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Short Communication

Biosynthesis of (*S*)-4-chloro-3-hydroxybutanoate ethyl using *Escherichia coli* co-expressing a novel NADH-dependent carbonyl reductase and a glucose dehydrogenase

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ABSTRACT

A novel NADH-dependent carbonyl reductase (PsCR II) gene with an open reading frame of 855 bp encoding 285 amino acids was cloned from *Pichia stipitis*. Analysis of the amino acid sequence of PsCR II revealed less than 55% identity to known reductases that produce (*S*)-4-chloro-3-hydroxybutanoates ethyl [(*S*)-CHBE]. When NADH was provided as an electron donor, *Escherichia coli* with pET-22b-PsCRII exhibited an activity of 15 U/mg protein using 4-chloro-3-oxobutanoate ethyl (COBE) as a substrate. This activity was the highest ever reported for reductases, with the exception of PsCR I, which in our previous analysis required NADPH for catalysis. Biocatalysis of COBE to (*S*)-CHBE was investigated using *E. coli* with a polycistronic plasmid pET-BP II co-expressing PsCR II and a glucose dehydrogenase in a water/butyl acetate system for 24 h. The transformants gave a molar yield of 91%, and an optical purity of the (*S*)-isomer of higher than 99% enantiomeric excess.

1. Introduction

(*S*)-4-Chloro-3-hydroxybutanoate ethyl [(*S*)-CHBE] is a chiral intermediate in the synthesis of pharmacologically active compounds (Asako et al., 2009). Asymmetric reduction of 4-chloro-3-oxobutanoate ethyl (COBE) to (*S*)-CHBE by reductases has several positive attributes, including low cost, mild reaction conditions, high yield, and remarkable enantioselectivity (Nakamura and Matsuda, 2006). For example, we previously described a novel carbonyl reductase (PsCR I) from *Pichia stipitis* that produces (*S*)-CHBE at greater than 99% enantiomeric excess (e.e.) (Ye et al., 2009a). Although several enzymes for the production of (*S*)-CHBE have been found that required NADPH for catalysis, few studies have addressed reductases that need the cofactor NADH as an electron donor. In this investigation, a novel NADH-dependent carbonyl reductase (PsCR II) was cloned from *P. stipitis* and expressed in *Escherichia coli* Rosetta (DE3). This enzyme had less than 55% identity to known reductases that produce (*S*)-CHBE. Biocatalysis of COBE to optically pure (*S*)-CHBE was examined using *E. coli* co-expressing PsCR II and a glucose dehydrogenase, in a water/butyl acetate system.

2. Methods

2.1. Cloning and expression of the PsCR II gene in E. coli

Genomic DNA was isolated from *P. stipitis* using a TIANamp Yeast DNA Kit (TIANGEN, China). Oligonucleotide primers specific for the full-length PsCR II gene were designed using GenBank Accession number XM_001387248.1. The forward primer was CRs, 5′-GGAATTCCATATGACTGTCGAAACCGCCACC-3′ (NdeI site is underlined). The reverse primer was CRr, 5′-CGCGGATCCCTAGACAGAACAGTAACCACCT-3′ (BamHI site is underlined). The amplified DNA fragment was double-digested with NdeI and BamHI and inserted into the expression vector pET-22b. Expression in *E. coli* transformants was as described previously (Ye et al., 2009a).

2.2. Construction of a polycistronic co-expressing plasmid

Cloning of a glucose dehydrogenase (BmGDH) gene from *Bacillus megaterium* was described previously (Ye et al., 2010a). A polycistronic plasmid pET-BP II encoding the PsCR II gene and BmGDH gene was constructed. PsCR II was cloned into the BamHI/XhoI site

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of pET-22b-BmGDH. The resulting plasmid (pET-BP II) with two enzyme genes was transformed into *E. coli* Rosetta (DE3). Details on construction and efficient co-expression of PsCR II and BmGDH were described previously (Ye et al., 2010a).

2.3. Biocatalysis of COBE to (S)-CHBE

Biocatalysis of COBE to (*S*)-CHBE was carried out by *E. coli* Rosetta containing pET-BP II. A 100-ml reaction mixture of 100 mM potassium phosphate buffer (pH 6.0), COBE (609 mM), NAD^+ (0.1 mM), glucose (1.5 M), Triton X-100 (1%, v/v), 5 g of dry cells and an equal volume of butyl acetate was incubated at 30 °C, 220 rpm for 24 h, in a 250-ml reacting vessel. The pH was adjusted to 6.0 with 2 M $Na(OH)_2$.

