Biosci. Biotechnol. Biochem., **64** (7), 1430–1436, 2000

Molecular Cloning and Overexpression of the Gene Encoding an NADPH-Dependent Carbonyl Reductase from *Candida magnoliae*, Involved in Stereoselective Reduction of Ethyl 4-Chloro-3-oxobutanoate

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Received January 21, 2000; Accepted March 7, 2000

An NADPH-dependent carbonyl reductase (S1) isolated from *Candida magnoliae* catalyzed the reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate (CHBE), with a 100% enantiomeric excess, which is a useful chiral building block for the synthesis of pharmaceuticals. The gene encoding the enzyme was cloned and sequenced. The S1 gene comprises 849 bp and encodes a polypeptide of 30,420 Da. The deduced amino acid sequence showed a high degree of similarity to those of the other members of the short-chain alcohol dehydrogenase superfamily. The S1 gene was overexpressed in *Escherichia coli* under the control of the *lac* promoter. The enzyme expressed in *E. coli* was purified to homogeneity and had the same catalytic properties as the enzyme from *C. magnoliae* did. An *E. coli* transformant reduced COBE to 125 g/l of (*S*)-CHBE, with an optical purity of 100% enantiomeric excess, in an organic solvent two-phase system.

Key words: carbonyl reductase; stereoselective reduction; *Candida magnoliae*; ethyl 4-chloro-3-hydroxybutanoate

Optically active 4-chloro-3-hydroxybutyric acid esters are useful chiral building blocks for the synthesis of pharmaceuticals.[1,2] The asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) is one of the economical ways of obtaining optically active ethyl 4-chloro-3-hydroxybutanoate (CHBE), because COBE is easily synthesized from cheaply available materials. In previous papers, we reported that *Candida magnoliae* AKU4643 produces several COBE-reducing enzymes. Three of them were purified and characterized in some detail; two, designated as S1 and S4, give (*S*)-CHBE from COBE, and the other, designated as R, gives (*R*)-CHBE.[3–6] Among them, the novel carbonyl reductase, S1, catalyzes the stereoselective reduction of COBE to (*S*)-CHBE, with a 100% enantiomeric excess.[4] There have been many reports on COBE-reducing enzymes and microorganisms, but there has been no report concerning the gene cloning of an enzyme that gives (*S*)-CHBE from COBE so far, although there is one on an aldehyde reductase from *Sporobolomyces salmonicolor* that gives (*R*)-CHBE.[7]

We report here the cloning, expression, and sequence analysis of the gene encoding a novel COBE-reducing carbonyl reductase, S1, from *C. magnoliae*. Comparison of its amino acid sequence with those of other known alcohol dehydrogenases is also described. We produced a carbonyl reductase S1-overproducing strain and used it for the synthesis of optically active (*S*)-CHBE in the presence of glucose dehydrogenase as a coenzyme regenerator.

Materials and Methods

Microorganisms and culture conditions. Candida magnoliae AKU4643 was used as the DNA donor. The organism was cultured at 30°C in a medium comprising 2% glucose, 2% Bacto-tryptone, 1% yeast extract, and 0.004% adenine hydrochloride (pH 7.0). *Escherichia coli* NM415, JM109, and HB101 were used as host cells. *E. coli* was cultured at 37°C in LB medium containing 1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 1% NaCl (pH 7.0), or in 2×YT medium containing 1.6% Bacto-tryptone, 1.0% Bacto-yeast extract, and 0.5% NaCl (pH 7.0).

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Abbreviations: COBE, ethyl 4-chloro-3-oxobutanoate; CHBE, ethyl 4-chloro-3-hydroxybutanoate; e.e., enantiomeric excess

When necessary, ampicillin (0.1 mg/ml) was added to the medium. Plasmid pUCNT was prepared from pUC19 and pTrc99A as described by Nanba *et al.*[8]

Partial amino acid sequence. The carbonyl reductase S1 was purified from cells of *C. magnoliae* AKU4643 as described previously.[4] The enzyme was digested with lysyl endopeptidase in 50 mM NH_4HCO_3, pH 7.8, containing 2 mM EDTA for 24 h at 37°C at a substrate/enzyme ratio of 100/1. The peptides obtained on lysyl endopeptidase digestion were reduced with dithiothreitol for 5 h at 50°C. Then the peptides were separated by HPLC on a YMC-Pack ODS AM302 column (4.6×170 mm; YMC, Kyoto, Japan) equilibrated with 0.1% trifluoroacetic acid, and eluted with a linear acetonitrile gradient (0 to 80%) at the flow rate of 1.0 ml/min. The sequence was analyzeded with a model 477A gas-liquid-phase protein sequencer (Applied Biosystems, USA).

