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A Novel Reductase from *Candida albicans* for the Production of Ethyl (*S*)-4-Chloro-3-hydroxybutanoate

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A novel NADPH-dependent reductase (CaCR) from *Candida albicans* was cloned for the first time. It catalyzed asymmetric reduction to produce ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE). It contained an open reading frame of 843 bp encoding 281 amino acids. When co-expressed with a glucose dehydrogenase in *Escherichia coli*, recombinant CaCR exhibited an activity of 5.7 U/mg with ethyl 4-chloro-3-oxobutanoate (COBE) as substrate. In the biocatalysis of COBE to (*S*)-CHBE, 1320 mM (*S*)-CHBE was obtained without extra $NADP^+$/NADPH in a water/butyl acetate system, and the optical purity of the (*S*)-isomer was higher than 99% enantiomeric excess.

Key words: biocatalysis; reductase; ethyl 4-chloro-3-hydroxybutanoate; ethyl 4-chloro-3-oxobutanoate; candida albican

The asymmetric reduction of 4-chloro-3-oxobutanoate ethyl (COBE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE], a promising chiral intermediate in the synthesis of pharmacologically active compounds such as hydroxymethylglutaryl-CoA reductase inhibitors[1] and 4-hydroxypyrrolidone, by reductases has several positive attributes, including low cost, mild reaction conditions, high yield, and remarkable enantioselectivity, and has received much attention in recent years. There are two points in the biosynthesis of (*S*)-CHBE. One is a reductase with high activity and remarkable enantioselectivity, and the other is an efficient coenzyme regeneration system.

Several reductases have been cloned and used in the asymmetric synthesis of chiral alcohols, including ScCR from *Streptomyces coelicolor*,[2] PsCR and PsCRII from *Pichia stipitis*,[3–5] KaCR from *Kluyveromyces aestuarii*,[3] and S1 from *Candida magnolia*.[4] Nearly all of these reductases use glucose/glucose dehydrogenase[5–7] as a enzyme-coupled coenzyme system to produce (*S*)-CHBE, but all of them require extra $NADP^+$/NADPH in the reaction, which greatly increases costs and is an impediment of their application.[4,8]

In this study, we identified a novel reductase CaCR by the genome data mining method from *Candida albicans* for the first time. This can produce (*S*)-CHBE at a very high enantioselectivity. Highly efficient synthesis of (*S*)-CHBE was achieved in an organic solvent–water biphasic system without adding extra $NADP^+$/NADPH.

Materials and Methods

Cloning and expression of the CaCR gene in E. coli. The gene of CaCR from *Candida albicans* was amplified by PCR with designed primers, 5′-GGAGGCTCATGAGCAAAGAAACAT-3′ (*BspHI* site underlined) and 5′-CGCGGATCCTTAAATAACTGTGTAACCACC-ATCAA-3′ (*BamHI* site underlined), based on the nucleotide sequence of P87218 in GenBank. The amplified DNA fragment was double-digested with *NdeI* and *BspHI* and inserted into expression vector pET-22b.

Construction of a polycistronic co-expressing plasmid. Cloning of a glucose dehydrogenase (GDH) gene from *Bacillus megaterium* was finished in our laboratory previously.[8,9] To construct an efficient polycistronic plasmid with a high level of enzyme co-expression, we changed the order of the genes, altering the Shine–Dalgarno (SD) regions, and aligned spacing (AS) between the SD sequence and the translation initiation codon. The optimal SD sequence was 5-TAAGGAGG-3, and the AS distance was eight nucleotides. A polycistronic plasmid, pET-22b-GDH-CaCR (22b-GC), encoding the GDH gene and the CaCR gene, was constructed (Fig. 1) and transformed into *E. coli* Rosetta (DE3).

*Biocatalysis of COBE to (*S*)-CHBE.* A 25-mL reaction mixture of 100 mM potassium phosphate buffer (pH 6.2), COBE (4,800 mM), glucose (4,800 mM), butyl acetate (12.5 mL), Triton X-100 (1%, v/v) and cells (300 mg dry weight, 100 mL culture broth) was incubated at 30 °C at 220 rpm for 25 h in a 50-mL vessel. The pH of the reaction mixture was controlled at 6.2 with 5 M sodium hydroxide. After mixing and centrifugation when the reaction finished, the upper phase (the organic layer) was assayed to identify the product (CHBE) and the substrate (COBE).

