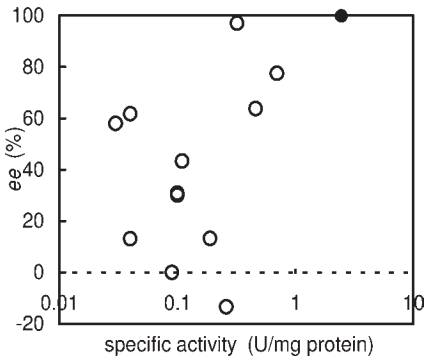

Figure 1. Specific activities and enantioselectivity of the screened reductases. (●) CgKR2; (○) other reductases with lower activity or selectivity.

CAG60239.1, CAG60240.1, CAG58278.1, CAG58834.1, EDK38638.2, CAH02579.1, EEQ44752.1, and EEQ47109.1) were cloned and heterologously expressed. Among the total 13 recombinant reductases obtained, CgKR2 was identified to be the best target biocatalyst because of its highest specific activity (2.4 U/mg of protein) and enantioselectivity (>99%) (Figure 1).

To investigate its basic catalytic properties, the recombinant reductase CgKR2 was purified to homogeneity by a Ni-NTA column from the cell free extract of *E. coli* containing the recombinant CgKR2 (*E. coli*/pCgKR2). The specific activity of the purified CgKR2 was 10.6 U/mg of protein, which was 4.3-fold higher compared to the crude extract. The optimum pH of purified CgKR2 was determined at pHs ranging from 4.0 to 11.0. CgKR2 displayed the maximum activity at pH 6.0 in sodium phosphate buffer. The effect of temperature on the activity of purified CgKR2 was examined from 25 to 55 °C. The optimal activity was detected at 45 °C. The thermostability of purified CgKR2 was also studied. Purified CgKR2 retained 48% of initial activity after incubation for 84 h at 30 °C and only 10% of activity after 36 h of preservation at 40 °C. The enzyme was unstable at 50 °C with a half-life of 2.6 min. The results indicate that CgKR2 is more stable under moderate reaction conditions.

The kinetic parameters of the purified CgKR2 were determined, giving V_{max} and K_m of 18.5 μmol min^{-1} mg^{-1} of protein and 0.1 mM, respectively. CgKR2 has a lower K_m toward OPBE than the carbonyl reductase from *Candida krusei* SW2026 toward OPBE[10] and YiaE toward 2-oxo-4-phenylbutanoic acid.[11]

The substrate specificity and stereoselectivity of CgKR2 for various α- and β-ketoesters as well as aromatic ketones were also explored. The specific activity of CgKR2 toward the reduction of ketones and ketoesters was determined spectrophotometrically. The stereoselectivity of CgKR2

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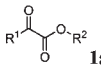
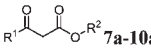
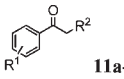
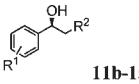
Table 1. CgKR2-Catalyzed Reduction of Various Ketones and Ketoesters

substrate	product	relative activity (%)[a]	conversion (%)[b]	*ee* (%)[c]
α-ketoesters				
1. $R^1 = CH_3$, $R^2 = C_2H_5$	 **1a-6a**	65.6	>99	97.2 (*R*)
2. $R^1 = C_6H_5$, $R^2 = CH_3$		68.8	>99	>99 (*R*)
3. $R^1 = C_6H_5$, $R^2 = C_2H_5$		25.3	91.8	94.0 (*R*)
4. $R^1 = C_6H_5(CH_2)_2$, $R^2 = C_2H_5$		100.0	>99	>99 (*R*)
5. $R^1 = o$-Cl-C_6H_4, $R^2 = CH_3$		162.5	>99	>99 (*R*)
6. $R^1 = o$-Cl-C_6H_4, $R^2 = C_2H_5$		94.8	99.0	>99 (*R*)
β-ketoesters				
7. $R^1 = C_2H_5$, $R^2 = C_2H_5$	 **7a-10a**	0.1	42.7	>99 (*R*)
8. $R^1 = CH_2Cl$, $R^2 = C_2H_5$		8.3	>99	94.5 (*S*)[d]
9. $R^1 = CH_2Br$, $R^2 = C_2H_5$		5.2	96.2	87.3 (*S*)[d]
10. $R^1 = CF_3$, $R^2 = C_2H_5$		0.7	>99	69.8 (*S*)
aromatic ketones				
11. $R^1 = H$, $R^2 = H$	 **11a-15a**	N.D.[e]	No conversion	
12. $R^1 = p$-Cl, $R^2 = H$		N.D.[e]	No conversion	
13. $R^1 = H$, $R^2 = F_3$		6.3	>99	97.6 (*R*)
14. $R^1 = H$, $R^2 = Cl$		0.3	81.0	28.6 (*R*)
15. $R^1 = H$, $R^2 = Br$	 **11b-15b**	0.9	86.2	27.5 (*R*)

