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Carbonyl reductase SCRII from *Candida parapsilosis* catalyzes anti-Prelog reaction to (S)-1-phenyl-1,2-ethanediol with absolute stereochemical selectivity

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ABSTRACT

An (S)-specific carbonyl reductase (SCRII) was purified to homogeneity from *Candida parapsilosis* by following an anti-Prelog reducing activity of 2-hydroxyacetophenone. Peptide mass fingerprinting analysis shows SCRII belongs to short-chain dehydrogenase/reductase family. Its coding gene was cloned and overexpressed in *Escherichia coli*. The recombinant SCRII displays the similar enzymatic characterization and catalytic properties to SCR. It catalyzes the enantioselective reduction of 2-hydroxyacetophenone to (S)-1-phenyl-1,2-ethanediol with excellent optical purity of 100% in higher yield than SCR. Based on the sequence-structure alignment, several single-point mutations inside or adjacent to the substrate-binding loop or active site were designed. With respect to recombinant native SCRII, the A220 and E228 mutations almost lost enantioselectivity towards 2-hydroxyacetophenone reduction. The catalytic efficiencies (k_{cat}/K_m) for the A220 or E228 variants are <7% that of the unmutated enzyme. This work provides an excellent catalyst for enantiopure alcohol preparation and the lethal mutations of A220 and E228 suggest their importance in substrate-binding and/or catalysis.

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1. Introduction

The enantiomerically pure alcohols are the versatile chiral building blocks for the synthesis of numerous products (Edegger et al., 2006; Nakamura et al., 2006). Recent special attention is focused on preparation of chiral alcohols by employing “green” enzymatic processes (Edegger et al., 2006; Nakamura et al., 2006). Many short-chain dehydrogenase/reductases (SDRs) are found to catalyze the stereoselective reduction of the carbonyl group to the corresponding chiral alcohol (Kroutil et al., 2004; Zhang et al., 2008). However, most SDR enzymes follow Prelog’s rule in the sense of the stereochemistry outcomes (Höfer et al., 2001), while very few catalysts were found to possess anti-Prelog selectivity (Bradshaw et al., 1992a,b; Ernst et al., 2005; Fantin et al., 1996; Wei et al., 2000). Moreover, only R-specific alcohol dehydrogenase from *Lactobacillus brevis* (LB-RADH) (Schlieben et al., 2005) and (S)-specific carbonyl reductase (SCR) from *Candida parapsilosis* CCTCC M203011 (Zhang et al., 2008) with anti-Prelog stereoselectivity were analyzed for their quaternary structures.

SDRs often exhibits high chemo-, region- and enantioselectivities (Jörnval et al., 1995; Oppermann et al., 2003; Tanaka et al., 2001; Zhu et al., 2008) due to its strict substrate recognition. If the residues inside or adjacent to the substrate-binding pocket of reductases were mutated, their substrate specificity and stereospecificity will significantly changes (Heiss et al., 2001); (Masuda et al., 2005). Recently, the enzyme SCR with unusual stereospecificity was overexpressed in *Escherichia coli* (Nie et al., 2007; Zhang et al., 2009) and its crystal structure was determined by our labs (Zhang et al., 2008). It catalyzes 2-hydroxyacetophenone to (S)-1-phenyl-1,2-ethanediol (PED) with enantiomeric purity of 96.2% in 83.8% yield (Zhang et al., 2009). For industrial application, the discovery of more efficient enzymes and the precise mechanism of enzymatic stereopreference would be of great significance.

In this study, the other novel (S)-specific carbonyl reductase SCRII was purified to homogeneity from *C. parapsilosis*. The gene *scrII* was cloned and overexpressed in *E. coli*. The enzyme shows the similar catalytic function to SCR except that it catalyzes an anti-Prelog reduction of 2-hydroxyacetophenone to (S)-PED with optical purity of 100% in high yield of 98.1%. Several single-mutations were introduced into the region inside or adjacent to the so-called hydrophobic pocket related substrate-binding or catalysis. The mutations on the alteration of product enantioselectivity in relation with their specific activities and kinetic properties were also discussed.