Construction of a genomic DNA library. Chromosomal DNA was prepared from *C. magnoliae* AKU4643 by the method of Hereford *et al.*[9] and partially digested with *Sau*3AI. The partial *Sau*3AI digest was fractionated by agarose gel electrophoresis. Fractions containing fragments 20 to 23 kb in length were recovered from the gels and inserted into *Bam*HI-digested λ EMBL3 with a Ligation Kit ver.1 (Takara Shuzo, Japan). The ligated DNA was packaged into λ phage capsids with an *in vitro* packaging kit (Gigapack II Gold; Stratagene, La Jolla, USA). After adsorption to *E. coli* NM415, the cells were plated onto NZCM agar plates containing 1% NZ amine, 0.5% NaCl, 0.5% yeast extract, 0.1% Casamino acids, and 0.2% $MgSO_4 \cdot 7H_2O$.

Screening of the library. On the basis of the amino acid sequence of the carbonyl reductase S1 from *C. magnoliae*, two oligonucleotides, 5′-AA(T/C)GT(T/C/A/G)GA(A/G)TA(T/C)CC(T/C/A/G)GC-3′ and 3′-CT(A/G)GT(T/C)CT(A/G)CT(A/G)CT(A/G)CT(A/G)TT-5′ were synthesized as probes to screen the λ EMBL3 library. The oligonucleotides were labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase. Hybridization was done at 35°C in 5× SSPE (1×SSPE is 0.18 M NaCl, 0.01 M Na_2PO_3, and 1 mM EDTA). Washing was done at 37°C in 2× SSPE containing 0.1% SDS three times and then at 45°C in 0.5×SSPE containing 0.1% SDS once.

Nucleotide sequencing. For nucleotide sequencing, the 1.3-kb *Hin*dIII-*Eco*RI fragment was digested with appropriate endonucleases and then cloned into pUC19 to provide templates. The nucleotides of the clones were sequenced by the dideoxy chain termination method[10] with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, USA) and an ABI 373A DNA sequencer (Applied Biosystems. U.S.A.). The S1-gene data has been deposited in the DDBJ DNA database under the accession number AB036927. Sequence data were analyzed by Genetyx-Mac (Software Development, Japan). Amino acid sequence alignment of S1 was done with the similarity searching program BLAST.

Construction of an expression vector. An expression vector, pNTS1, which contains the S1 gene, and the *lac* promoter and a terminator inserted in pUC19 was constructed. An N-terminus DNA primer (5′-TAGTCGTTAACCATATGGCTAAGAACTTCTCCAAC-3′) having an *Nde*I site added to the initiation codon of the S1 gene and a C-terminus DNA primer (5′-TCTGAGTTAACGAATTCTTAGGGAAGCGTGTAGCCACCGT-3′) having an *Eco*RI site added immediately after the termination codon were synthesized. Using the above two primers, double-stranded DNA was synthesized using the plasmid pUCHE given by inserting the 1.3-kb *Hin*dIII-*Eco*RI fragment into the *Hin*dIII-*Eco*RI site of plasmid pUC19, as a template. The resultant DNA fragment was digested with *Nde*I and *Eco*RI, and then inserted into the *Nde*I-*Eco*RI sites of pUCNT[8] to obtain the recombinant plasmid pNTS1.