Enzyme assay and analysis of COBE and CHBE. The assay mixture comprised 100 mM potassium phosphate buffer (pH 6.2), 1 mM NADPH, and 10 mM COBE. The reaction was monitored at 30 °C by the absorbance at 340 nm. CaCR and GDH activity were determined spectrophotometrically, as described previously.[10] One unit of CaCR was defined as the amount catalyzing the reduction of 1 μmol NADPH per min. The protein concentration was measured by the Bradford method, using bovine serum albumin (BSA) as standard. The concentrations of COBE and CHBE were determined by gas chromatography, and the optical purity of (*S*)-CHBE was determined by HPLC.[11] The method of measuring intracellular $NADP^+$/NADPH was followed ref. 20.

Results and Discussion

Amino acid sequences analysis

According to amino acid sequence analysis with other COBE-catalyzed reductases (Fig. 2), CaCR appeared to belong to the SDR superfamily based on three conserved sequences in the black boxes. The first was a conserved

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sequence region covering variable N-terminal Thr-Gly-X-X-X-Gly-X-Gly as cofactor binding motif. The second was a P-/G motif followed by a conserved Thr, concerned with reaction direction. The last was a conserved Tyr-X-X-X-Lys segment essential for the catalytic activity of SDR proteins.[12] All previous studies confirm that Tyr functions as a catalytic base and is the most conserved residue within the whole family, whereas Lys interacts with the nicotinamide ribose and lowers pKa of the Tyr-OH.[13–15] Amino acid sequence analysis of CaCR indicated that the homology was 44.0% with KpCR from *Kluyveromyces aestuarii*,[3] 55.1% with PsCRII from *Pichia stipiti*,[16,17] 55.8% with S1 from *Candida magnolia*,[18] 66.7% with PsCR from *Pichia stipiti*,[11] and 68.2% with CpCR from *Candida parapsilosis*.[19] Amino acid sequence alignments of CaCR with other SDRs are shown in Fig. 2. Despite low sequence identity, three conserved segments in CaCR showed a high degree of similarity to those of other SDRs.

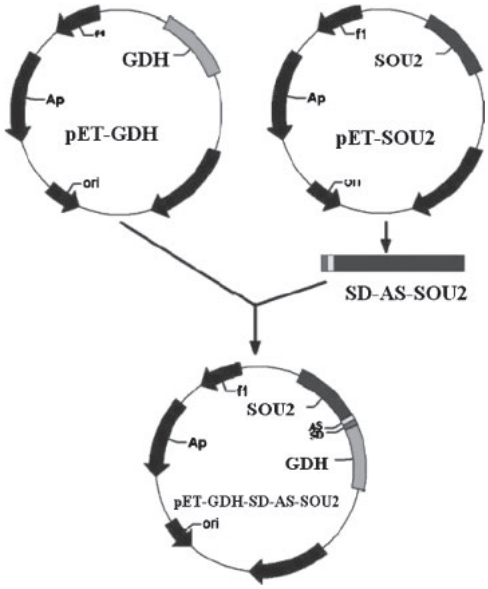

Fig. 1. Construction of Polycistronic Plasmids.

Enzyme activity analysis and cofactor specificity of CaCR

CaCR is a oxido-reductase in *Candida albicans* that is annotated as sorbose reductase in the NCBI, inferred from homology, but the enzyme has no activity toward sorbose, and any function remains unknown as to this novel reductase. Recombined *E. coli* Rosseta (pET-15b-CaCR) exhibited an activity of 5.7 U/mg for COBE with NADPH as electron donor, but exhibited only 0.12 U/mg with NADH as electron donor. Hence, we consider CaCR an NADPH-dependent reductase. When CaCR and GDH coexpressed in plasmid pET-15b-GDH-CaCR, CaCR exhibited an activity of 4.2 U/mg for COBE and GDH an activity of 3.7 U/mg for Glucose. *E. coli* Rosseta (pET-15b) showed no activity for COBE.

*Bioreduction of COBE to (*S*)-CHBE*

Most researchers use glucose/glucose dehydrogenase as the coenzyme regeneration system for the low cost of glucose and high activities of glucose/glucose towards both NADH and NADPH, but the extra NAD(P)/NAD(P)H added in these system is the biggest obstacle for them to be applied in industry. Our goal was to find a novel reductase with high activity and remarkable enantioselectivity and to build a coenzyme regeneration system for the biosynthesis of (*S*)-CHBE without adding extra NAD(P)/NAD(P)H.