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2. Methods

2.1. Microorganisms and chemicals

C. parapsilosis strain CCTCC M203011 was obtained from American Type Culture Collection (ATCC, USA). The organisms were cultivated as described previously (Nie et al., 2007; Zhang et al., 2009). The cofactors, 2-hydroxyacetophenone, (R)- and (S)-PED were purchased from the Sigma–Aldrich Chemical Co. Inc. The restriction enzymes were purchased from Takara Shuzou Co. (Kyoto, Japan). All other chemicals were of the analytical grade that could be obtained commercially.

2.2. Enzyme assay

The enzyme assay mixture in 250 μ l comprised 0.1 M potassium phosphate buffer (pH 6.5), 0.5 mM NADPH, 5 mM 2-hydroxyacetophenone, and appropriate enzyme. The reductive activity of 2-hydroxyacetophenone was measured at 35 °C by recording the rate of change in NAD(P)H absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation and oxidation of 1 μ mol of NAD(P)H per minute under measurement condition.

2.3. Biotransformation and analytical methods

The biotransformation reaction was carried out as described previously (Nie et al., 2007) with minor modification. For asymmetric reaction with the recombinant *E. coli* cells, the reaction mixture in a 2-ml volume consisted of 0.1 M potassium phosphate buffer (pH 6.5), 5 g/l 2-hydroxyacetophenone and 0.1 g of washed wet cells. When the purified protein was used as biocatalyst, the reaction mixture in a 2-ml volume consisted of 0.1 M potassium phosphate buffer (pH 6.5), 2 mM NADH or NADPH, 5 g/l 2-hydroxyacetophenone, and an appropriate amount of purified protein. The reactions were carried out at 30 °C for 48 h and 8 h with shaking at 150 rpm respectively, when using the wet recombinant cells and purified enzyme as biocatalysts. The product (S)-PED was extracted with ethyl acetate, and the organic layer was used for analysis. The optical purity and yield of product were determined by high-performance liquid chromatography on a Chiralcel OB-H column (Daicel Chemical Ind. Ltd., Japan).

2.4. Isolation of SCRII from *C. parapsilosis*

The microorganism *C. parapsilosis* was incubated as described previously (Nie et al., 2007). The cells were suspended in a buffer containing 20 mM Tris–HCl (pH 8.0) and 150 mM NaCl and disrupted by sonication with an ultrasonic oscillator (Sonic Materials Co., USA). The cell debris was removed by centrifugation (40,000 $\times$ g, 40 min, 4 °C). Solid (NH₄)₂SO₄ was slowly added to the supernatant to a final concentration of 70% (w/v). The precipitated protein was collected by centrifugation (40,000 $\times$ g, 30 min, 4 °C), and immediately dissolved in buffer A (20 mM Tris–HCl, pH 8.5). After dialysis against the same buffer overnight, the sample was filtered (0.22 μ m) and loaded onto a Q Sepharose Fast Flow column (Pharmacia) pre-equilibrated with buffer A. The enzyme was eluted with a linear gradient of 0–1.0 M NaCl and condensed to 5 ml by ultrafiltration with a Centricon YM-10 concentrator (Millipore). The active fraction was purified with a Blue Sepharose (Pharmacia Biotech) Fast Flow column (1.0 by 10 cm) equilibrated with the same buffer. After the column was washed with 50 mM NaCl, then 2 mM NAD⁺, and finally 1 mM NADP⁺, the fraction was eluted by 1 mM NADP⁺, and further applied to a Superdex 200 (Hi-Load 26/60, preparation grade) for chromatography in a buffer con-

taining 20 mM Tris–HCl (pH 8.5) and 150 mM NaCl. The active fractions with a single protein peak were concentrated by ultrafiltration and stored at –80 °C.