Preparation of a cell-free extract from the E. coli transformant. An *E. coli* transformant was grown in 2 ml of 2×YT medium containing 0.2 mg ampicillin with shaking at 37°C for 12 h. When necessary, 1 mmol of isopropyl-β-D-thiogalactopyranoside (IPTG) per liter was added to the medium. The cells were harvested by centrifugation, suspended in 10 ml of 100 mM potassium phosphate buffer (pH 6.5), and then disrupted by sonication. The cell debris was removed by centrifugation. The supernatant was recovered as the cell-free extract. Protein concentrations were measured with a Protein Assay Kit containing Coomassie Brilliant Blue (Nacalai Tesque, Japan), with bovine serum albumin as the standard.[11]

Purification of the carbonyl reductase S1 expressed in E. coli. All purification procedures were done at 0–4°C in 10 mM potassium phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol. The washed cells, isolated from 1,000 ml of culture broth of *E. coli* [pNTS1], were suspended in 100 ml of the buffer and then disrupted by sonication. The supernatant obtained on centrifugation was used as the cell-free extract. The latter was fractionated with solid ammonium sulfate. The precipitate obtained at 40–75% saturation was collected by centrifugation and dissolved in 40 ml of the buffer. This enzyme solution was applied to a phenyl-Sepharose column (2.5× 26.5 cm) previously equilibrated with the buffer containing 4 M NaCl. The enzyme was eluted with a

1,200 ml cross-linear gradient of NaCl and ethylene glycol (from 4 M NaCl and 0% ethylene glycol to 0 M and 50% (w/v), respectively, in the buffer). The active fractions were collected and concentrated to 51 ml with a membrane filter apparatus (Amicone, USA). The concentrate was put on a Q-Sepharose column (1.5 × 28 cm) previously equilibrated with the buffer, and the unabsorbed active fractions were collected. The enzyme solution was concentrated by 0–80% ammonium sulfate precipitation. The precipitate was collected by centrifugation, dissolved in 9 ml of the buffer, and then put on a Sephacryl S-200 column (1.25 × 120 cm) previously equilibrated with the buffer. The active fractions were collected and used as the purified enzyme.

Enzyme assay. The COBE-reducing enzyme assay was done as follows, the assay mixture comprising 100 mM potassium phosphate buffer (pH 6.5), 0.1 mM NADPH, and 1 mM COBE, and the reactions at 30°C were monitored as the decrease in absorbance at 340 nm. One unit of carbonyl reductase S1 was defined as the amount catalyzing the oxidation of 1 μmol of NADPH per min.

Bioconversion of COBE to CHBE in a two-phase system reaction. A reaction mixture of 25 ml of culture broth of *E. coli* HB101 [pNTS1], which was prepared from 25 ml of culture broth, 3.75 g of COBE, 5.5 g of glucose, 1.56 mg of $NADP^+$, and 293 units of glucose dehydrogenase, was mixed with 25 ml of *n*-butyl acetate in a reaction vessel, and then the mixture was stirred at 30°C. The pH of the mixture was adjusted to 6.5 by adding 5 M NaOH automatically.

Analysis. The concentrations of CHBE and COBE were measured by gas chromatography on a column (3.2 mm × 1.0 m) of 10% PEG-20M on 80/100 mesh Chromosorb WAW DMCS (GL Science, Japan). The optical purity of CHBE was measured by HPLC on a Chiralcel OB (4.6 × 250 mm; Daicel Chemicals, Japan). The HPLC conditions were hexane/2-propanol (9/1, v/v) as the mobile phase, ambient column temperature, and detection at 215 nm.

Enzymes and chemicals. Achromobacter lyticus lysyl endopeptidase was purchased from Wako Pure Chemicals, Japan. Restriction enzymes, alkaline phosphatase (from *E. coli* C75), T4 DNA ligase, and DNA polymerase were purchased from Takara Shuzo, Japan. Glucose dehydrogenase from *Bacillus megaterium* was purchased from Amano Pharmaceuticals, Japan. Ethyl 4-chloro-3-oxobutanoate was purchased from Tokyo Kasei Kogyo, Japan. (*R*, *S*)-CHBE was prepared from COBE by $NaBH_4$ reduction. All other chemicals were of reagent grade.

Results

Partial amino acid sequence

The carbonyl reductase S1 purified from cells of *C. magnoliae* was digested with lysyl endopeptidase. The seven peptides (K-1 to K-7) obtained were separated by reverse phase HPLC, and then the amino acid sequences of these peptides were analyzed (Table 1).