We found that 300 mg dry cells in the reaction is sufficient to produce 4500 mM (*S*)-CHBE without adding extra NAD(P)/NAD(P)H. The e.e. value of the product was excellent (>99%). The transformants gave a molar yield of 93.8%. The process is shown in the Fig. 3. The

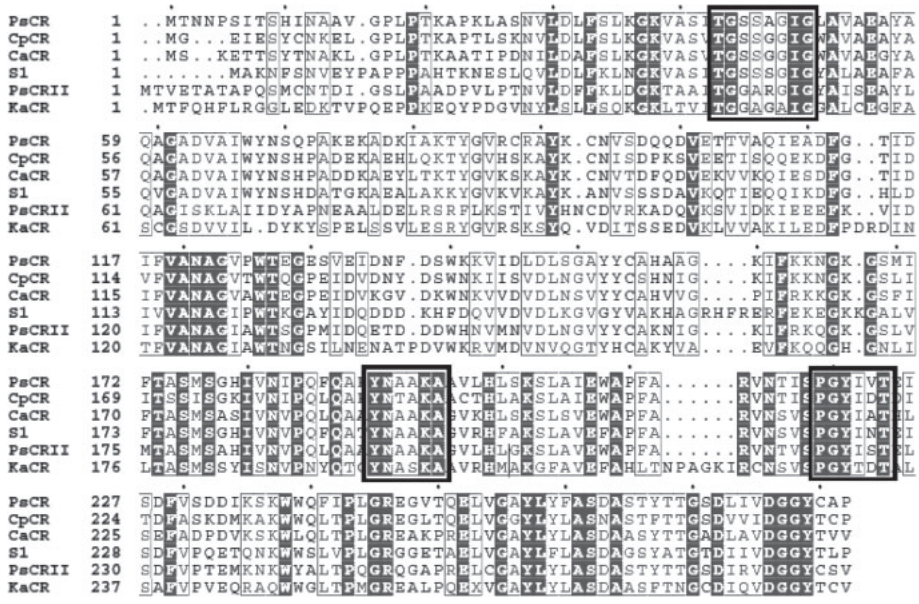

Fig. 2. Amino Acid Sequence Alignments of Short-Chain Alcohol Dehydrogenase/Reductase Using the Clustalx Tool.

Sequences are CaCR from *Candida albicans* in this study (GenBank accession no. P87218), Pichia. stipitis (PsCR, GenBank accession no. XP_001387287), *Pichia. stipiti* (PsCRII, GenBank accession no. XP_001387285), *Candida magnoliae* (S1, GenBank accession no. BAB21578), *Kluyveromyces aestuarii* (KaCR, GenBank accession no. BAD01116), *Candida parapsilosis* (CpCR, GenBank accession no. GI: 189096232).

Table 1. Comparison of Different Reductases in (*S*)-CHBE Product

Enzyme	S1	PsCRII	ScCR	KaCR	CmMR	CaCR
Coenzyme specificity	NADPH	NADH	NADH	NADH	NADPH	NADPH
Biomass (mg, dry weight)	200	1,000	1,000	40	2,000	300
Time of reaction (h)	34	30	22	6	36	25
Production of (*S*)-CHBE (mm)	2,600	1,398	3,600	300	1,550	1,320
(*S*)-CHBE/biomass (mm/mg)	13.0	1.4	3.6	7.5	0.8	15.0
Turnover number	21,600	13,980	12,900	—	12,900	38,200
Add coenzyme or not	YES	YES	YES	NO	NO	NO
e.e.%	>99%	>99%	>99%	>99%	92.1%	>99%
References	4)	17)	2)	3)	5)	This study

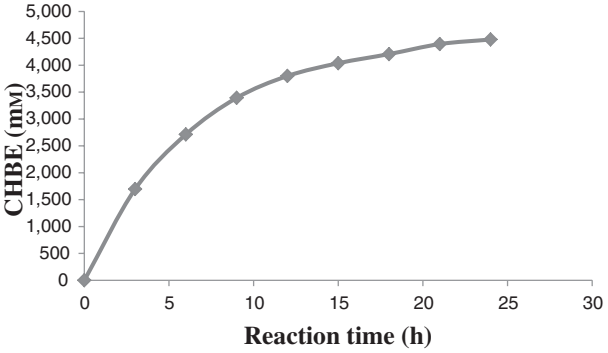

Fig. 3. Asymmetric Reduction of (*S*)-4-Chloro-3-hydroxybutanoate Ethyl.

highlight of our work is that we obtained the highest product concentration without adding NAD(P)/NAD(P)H to the reaction, got the highest turnover number (TTN), and used the least biomass to produce (*S*)-CHBE as compared with other work (Table 1). All of this greatly reduced costs and indicate applicability industrialization.

Conclusions

We found a novel reductase CaCR, a competitive catalyst for (*S*)-CHBE, with high activity and remarkable enantioselectivity through genome data mining for the first time. In our biocatalysis system, it yielded a highest product concentration without extra NAD(P)/NAD(P)H and the highest turnover number.

Acknowledgments

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