2.5. Peptide mass fingerprinting analysis and N-terminal amino acid sequencing

The purified protein was subjected to SDS–PAGE gel and cut for peptide mass fingerprinting analysis and N-terminal amino acid sequencing. Peptide mass fingerprinting analysis was performed by Proteomics solution I system and the N-terminal residue was sequenced with Edman method by Applied Biosystems Model 492cLC protein sequencer. The peptide mass data were used to query the Mascot database (<http://www.matrixscience.com>). Both analyses were performed by Shanghai Gene Core Biotechnologies Co. Ltd.

2.6. Cloning and expression of *scriI*

The enzyme SCRII coding gene *scriI* was amplified from *C. parapsilosis* genomic DNA by standard polymerase chain reaction (PCR) methodologies. Since the N-terminal amino acid sequence of *scriI* is identical to *scr*, the same primer was utilized for *scriI*. The forward primer was SCRII_F1: 5'-ATGGGCGAAATCGAATCTTATTG-3'. Based on C-terminal amino acid sequences, degenerate primer was designed as follow: SCRII_R1: 5'-RGANCCNCRTCNACNGCNTR-3' (with R representing A or G and N representing A, C, G, or T). The purified amplification product was ligated into the vector pMD18-T and the plasmid was verified by PCR amplification. After the gene sequence was confirmed by DNA sequencing, the primer was redesigned as follows: SCRII_F2: 5'-ATCGGATCCATGGGCGAAATCGAATCTTATTG-3' (*Bam*HI, underlined) and SCRII_R2: 5'-TGACTCTCGAGTGGACAAGTGTAACCACCATCGAC-3' (*Xho*I, underlined). The purified fragment of *scriI* was digested with *Bam*HI and *Xho*I and ligated into restriction-digested expression vector pET28c(+) similarly. The recombinant plasmid pETSCRII was transformed into the competent *E. coli* BL21 (DE3) cells chemically, and the positive clones were verified by DNA determination.

2.7. Purification of the recombinant enzymes

All recombinant proteins were expressed in *E. coli* strain BL21 (DE3) as His₆-tagged proteins. The protein purification was carried out as described previously (Zhang et al., 2008).

2.8. Temperature and pH dependence of SCRII

The temperature dependence of enzyme activity was determined at various temperatures ranging from 20 to 70 °C. The pH dependence of the reductive activity was determined between pH 4.0 and 9.0 using 100 mM NaAC–HAC (pH 4.0–6.0), 100 mM potassium phosphate (pH 6.0–7.0) or 100 mM Tris–HCl buffer (pH 7.0–9.0) at 35 °C. The enzyme activity was measured with the standard assay method described above.

2.9. Thermostability of SCRII

For thermal inactivation, the enzymes (0.2 mg/ml) were incubated in a buffer containing 100 mM NaAC–HAC, pH 6.0 at temperatures between 10 and 60 °C. The pH stability of enzyme was determined by incubating the purified enzyme at pH 4.0–9.0 at 35 °C. After exposure of enzyme to the indicated conditions for 1 h, the samples were taken and assayed for residual activity using 2-hydroxyacetophenone as substrate.

2.10. Effects of metal ions on enzyme activity

The enzyme activity was examined in the presence of 1 mM of various metal salts: CaCl₂, CoCl₂, CuSO₄, Fe₂(SO₄)₃, FeSO₄, MgCl₂, MnCl₂, NiCl₂, ZnSO₄ or EDTA using the assay method.

2.11. Determination of kinetic parameters

To determine the kinetic parameters, various concentrations of substrate 2-hydroxyacetophenone (0.5–20 mM), enzyme (10–200 μM), and cofactors NADPH (0.5–5.0 mM) in 100 mM phosphate buffer (pH 7.5) were used (Zhang et al., 2009). The data were fitted to the Michaelis–Menten equation by using a nonlinear least-square iterative method using KaleidaGraph (Synergy Software, Reading, PA). Three sets of kinetic parameters were obtained from three independent experiments and then simply averaged to yield the final estimates. The final estimates are shown with the standard errors for the three sets.