2.4. Enzyme assay and analysis of COBE and CHBE

Transformed *E. coli* cells were harvested by centrifugation and disrupted with an ultrasonic oscillator. Cell debris was removed by centrifugation, and the supernatant used as a crude enzyme extract. PsCR II and BmGDH activities were determined spectrophotometrically as described previously (Ye et al., 2009b). The ability of NADH and NADPH to act as electron donors was tested by recombinant PsCR II. The concentrations of COBE and CHBE were determined by gas chromatography, and the optical purity of (*S*)-CHBE determined by HPLC (Ye et al., 2010b).

3. Results and discussion

3.1. Gene cloning, expression and cofactor specificity of PsCR II

The PsCR II gene was amplified from *P. stipitis* using specific primers. Sequence analysis indicated that the 855 bp gene encoded 285 amino acids, with a calculated molecular mass of 30.7 kDa and an estimated p*I* of 5.21. Fig. 1 shows SDS–PAGE of soluble proteins after sonication from *E. coli* Rosetta (DE3) with pET-22b-PsCR II (Fig. 1, lane 3). Enzyme activities were examined using COBE as the substrate. The recombinant PsCR II exhibited an activity of 15 U/mg protein with NADH as an electron donor, which was about 3-fold higher than the activity of another carbonyl reductase, *E. coli* HB101 harboring pSE-KaCR, which also uses NADH for catalysis (Yamamoto et al., 2004). The activity of *E. coli* with pET-22b-PsCR II was the highest ever reported for reductases producing (*S*)-CHBE, with the exception of PsCR I, which required NADPH as an electron donor in our previous studies (Ye et al., 2009b). When NADH was replaced with an equimolar concentration of NADPH, the recombinant PsCR II showed only 1.7 U/mg activity.

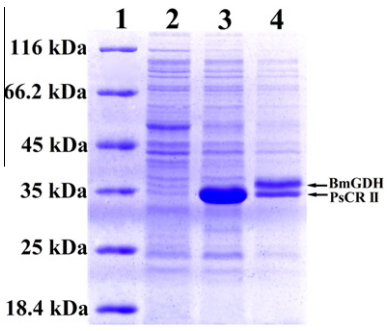

Fig. 1. SDS–PAGE analysis of recombinant protein. Lane 1: protein molecular weight marker. Lane 2: supernatant (soluble proteins) after sonication of *E. coli* Rosetta (DE3) with pET-22b. Lane 3: supernatant (soluble proteins) after sonication of *E. coli* Rosetta (DE3) with pET-22b-PsCR II; Lane 4: supernatant (soluble proteins) after sonication of *E. coli* Rosetta (DE3) with pET-22b-BP II.

3.2. Amino acid analysis

By amino acid sequence analysis, PsCR II appeared to belong to the short-chain dehydrogenase/reductase (SDR) superfamily, based on two conserved sequences. One is the Gly-X-X-X-Gly-X-Gly cofactor binding motif, where X denotes any amino acid and the glycines are Gly-46, Gly-50 and Gly-52 with numbering according to PsCR II. The other is the Tyr-X-X-X-Lys segment at residues 193–197 that is essential for the catalytic activity of SDR proteins (Persson et al., 2009). Amino acid sequence alignments of PsCR II with other SDRs from both prokaryotes and eukaryotes are shown in Fig. 2. The results show that the highest level of homology is with CpCR from *Candida parapsilosis* (55% identity), while the amino acid sequence identity between PsCR II and the SDRs that are not from yeast was less than 33%. Several enzymes in the SDR family that produce (*S*)-CHBE with 99% e.e. have been found, including PsCR I from *P. stipitis* (Ye et al., 2009a), S1 from *Candida magnoliae* (Yasohara et al., 2000), and KaCR from *Kluyveromyces aestuarii* (Yamamoto et al., 2004). Amino acid sequence alignment showed only 54% identity between PsCR II and PsCR I, 53% identity between PsCR II and S1, and 46% between PsCR II and KaCR. Of these four SDRs, PsCR II and KaCR preferred NADH to NADPH for activity. Although the lowest identity was between PsCR II and KaCR, a conserved N-terminal coenzyme binding motif, Thr-Gly-Gly-Ala-X-X-Lle-Gly-X-Ala (positions 45–52), was found in both PsCR II and KaCR, (Persson et al., 2009). Interestingly, the functional annotation information of PsCR II suggests that PsCR II requires the cofactor NADPH for activity with a physiological substrate. In our case, however, PsCR II preferred NADH to NADPH with a non-natural substrate (COBE).