Isolation of the S1-clone

Total DNA was isolated from *C. magnoliae* cells, and then partially digested DNA fragments of predominantly 15 to 20 kb and 20 to 23 kb in length were inserted into λ EMBL3. To screen for the S1 gene, two oligonucleotides, 5′-AA(T/C)GT(T/C/A/G)GA(A/G)TA(T/C)CC(T/C/A/G)GC-3′, encoding NVEYPA in the N-terminus region, and 3′-CT(A/G)GT(T/C)CT(A/G)CT(A/G)CT(A/G)TT-5′, encoding DQDDDK in peptide K-4 were synthesized as probes to screen the λ EMBL3 library. The library was screened, and one clone, to which both oligonucleotides hybridized, was obtained. DNA extracted from the phages was digested with restriction enzymes, and then Southern hybridization analysis was done with the two oligonucleotide probes. A 1.3-kb DNA fragment double digested with *Eco*RI and *Hin*dIII was hybridized with these synthetic DNA probes. The 1.3-kb DNA fragment was inserted into the *Eco*RI-*Hin*dIII site of plasmid pUC19 to give the recombinant plasmid pUCHE, which was selected as the chromosome DNA clone including the S1 gene. A variety of restriction enzymes were reacted with the recombinant plasmid pUCHE, and the digested fragments produced during the reactions were analyzed to create a restriction enzyme cleavage map. Then, various DNA fragments obtained during the analysis were inserted into the multi-cloning site of plasmid pUC19 to obtain recombinant plasmids. Using these recombinant plasmids, the nucleotide sequence of the 1.3-kb DNA fragment was analyzed.

Nucleotide and deduced amino acid sequences

Figure 1 shows the nucleotide sequence of the

Table 1. Partial Amino Acid Sequence of the Carbonyl Reductase S1 from *C. magnoliae* AKU4643

	Amino acid sequence[a]
N-terminus	AKNFSNVEYPAPPPAHTKNESLQV
K-1	KYGVK
K-2	YVAK
K-3	NFSNVEYPAPPPAHTK
K-4	GAYIDQDDDK
K-5	GALVFTASMSGHIVNVPQFQA
K-6	DFGHLDIVVANAGIPWTK
K-7	SLAVEFAPFARVNSVSPGYINTEIS

[a] The amino acids are given in the one-letter code. The underlined amino acids comprise the sequences used for the construction of oligonucleotides.

1.3-kb *Hin*dIII-*Eco*RI fragment and the deduced amino acid sequence. There was one open reading frame (849 bp, 283 amino acids), and the deduced amino acid sequence was identical to the partial amino acid sequence found on peptide sequencing (the underlined amino acid sequence in Fig. 1). The predicted molecular mass (30,420 Da) was nearly identical to the molecular mass (33,000 Da) estimated by SDS-PAGE. A TATA box[12] sequence was found 60 bp upstream of the ATG codon. The sequence of TATATA found 27 bp downstream of a TAA stop codon may be an additional sequence of poly-adenylate.[13]