2.12. Protein structure homology modeling

On the basis of a sequence alignment between the protein SCR_{II} and the template structure SCR (PDB ID: 3CTM), a three-dimensional model for SCR_{II} is generated using SWISS-MODEL workspace (<http://swissmodel.expasy.org/SWISS-MODEL.html>).

2.13. Gene Bank accession code

The *scrII* gene sequence has been deposited in the GenBank database with the accession number GQ411433.

3. Results and discussion

3.1. Isolation and identification of SCR_{II} from *C. parapsilosis*

Because of the scarcity of reductases with anti-Prelog selectivity, the discovery of enzymes would be valuable not only for asymmetric synthesis, but also for asymmetric reduction mechanism (Heiss et al., 2001). In searching for new biocatalysts, *Candida* species have been attractive as highly stereospecific oxidoreductase donors (Kamble et al., 2005; Nakamura and Matsuda, 2006; Pollard et al., 2006). In this work, the enzyme was purified from the culture supernatant of *C. parapsilosis* through ammonium sulfate precipitation, anion exchange and gel filtration chromatography by moni-

toring the reduction of 2-hydroxyacetophenone. The purified samples exhibit one single peak on Superdex™ 200 (10/300 GL) chromatography, and its molecular weight was around 31 kDa by SDS–PAGE analysis.

The purified samples exhibited a specific activity of 5.98 U/mg with 2-hydroxyacetophenone as substrate. When using the purified protein as catalyst, the product was confirmed to comprise only (S)-PED with absolute configuration (optical purity >99%) in a high yield of 98%. The SCR from *C. parapsilosis* was reported to reduce 2-hydroxyacetophenone into (S)-PED (Nie et al., 2007) efficiently, while the recombinant *E. coli*-SCR catalyzed the transformation to (S)-PED with the optical purity of 96.2% in yield of 83.8% (Zhang et al., 2009). The novel enzyme displays the similar catalytic function to SCR except that it shows much better performance. So the enzyme was named SCR_{II}.

3.2. Gene cloning, overexpression and purification of SCR_{II}

The purified sample (31 kDa) was used for chymotrypsin digestion, and the resulting peptides were analyzed by mass spectrometry. For comparison, the recombinant SCR was purified and assayed under the same conditions. The above results showed that two significant matches were found between SCR and SCR_{II}. Among these matched peptides, eight of them were shared by both proteins, four of them were unique for SCR_{II} and six of them were unique for SCR (Table 1). Therefore, the enzyme SCR_{II} has approximate 280 amino acids and shares nearly 85% identity with SCR. It was annotated as carbonyl reductase due to its containing the consensus sequence Gly-X-X-X-Gly-X-Gly (Oppermann et al., 2003; Tanaka et al., 2001).

The gene encoding SCR_{II} was cloned in *E. coli*. Nucleotide sequencing analysis reveals an open reading frame of 837 bp and a polypeptide of 278 amino acid residues. Comparison of the coding gene and amino acid sequence, SCR_{II} shows 85.4% and 88.5% identities with SCR, respectively. The gene sequence of *scrII* was further identified in the website (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/c_parapsilosis). The enzyme SCR_{II} shares significant similarity (between 46% and 85%) with other SDR proteins in the NCBI database (Fig. 1). So it belongs to the SDR family (Oppermann et al., 2003; Tanaka et al., 2001; Ye et al., 2009).

When the recombinant cells were induced with 0.1 mM isopropyl-β-thiogalactopyranoside (IPTG), a predominant band was observed with the approximate size of 34 kDa including the 6 × Histidine and T₇ tag. The protein SCR_{II} was overproduced at a

Table 1
Comparison of SCR and SCR_{II} on MALDI peptide mass data.