3.3. Biocatalysis of COBE to (S)-CHBE in a two-phase system reaction

(*S*)-CHBE is a precursor for enantiopure intermediates for chiral drugs like Pfizer's Lipitor (atorvastatincalcium) (Lee and Park, 2009). The reduction of COBE is a more economical way of producing (*S*)-CHBE because COBE is inexpensive and easily synthesized. *P. stipitis*, however, produces (*S*)-CHBE with low enantiomeric excess (Ye et al., 2009a). This non-satisfactory value was obtained mainly because wild strains contain several enzymes with variable or opposing stereospecificities that reduce COBE to products of different chirality (Stewart 2001). A simple and effective way to circumvent this is to apply recombinant DNA technology to clone a reductase gene into a host that is less prone to side-product formation. In this study, the carbonyl reductase PsCR II was discovered to have high enantioselectivity using COBE as a substrate. *E. coli* Rosseta (DE3) carrying the vector pET-22b exhibited no activity with COBE as a substrate (Fig. 1, line 2), so the PsCR II gene was cloned and expressed in this host. The transformants gave an optical purity of the (*S*)-isomer of higher than 99% (Supplementary Fig. S1).

PsCR II requires a coenzyme to produce (*S*)-CHBE, and these are too expensive to be used as stoichiometric reagents. To lower cost, PsCR II and BmGDH were co-expressed to create a coenzyme regeneration system. BmGDH can regenerate both NADH and NADPH, and the GDH substrate, glucose, is inexpensive (Kataoka et al., 1999). Fig. 1 shows SDS–PAGE of soluble proteins from *E. coli* Rosetta (DE3) with pET-BP II (Fig. 1, lane 4). *E. coli* Rosetta (DE3) containing the polycistronic plasmid pET-BP II exhibited an activity of 10 U/mg with COBE as a substrate, and 11 U/mg with glucose as a substrate. Since COBE and CHBE were both inhibitory for enzymes and cells in an aqueous mono-phase system (Ye et al., 2009a), biosynthesis of (*S*)-CHBE using recombinant *E. coli* harboring pET-BP II was examined using a water/butyl acetate diphasic system for efficient partitioning of COBE and CHBE (Ye et al.,