Comparison of the S1 amino acid sequence with those of other proteins

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AAGCTTGGGGAACCGACGTCCCCGCCCTCGTACATGCAGTGCATACAGCATTGCCCAAAC   60
CCCACATTGTGCCCCCACCCCCCCGCGGATTCCGTAACTATATAAAGGCCGCCAGTGCCG  120
ACTATGGACCATCATCCCGAAATCACCAAGAACTAACAATGGCTAAGAACTTCTCCAACG  180
                                   M  A  K  N  F  S  N  V
TCGAGTACCCCGCCCCGCCTCCGGCCCACACCAAGAACGAGTCGCTGCAGGTCCTTGACC  240
 E  Y  P  A  P  P  P  A  H  T  K  N  E  S  L  Q  V  L  D  L
TGTTCAAGCTGAATGGCAAGGTTGCCAGCATCACTGGCTCGTCCAGCGGTATTGGCTACG  300
 F  K  L  N  G  K  V  A  S  I  T  G  S  S  S  G  I  G  Y  A
CTCTGGCTGAGGCCTTCGCGCAGGTCGGCGCTGACGTCGCCATCTGGTACAACAGCCACG  360
 L  A  E  A  F  A  Q  V  G  A  D  V  A  I  W  Y  N  S  H  D
ACGCTACTGGCAAGGCTGAGGCCCTCGCCAAGAAGTACGGCGTCAAGGTCAAGGCCTACA  420
 A  T  G  K  A  E  A  L  A  K  K  Y  G  V  K  V  K  A  Y  K
AGGCGAACGTGAGCAGCTCTGACGCCGTGAAGCAGACGATCGAGCAGCAGATCAAGGACT  480
 A  N  V  S  S  S  D  A  V  K  Q  T  I  E  Q  Q  I  K  D  F
TCGGCCACCTCGACATTGTCGTGGCGAACGCCGGCATTCCCTGGACGAAGGGTGCCTACA  540
 G  H  L  D  I  V  V  A  N  A  G  I  P  W  T  K  G  A  Y  I
TCGACCAGGACGACGACAAGCACTTCGACCAGGTCGTTGACGTCGATCTGAAGGGTGTTG  600
 D  Q  D  D  D  K  H  F  D  Q  V  V  D  V  D  L  K  G  V  G
GATACGTCGCGAAGCACGCTGGCCGTCACTTCCGCGAGCGCTTCGAGAAGGAGGGCAAGA  660
 Y  V  A  K  H  A  G  R  H  F  R  E  R  F  E  K  E  G  K  K
AGGGCGCCCTTGTGTTCACGGCCTCCATGTCTGGCCACATTGTGAACGTGCCCCAGTTCC  720
 G  A  L  V  F  T  A  S  M  S  G  H  I  V  N  V  P  Q  F  Q
AGGCCACGTACAACGCGGCCAAGGCTGGCGTGCGCCACTTCGCGAAGTCGCTGGCCGTCG  780
 A  T  Y  N  A  A  K  A  G  V  R  H  F  A  K  S  L  A  V  E
AGTTCGCGCCGTTCGCGCGCGTGAACTCTGTGTCGCCGGGCTACATCAACACGGAGATCT  840
 F  A  P  F  A  R  V  N  S  V  S  P  G  Y  I  N  T  E  I  S
CGGACTTCGTGCCCCAGGAGACGCAGAACAAGTGGTGGTCGCTCGTGCCCCTTGGCCGCG  900
 D  F  V  P  Q  E  T  Q  N  K  W  W  S  L  V  P  L  G  R  G
GCGGAGAGACGGCCGAGCTCGTTGGCGCCTACCTGTTCCTTGCATCTGACGCCGGCTCGT  960
 G  E  T  A  E  L  V  G  A  Y  L  F  L  A  S  D  A  G  S  Y
ACGCCACTGGTACGGACATCATTGTTGACGGTGGCTACACGCTTCCCTAAGCGGCGTGCC 1020
 A  T  G  T  D  I  I  V  D  G  G  Y  T  L  P  *
GGAAACATAGAGCTATCTATATAACCATAATGATGCGCATATTATGATCTACTACCTTGA 1080
CTTCGATCGGAACTTAGGAACGATAAGGGTGGAATGCGTGAAAGCGTGCATGCTGCAGAG 1140
CGGTGTAATCGGCAGGGCTGTAGGGTGCCTGAGGCGGCGGGCCAGCAGTGCATGTAACCG 1200
GAGCTGAAGCGGAGGCACACATTGCGATGCAGCGAAGCACGGCCGCCAGAACTCTTTGAG 1260
AACAAGCGCGGCCCTCGACTATGCAGCGGCAACAAGCGAATTC
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Fig. 1. Nucleotide and Deduced Amino Acid Sequence of Carbonyl Reductase S1 Gene from *Candida magnoliae*.

The nucleotide sequence of 1,303 bp *Hin*dIII-*Eco*RI fragment containing the S1 gene in numbered from the first base of the *Hin*dIII site. The deduced amino acid sequence is given in the one letter code above the nucleotide sequence at the first position of each codon. The stop codon is indicated by an asterisk (*). Amino acids matching ones identified on partial amino acid sequence analysis, a TATA box, and a polyadenylation signal are underlined.