Peptide sequence	Sequence length	MW _{observed}	MF _(calculated)	Comments
VANAGVPW	8	813.0253	812.4181	Unique for SCR _{II}
KKNGKGSVLITSSMSGTIVNVPQLQAAY	28	2891.0039	2890.5586	
IATEISDF	8	894.9277	894.4334	
TTGANLAVDGGY	12	1137.8962	1137.5302	
GVHSKAY	7	760.4565	760.3868	Unique for SCR
VANAGVTW	8	816.8921	816.4130	
TQGPEDVDNY	10	1249.6524	1249.5462	
CSHNIGKIF	9	1018.3057	1017.5066	
IDTDITDF	8	938.5943	938.4233	
TTGSDVVIDGGY	12	1137.5591	1182.5404	
MGEIESY	7	827.8331	827.3371	Shared by SCR and SCR _{II}
SLKGKVASVTGSSGGIGW	18	1690.3912	1689.9049	
AVAEAY	6	622.6936	622.2962	
AQAGADVAIW	10	1000.7679	1000.4978	
AQAGADVAIWY	11	1163.5619	1163.5611	
NSHPADEKAEHLQKTY	16	1867.3301	1866.8860	
KCNISDPKSVETISQQEKDF	21	2424.7871	2424.1478	
GTIDVF	6	650.8225	650.3275	