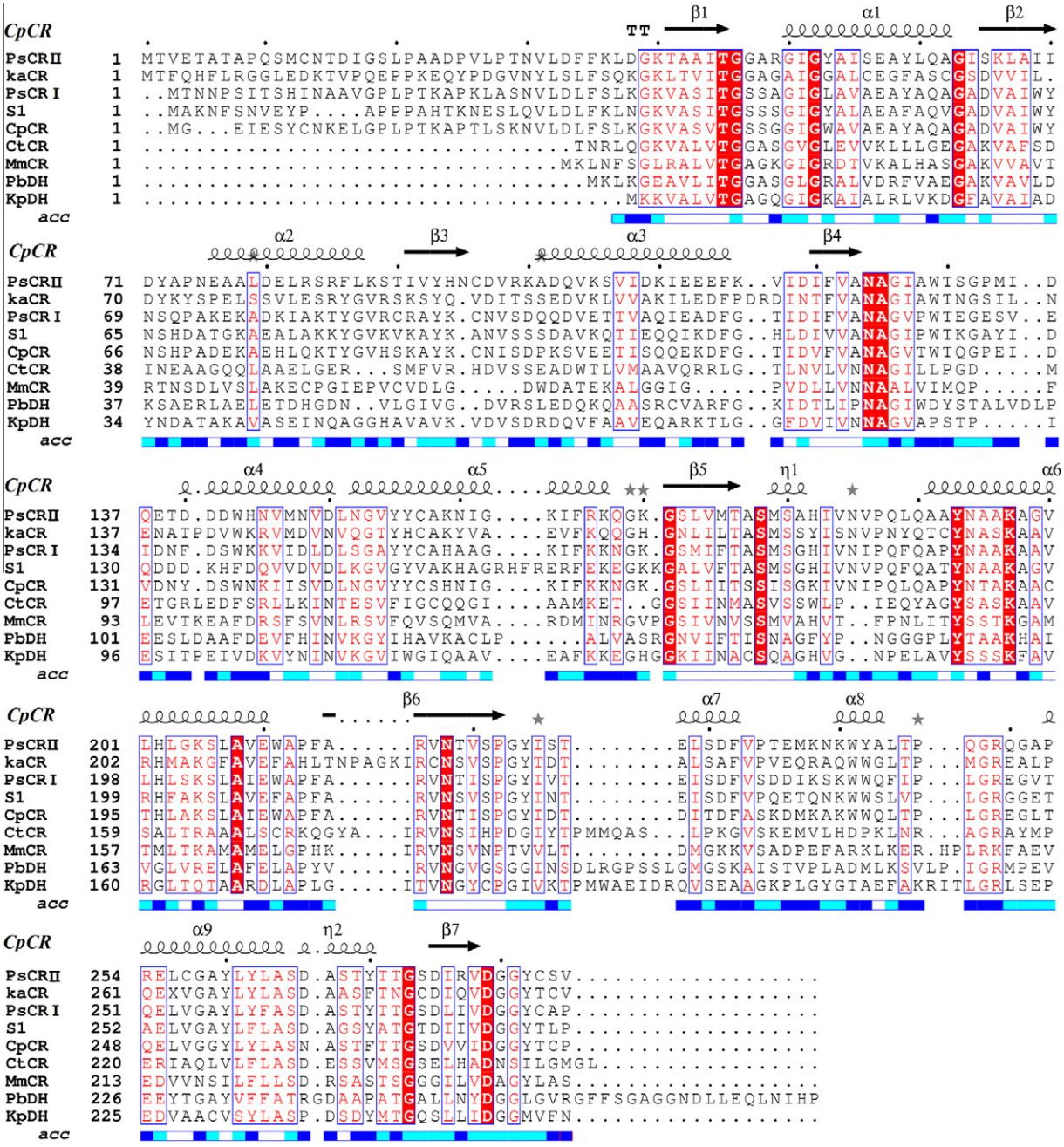

Fig. 2. Amino acid sequence alignments of short-chain alcohol dehydrogenase/reductase using ESPript tool (Gouet et al., 2003). Sequences are *P. stipitis* (PsCR II) from this study (GenBank Accession No. XP_001387285), *P. stipitis* (PsCR I) (GenBank Accession No. XP_001387287), *Candida magnoliae* (S1, GenBank Accession No. BAB21578), *Kluyveromyces aestuarii* (KaCR, GenBank Accession No. BAD01116), *Candida parapsilosis* (CpCR, GenBank Accession No. GI:189096232), *Comamonas testosterone* (CtCR, GenBank Accession No. GI:27573636), mouse (MmCR, GenBank Accession No. GI:1827689), *Pseudomonas* sp. (PbDH, GenBank Accession No. GI:157830235), *Klebsiella pneumoniae* (KpDH, GenBank Accession No. GI:13399598). Secondary structure elements of CpCR (PDB Accession No. 3CTM) are indicated on top of the sequence with arrows for β-stands and cylinders for α-helices.

2010a). In this reaction system, NADH was regenerated by coupling glucose oxidation with BmGDH. The resulting gluconate was neutralized with NaOH to maintain an initial pH of 6.0 (Ye et al., 2009a). The maximum reaction rate was observed from 0 to 2 h and at 0.5 h, 117 mM CHBE was obtained (Fig. 3). At 24 h, transformants produced 554 mM CHBE in the organic phase with a molar yield of 91%, and an optical purity of higher than 99% e.e. for the (*S*)-isomer. The remaining COBE substrate was not detectable. The molar yield of CHBE was 91% because of the partitioning of CHBE in the water/butyl acetate system.

4. Conclusions

In summary, we report a novel carbonyl reductase, PsCR II, from *P. stipitis* for production of (*S*)-CHBE with greater than 99% e.e. PsCR II was grouped with SDRs, and had less than 55% identity to known reductases that produce (*S*)-CHBE. The recombinant PsCR II showed 15 U/mg activity with COBE as substrate. This was the highest reported activity for reductases that need NADH as an electron donor. The purification and characterization of PsCR II are now in progress in our laboratory.

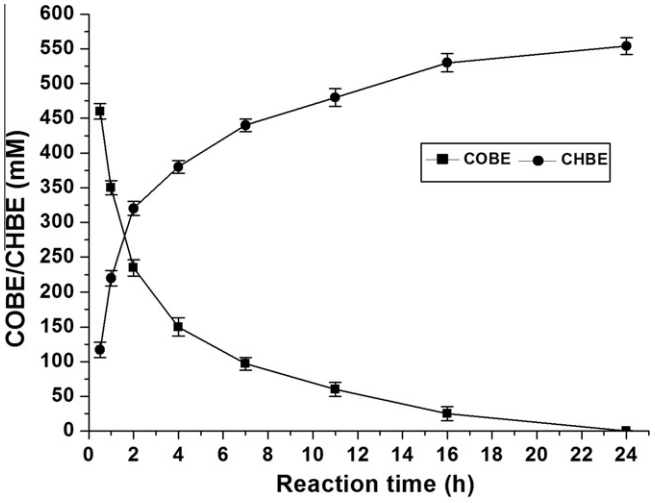

Fig. 3. Asymmetric reduction of 4-chloro-3-oxobutanoate ethyl (COBE) to (*S*)-4-chloro-3-hydroxybutanoate ethyl [(S)-CHBE] using *E. coli* Rosetta (DE3) with pET-22b-BP II in a water/butyl acetate system. For details, see Section 2. Reactions were performed in triplicate, and error bars represent standard deviations (StDev).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.06.098.

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