The deduced amino acid sequence of S1 was compared with other protein sequences in a database (PIR). A high level of identity was found with other proteins belonging to the short-chain alcohol dehydrogenase/reductase (SDR) superfamily, *i.e.*, D-arabinitol dehydrogenase from *Piciha stipitis*,[14] gluconate dehydrogenase from *Gluconobacter suboxydans*,[15] 3-oxoacyl-[acyl-carrier-protein] reductase from *E. coli*,[16] glucose dehydrogenase from *Bacillus megaterium*,[17] acetoacetyl-CoA reductase from *Zoogloea ramigera*,[18] and short-chain alcohol dehydrogenase Ke6 from mouse. Alignment of carbonyl reductase S1 with these six enzymes is done in Fig. 2. Two conserved sequences of the SDR superfamily were found, *i.e.* a Gly-X-X-X-Gly-Gly cofactor binding motif (where X denotes any amino acid. Gly-40, Gly-44 and Gly-46; numbering according to S1 from *C. magnoliae*), and a Tyr-X-X-X-Lys segment (residues 204–208) essential for the catalytic activity of SDR proteins.[19–22]

Construction of an expression vector

Using the two primers described in Materials and methods, PCR was done by using plasmid pUCHE as a template. The amplified DNA fragments was digested with *Nde*I and *Eco*RI, and then inserted into the *Nde*I-*Eco*RI sites of pUCNT[8] to obtain the recombinant plasmid pNTS1. The specific COBE-reducing activity in a cell-free extract of *E. coli* JM109 carrying pNTS1 was high (13 units/mg-protein) with induction by IPTG; it was 16-fold higher than that of *E. coli* JM109 carrying pUCHE (Table 2), and 19-fold higher than that of a cell-free extract of *C. magnoliae*.

Overproduction and purification of S1

The recombinant carbonyl reductase S1 was purified 2-fold from *E. coli* HB101 [pNTS1] cells, with a yield of 6%, to apparent homogeneity on SDS-PAGE (Table 3). The N-terminus amino acid sequence was conserved in the purified enzyme from *C. magnoliae*. The substrate specificity for typical carbonyl compounds, stereoselectivity for COBE, and some other characteristics of the enzyme from *E. coli*

Table 2. The Carbonyl Reductase S1 activity of *E. coli* Transformants

Plasmid	Host	IPTG (mM)	Specific activity (units/mg)
pUCNT	JM109	1	0.004
pUCHE	JM109	1	0.83
pUCHE	JM109	0	0.015
pNTS1	JM109	1	13.1
pNTS1	JM109	0	0.29
pNTS1	HB101	0	8.1

The *E. coli* transformant was cultured in 2 × YT medium with or without added IPTG. Specific activity is expressed as units per mg protein.