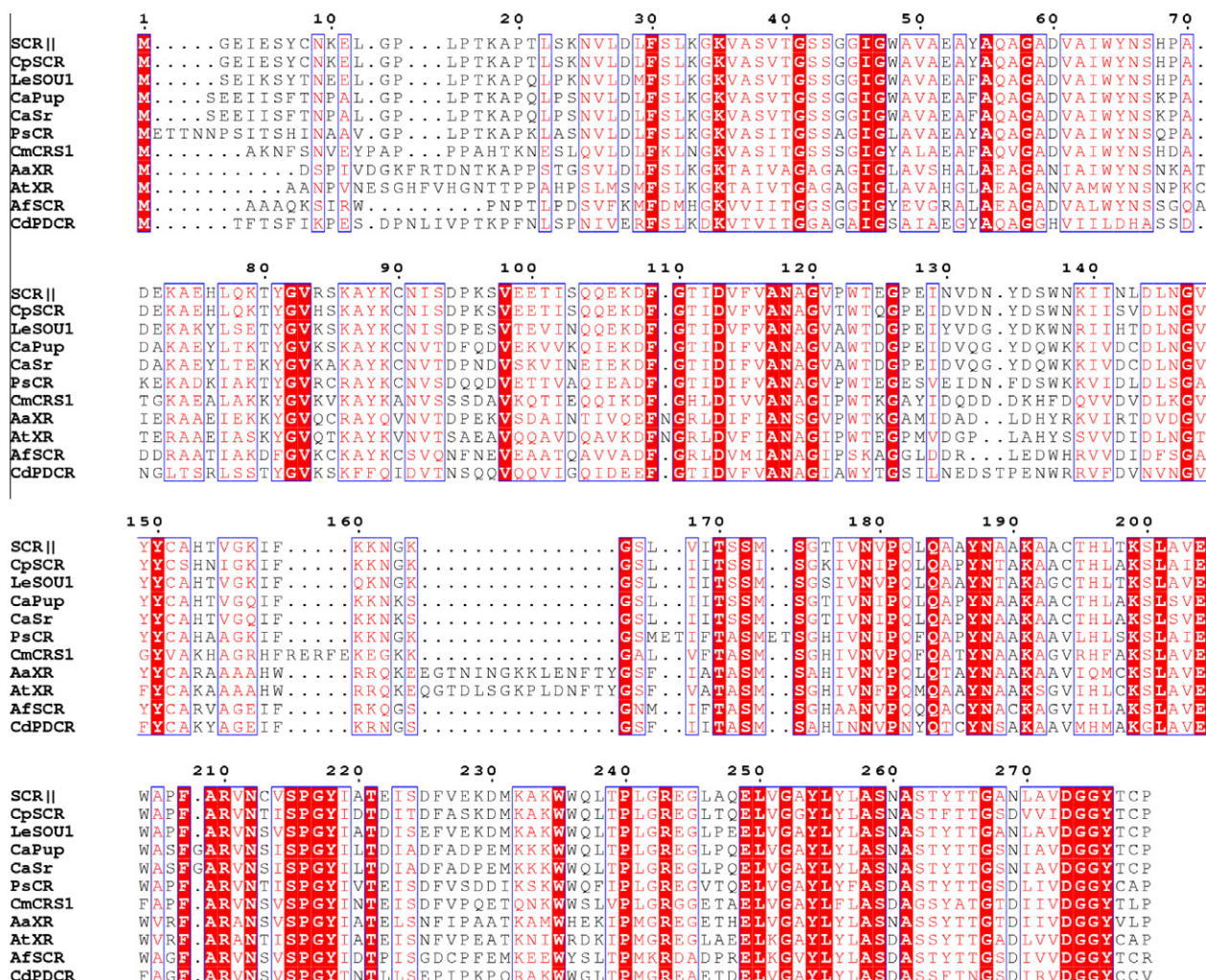


Fig. 1. Amino acid sequence alignment of SCR|| from *C. parapsilosis* (GenBank ID: GQ411433), (S)-specific carbonyl reductase from *C. parapsilosis* (SCR, GenBank ID: DQ675534), sorbitol utilization protein SOU1 from *Lodderomyces elongisporus* (LeSOU1, 5230205), putative uncharacterized protein from *C. albicans* (CaPUP, EAK96529), sorbose reductase from *C. albicans* SC5314 (CaSR, 3642799), carbonyl reductase S1 from *C. magnoliae* (CmCRS1, BAB21578), L-xylulose reductase from *Ajellomyces capsulatus* H143 (AaXR, 24027753), short-chain dehydrogenase from *Aspergillus flavus* (AfSCR, 7915993), L-xylulose reductase from *Aspergillus terreus* (AtXR, 4317250), peroxisomal 2,4-dienoyl-coa reductase from *C. dubliniensis* CD36 (CdPDCR, 8049375) and carbonyl reductase from *Pichia stipitis* (PsCR, 4850856). Gaps in the aligned sequences are indicated by dashes. Identical amino acid residues are highlighted in red. Conserved residues are boxed with blue lines. Selected residue numbers of the SCR|| are labeled above the sequence. This figure was prepared with the program Esript (<http://esript.ibcp.fr/ESript/cgi-bin/ESript.cgi>).

level equal to that of SCR (Zhang et al., 2009). SDS-PAGE analysis showed that the purified SCR|| was around 34 kDa. Calculations of the corresponding A_{280} value and SDS-PAGE analysis value revealed SCR|| was produced more than 40 mg/l of culture.

3.3. Catalytic properties of recombinant SCR||

The enzyme SCR|| displays an optimal temperature at 35 °C and a pH optimum of 6.0 for 2-hydroxyacetophenone. The catalytic properties of SCR|| are very similar to that of *C. parapsilosis* whole-cell catalyst (pH 6.5, temperature 35 °C) (Nie et al., 2004). The thermostability of SCR|| was performed between 10 and 60 °C. The results showed enzyme SCR|| was fairly stable up to 40 °C. More than 70% of the whole activity was retained after 1 h at 40 °C. But residual activity decreased sharply and almost 90% of activity was lost at 50 °C. The recombinant enzyme SCR|| shows good pH stability and keeps more than 75% of the whole activity after 1 h at pH 5.0–7.0.

The effects of different metal ions and EDTA on the activity of SCR|| in vitro were investigated. The results showed 1 mM Co^{2+} , Cu^{2+} , Ni^{2+} and Zn^{2+} had negative effect on the enzyme activity,

causing over 85% of the total activity lost. When 1 mM Ca^{2+} , Fe^{3+} , Fe^{2+} , Mg^{2+} or Mn^{2+} was added into the protein, the activity remained almost constant. The metal-chelating reagent EDTA did not influence the enzyme activity. From these results, SCR|| shows similar catalytic properties to SCR, only with slight difference on a pH optimum (Nie et al., 2007).