[a] The enzyme activity was measured using the standard assay protocol. To calculate the relative activity, the activity for 2 mM OPBE was taken as 100%. [b] Measured by chiral GC analysis. [c] The *ee* values were measured by chiral GC or HPLC analysis. [d] Determined by chiral GC analyses after acetylation of the product. [e] Not detected.

was assayed by GC or HPLC after the reduction of ketones and ketoesters with a NADPH recycling system containing glucose and glucose dehydrogenase (GDH). As described in Table 1, α-ketoesters served as good substrates with relative activities of more than 60% except for ethyl benzoylformate, **3a**. CgKR2 exhibited higher activity toward aromatic α-ketoesters with an *ortho*-chloro substituent on the benzene ring, and methyl esters gave higher activities than ethyl esters. The highest activity was observed when using methyl *o*-chlorobenzoylformate, **5a**. Moreover, stereoselectivity of >99% *ee* and conversion of >99% were achieved for the product methyl (*R*)-*o*-chloro-mandelate, (*R*)-**5b**, a key intermediate for clopidogrel,[12] expanding the scope of possible CgKR2 applications. All α-ketoesters were reduced to the corresponding (*R*)-chiral alcohols with high stereoselectivity and high conversion.

For β-ketoesters and aromatic ketones, CgKR2 was less active, with <10% relative activity. Stereoselectivity of >97% *ee* as well as conversion of >99% were obtained only for the product of (*R*)-1-phenyl-2,2,2-trifluoroethanol, (*R*)-**13b**, which is a synthon for liquid crystals.[13] CgKR2 catalyzed the reduction of ethyl propionylacetate, **7a**, with excellent stereoselectivity, while other β-ketoesters with a halogen group were reduced with moderate to high selectivity. It has been found that the substitution of a halogen group at the 4-position of ethyl 3-oxo-butyrate influences the enantioselectivity of the reported reductases, YtbE[14] and SSCR.[15] For CgKR2, the effect is more obvious and it seems that the enantioselectivity decreases as the substituent group becomes bulkier.

Table 2. Asymmetric Reduction of 1 M OPBE with Cells of *E. coli*/pCgKR2 and GDH[a]

entry	CgKR2 (kU/L)	$NADP^+$ (mM)	time (h)	conv (%)[b]	yield (%)[c]	*ee* (%)[b]
1	60	0.5	5	>99	91	>99
2	60	0.3	6.5	>99	86	>99
3	60	0	24	97.1		>99
4	120	0	7	98.4		>99
5[d]	120	0	6	>99	84	>99

[a] Reaction conditions: OPBE (1 M), D-glucose (1.5 M), lyophilized cells of *E. coli*/pCgKR2 (activity was indicated above, 1200 U equals about 0.5 g of lyophilized cells (about 2 g of wet cells)), lyophilized GDH powders (1 equiv activity with respect to CgKR2, 1200 U equals about 0.14 g of enzyme powders), $NADP^+$ (concentration was indicated above), 10 mL of sodium phosphate buffer (pH 6.0, 100 mM), 30 °C. pH was kept at 6.0 with 2 M Na_2CO_3. [b] Determined by GC analysis. [c] Isolated yield of (*R*)-HPBE. [d] 100 mL of sodium phosphate buffer.