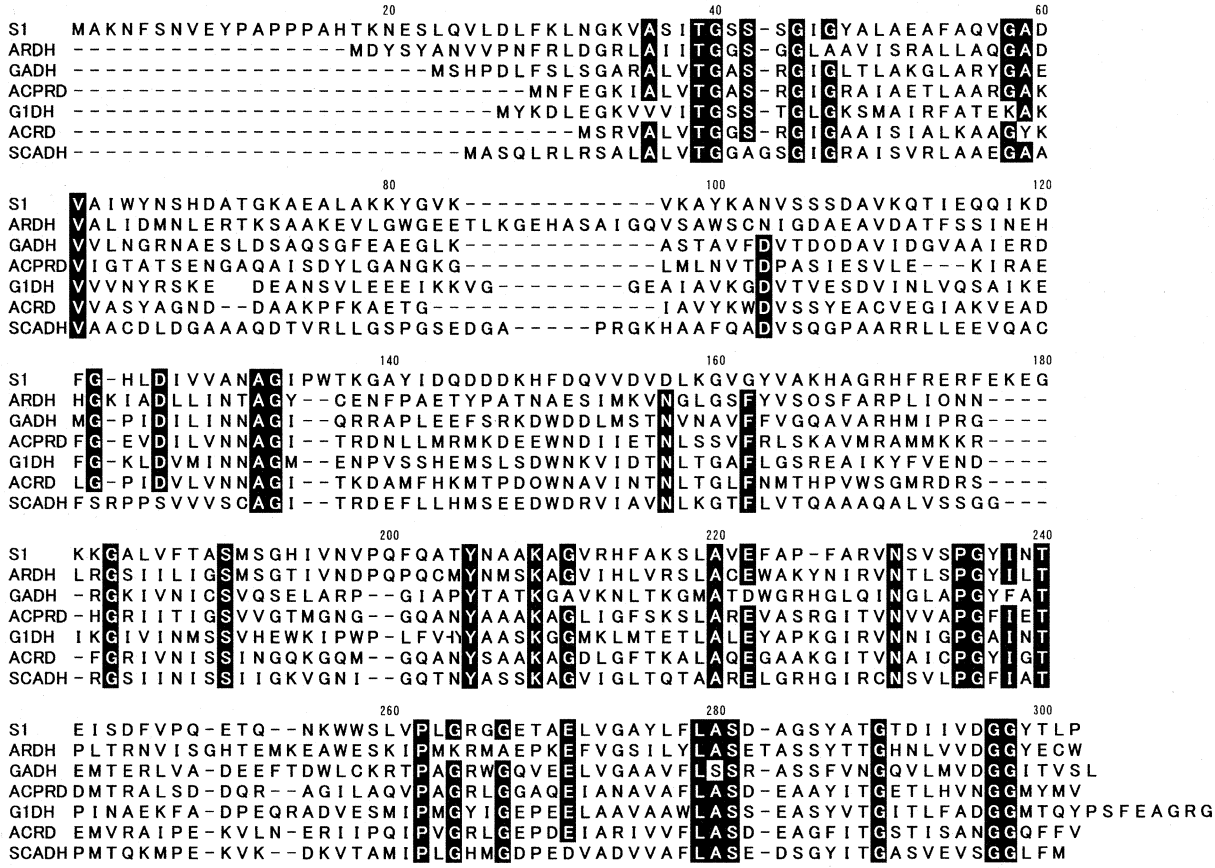

Fig. 2. Amino Acid Sequence Alignment of the Carbonyl Reductase S1 from *Candida magnoliae*.

The carbonyl reductase S1 from *Candida magnoliae* (S1), D-arabinitol 2-dehydrogenase from *Pichia stiptis* (ARDH), gluconate 5-dehydrogenase from *Gluconobacter suboxydans* (GADH), 3-oxoacyl-[acyl-carrier-protein] reductase from *Escherichia coli* (ACPRD), glucose 1-dehydrogenase from *Bacillus megaterium* (G1DH), acetoacetyl-CoA reductase from *Zoogloea ramigera* (ACRD), and short-chain alcohol dehydrogenase Ke-6 from mouse (SCADH) are aligned. Strictly conserved residues are outlined in black.

Table 3. Purification of carbonyl reductase S1 from *E. coli* [pNTS1][a]

	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cell-free extract	18420	1350	13.6	100
Ammonium sulfate	16440	1050	15.7	89
Phenyl-Sepharose	4990	248	20.1	27
Q-Sepharose	4370	176	24.8	24
Sephacryl S-200	1120	40	27.8	6

[a] The enzyme activity was measured with 1 mM COBE.

were the same as those of *C. magnoliae*.

Production of (S)-CHBE with E. coli cells in a two-phase system reaction

It is more effective to done the enzymatic COBE reduction reaction in a water-organic two-phase system, because unstable COBE in water is extracted into the organic phase and strong inactivation of the enzyme by COBE is thereby avoided. To regenerate the coenzyme, NADPH, we used glucose dehydrogenase from *Bacillus megaterium*, which reduces $NADP^+$ to NADPH and oxidizes glucose to gluconolactone. The culture broth of *E. coli* HB101 [pNTS1] reduced 15% of COBE to (*S*)-CHBE with coenzyme regeneration in a water-butyl acetate two-phase system. The concentration in the organic solvent reached 1,060 mM (125 g/l), and COBE was not detected after 30 h reaction. The molar conversion yield and enantiomeric excess (e.e.) of (*S*)-CHBE were 87.5% and 100% e.e., respectively. The cuture broth of *E. coli* HB101[pNTS1] did not reduce COBE in the absence of an NADPH regeneration system under the same reaction conditions.

Discussion

C. magnoliae produces at least two NADPH-dependent COBE-reducing enzymes which give (*S*)-CHBE, one carbonyl reductase S1 and the other carbonyl reductase S4.[4] S1 reduces COBE to (*S*)-CHBE with a 100% e.e., but S4 reduces COBE to (*S*)-CHBE with a 50.7% e.e.[4,5] These two enzymes show different substrate specificities.[4,5] Also, Shimizu *et al.* purified and characterized a NADPH-dependent aldehyde reductase which reduces COBE to (*R*)-CHBE from *Sporobolomyces salmonicolor*.[23] The gene for

this COBE-reducing enzyme from *S. salmonicolor* was cloned and its deduced amino acid sequence showed a high degree of similarity to those of other members of the aldo-keto reductase superfamily.[7] Here we cloned the S1 gene from *C. magnoliae* and compared it with other carbonyl reductases.