3.4. Identification of residues for mutation by sequence-structure analysis

By structure-based sequence alignment (Fig. 2A), SCR|| shares the high similarity with SCR (PDB 3CTM). However, the Asn142, Ala220 and Glu228 inside or adjacent to the substrate-binding pocket (Benach et al., 1999; Jörnval et al., 1995) in SCR|| are very different with those corresponding residues in SCR. We speculated that the Asn142, Ala220 and Glu228 of the enzyme SCR|| may play roles on catalyzing 2-hydroxyacetophenone to (S)-PED efficiently.

The three-dimensional structure of SCR|| was built using SWISS-MODEL workspace and SCR as homology model (Zhang et al., 2008). The residues Asn142, Ala220 and Glu228 are close to the catalytic group Ser172-Tyr187-Lys191, and the distances be-

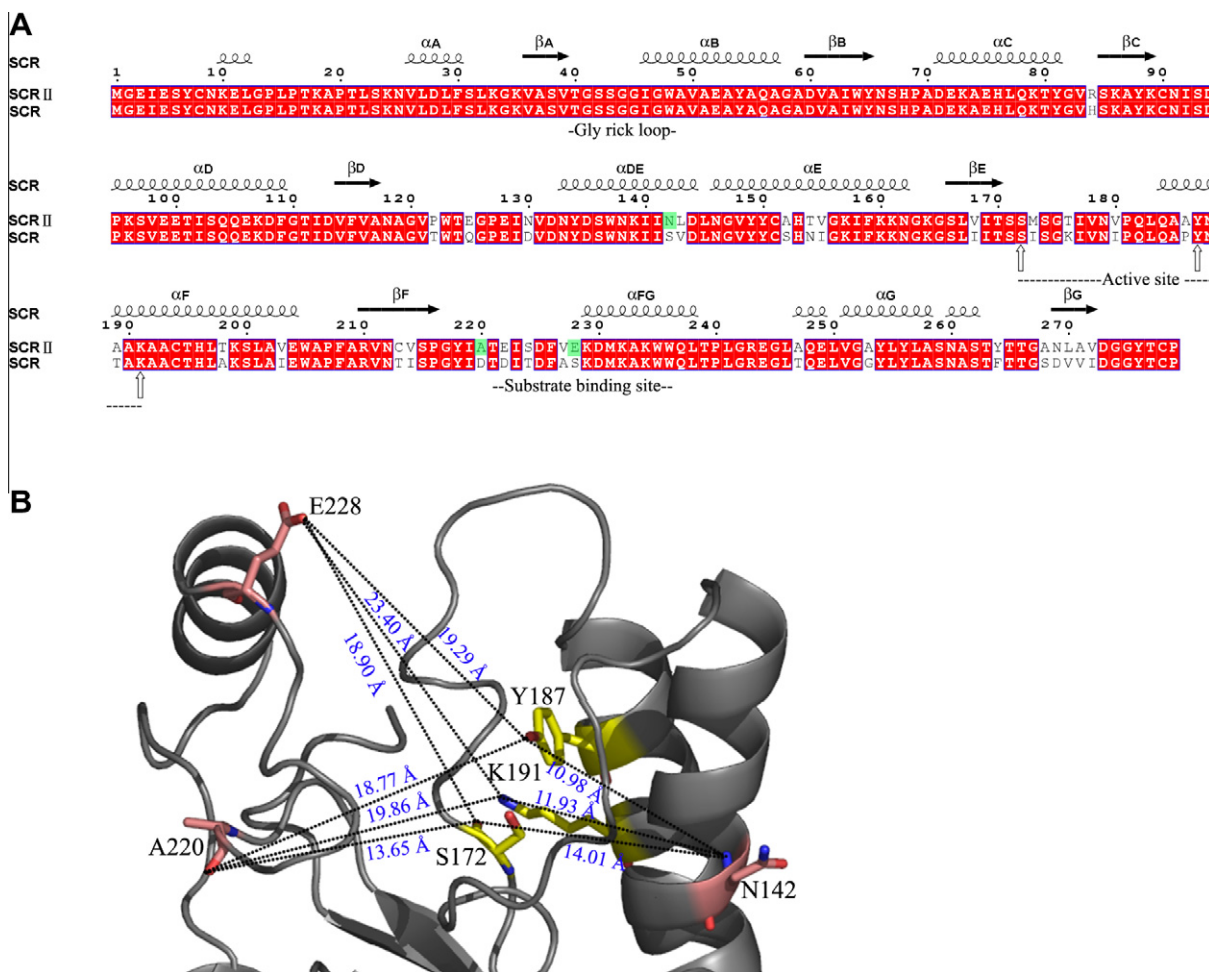


Fig. 2. Mutation analysis in SCR II. (A) Structural alignment of the SCR II with SCR (PDB 3CTM). Identical residues are highlighted in red, and conserved residues are boxed with blue lines. The glycine-rich consensus sequence, the active site and the substrate-binding site are indicated. The three residues adjacent to or inside the active site and the substrate-binding site for mutation in SCR II are marked with solid triangles. This figure was prepared with the program Esprpt (<http://esprpt.ibcp.fr/ESPrpt/cgi-bin/ESPrpt.cgi/>). (B) A closer look at the location of the N142, A220 and E228. The distances from the guanidyl nitrogen of N142, A220 and E228 to the catalytic residues (S172, Y187 and K191) are shown. The molecular graphics image was drawn using PyMOL software (Brunger et al., 1998).

tween them are less than 20 Å (except that the distance between Glu228 and Lys191 is 23.40 Å) (Fig. 2B). These results further suggested that Asn142, Ala220 and Glu228 are possibly relative to enzyme function.

3.5. The A220 and E228 variants tend to lose enantioselectivity

Several mutations of Asn142, Ala220 and Glu228 were introduced in SCR II. SDS-PAGE analysis showed that all mutant proteins were overproduced at levels equal to that of WT. The N142S and N142G present similar specific activities and biotransformation efficiency with WT SCR II. The A220 and E228 variants resulted in almost 20-fold decrease in the specific activity, and significant decrease of enantioselectivity toward the (S)-configuration with poor yields (<5%) (Table 2), which suggested that most of the substrate was not converted into (S)-PED (Fig. 3).