To further utilize the newly discovered biocatalyst for practical synthesis of (*R*)-HPBE, initial experiments were performed at a higher substrate loading, 0.1 M, in 1 mL of sodium phosphate buffer. Lyophilized cells of *E. coli*/pCgKR2 were employed to carry out the reduction, and lyophilized GDH (crude powders) was used to complete the requisite regeneration of cofactor by catalyzing the oxidation of glucose. The conversion reached 98.4% after a 20 h reaction with >99% *ee*. The increase of substrate loading to 0.4 M led to a 34% decrease of conversion but had no effect on stereoselectivity. These results displayed great potential for industrial applications. Then a series of optimizitions were carried out, including autotitrating the reaction mixture with 2 M Na_2CO_3, increasing the substrate loading and the dosage of reductase and scaling up the reaction. It was found that a nearly complete

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Table 3. Comparison between CgKR2 and Other Reported Biocatalysts

biocatalyst	OPBE conc (M)	reaction media	reaction time (h)	*ee* (%)	space-time yield ($g \cdot L^{-1} \cdot d^{-1}$)
CgKR2[a]	1.00	aqueous phase	6	>99 (*R*)	700
By cell[b] [18]	0.40	two-liquid phase systems	48	87.5 (*R*)	17
Ck cell[c] [2c]	0.10	biphasic system	16	97.4 (*R*)	25
	0.01	aqueous phase	14	99.7 (*R*)	4
ADH[d] [19]	0.02	aqueous phase	24	>99 (*R*)	<5
Cb cell[e] [2b]	0.02	aqueous phase	12	99.0 (*R*)	8

[a] Lyophilized cells of *E. coli*/pCgKR2. [b] Pretreated Baker's yeast. [c] Wet cells of *Candida krusei* SW2026. [d] Alcohol dehydrogenase from *Paracoccus pantotrophus* DSM 11072. 1 mM NADH was added. [e] Wet cells of *Candida boidinii* CIOC21.

conversion of OPBE was achieved within 5 h with >99% *ee* in 10 mL of sodium phosphate buffer (Table 2, entry 1) employing 600 U of CgKR2 while the loading of OPBE was increased up to 1 M, which is a high concentration from the viewpoint of industrial practice.

As the expensive nicotinamide cofactors are a major hindrance to the practical application of reductases,[16] a further attempt was made to reduce the amount of external addition of $NADP^+$. It should be noted that even if only the internal cofactors from the cells of *E. coli*/pCgKR2 were utilized to carry out the reduction (Table 2, entry 3), CgKR2 also gave 97.1% conversion in 24 h under the same conditions. After increasing the cells of *E. coli*/pCgKR2 and GDH to 120 U/mL, the reaction was almost complete within 7 h with 98.4% conversion and >99% *ee* (Table 2, entry 4). Then the bioreduction of OPBE was scaled up by 10-fold (Table 2, entry 5). After 6 h reaction and subsequent workup, the desired product, (*R*)-HPBE, was isolated in 84% yield and >99% *ee*, indicating the potential of CgKR2 for practical usage.

To work as an alternative to chemical catalysts, the biocatalyst must tolerate a high substrate concentration for practical application on an industrial scale.[16a,17] To the best of our knowledge, there is only one example of the asymmetric reduction of OPBE at as high as 0.4 M in a two-liquid phase system of water/organic solvent, with 41.9% conversion of OPBE and 87.5% *ee* of (*R*)-HPBE.[18] This is the first report of the biocatalytic synthesis of (*R*)-HPBE with >99% *ee* via reduction of OPBE at 1 M without the addition of any external cofactors. One can see from Table 3, when the CgKR2-catalyzed reduction was carried out in an aqueous monophase reaction medium, the shortest reaction time was achieved. The space-time yield of (*R*)-HPBE production reached 700 $g \cdot L^{-1} \cdot d^{-1}$, which was 27 times higher than the highest record reported so far. Compared with other enzymes exhibiting excellent enantioselectivity, CgKR2 provides several additional advantages, making it a more competitive and promising biocatalyst. Further work including optimization of the reaction for large scale applications is still in progress.

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Supporting Information Available. Experimental methods and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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