Previously, we reported that 35 amino acids residues of the N-terminus of S1 showed no similarity to those of other oxidoreductases.[4] But we found that the whole deduced amino acid sequence showed similarity to those of other members of the short-chain alcohol dehydrogenase/reductase (SDR) superfamily. There were about twenty extra amino acid residues between the N-terminus and a cofactor binding motif, Gly-X-X-X-Gly-X-Gly sequence (residues 40–46 in the S1 sequence), than in the other SDR superfamily enzymes. Another essential segment for the catalytic activity of SDR enzymes, Tyr-X-X-X-Lys[19,21] (residues 191–195) was also found. There were five, Gly-40, Gly-44, Gly-168, Tyr-191, and Lys-194 (numbering according to S1), of six strictly conserved important residues for all group II alcohol dehydrogenases.[21] There were some strictly conserved residues in the C-terminus parts of the proteins, which would reflect the wide substrate specificity of SDRs and group II alcohol dehydrogenases.[15] As the three enzymes shown in Fig. 2 are involved in the oxido-reduction of carbohydrates and their derivatives,[14,15,17] S1 may play a role in carbohydrate metabolism in *C. magnoliae*.

The members of the SDR superfamily and group II alcohol dehydrogenases appear to have no metal requirements, which is in contrast to in the case of many other pyridine-coenzyme-linked dehydrogenases.[21] S1 does not require metals for its catalysis.[4] S1 may be close to SDRs or group II alcohol dehydrogenases.

To overexpress the S1 gene in *E. coli*, we constructed a plasmid vector, pNTS1, comprising pUCNT, which was used for overexpression of the *N*-carbamoyl-D-amino acid amidohydrolase gene,[8] and a DNA fragment which only contains the ORF for S1. In the absence of an inducer, *E. coli* JM109 [pNTS1] containing the S1 gene showed only low specific activity (0.29 units/mg), which corresponded to about 1% of the soluble protein. On the other hand, the specific activity of S1 in the cell-free extract of *E. coli* HB101 [pNTS1] without the inducer was 8.1 units/mg-protein, corresponding to about 29% of the soluble protein, which was 12-fold higher than in the case of *C. magnoliae*, which was the original producer of this enzyme. It is very important to obtain broth containing high enzyme activity without an expensive inducer for industrial use of this enzyme from both economical and technical points of view.

In a previous study, we showed that cells of *C. magnoliae* produced 90 g/l (*S*)-CHBE in the presence of a coenzyme regenerating system in a water-solvent two-phase system. But its optical purity was only 96.6% e.e. because of the occurrence of multiple COBE-reducing enzymes.[3] It is very difficult to increase its optical purity by a general purification procedure, such as recrystallization, because CHBE is an oily compound. Thus we have studied a COBE-reducing enzyme, S1, giving optically pure (*S*)-CHBE, aiming at stereoselective reduction by means of a single COBE-reducing enzyme.

The culture broth of *E. coli* [pNTS1] produced 125 g/l (*S*)-CHBE from COBE in the presence of an NADPH-regeneration system in a two-phase system. The calculated turnover of $NADP^+$, based on the amounts of $NADP^+$ added and CHBE formed, was about 13,000 mol/mol. This turnover number of the coenzyme may be increased through further research. Shimizu *et al.* reported the reduction of COBE to (*R*)-CHBE by a recombinant *E. coli* producing an aldehyde reductase from *S. salmonicolor* in the presence of an NADPH-generating system in a two-phase system in the same manner as that observed in this study. They reported that 250 g/l CHBE was formed, and that the optical purity of (*R*)-CHBE formed was 91% e.e. and the calculated turnover of $NADP^+$ was 5,100 mol/mol.[24] In the case of the use of *E. coli* [pNTS1] cells, the accumulation of (*S*)-CHBE in the organic-phase was lower (125 g/l), but with a higher turnover of $NADP^+$ and a higher optical purity of (*S*)-CHBE (100% e.e.). The use of *E. coli* [pNTS1] had advantages, such as the synthesis of (*S*)-CHBE of high quality and the use of a smaller amount of expensive $NADP^+$, for the stereoselective reduction of COBE.

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