The bioreduction was further performed using equivalent purified mutant enzymes. The results showed PED was not detected in the NADH-linked reaction catalyzed using the purified protein (data not shown). Although all the mutant proteins produced (S)-PED with higher optical purity and yield during the NADPH-mediated reaction than their corresponding transformants, they exhibited similar trends in the transformation efficiency. Based on these above results, it was strongly suggested the mutants A220D and

E228S almost lost the enantioselectivity efficiency, indicating the possible importance of A220 and E228 in the reduction of ketone. The altered activity and biotransformation efficiency toward ketone reduction were also observed by Cys295Ala substitution in other secondary alcohol dehydrogenase (SADH) (Heiss et al., 2001); (Leblanc et al., 1998).

3.6. Dramatic decrease in catalytic efficiency (k_{cat}/K_m) by A220 or E228 mutations

For further examination of the mutations on alteration of catalytic properties, the kinetic effect of the mutations was assessed between WT SCR II and its variants. Compared with WT SCR II, the N142 mutant maintained K_m and k_{cat} (Table 3) essentially at the similar levels, suggesting the N142S and N142G mutations did not attenuate the catalytic function or are not detrimental to substrate-binding and/or catalysis. The A220 or E228 variants resulted in approximate twofold increase in the K_m values and 7.8–12.3 folds decrease in the k_{cat} values with respect to WT SCR II in the NADPH-mediated reduction. The k_{cat}/K_m values of A220 or E228 variants were less than 7% of WT form, indicating the catalytic efficiency decreases dramatically by the mutagenesis. Although the kinetic parameters in the NADH-linked reaction did not change significantly comparable to the NADPH-linked one, the similar

Table 2

The bioreduction of 2-hydroxyacetophenone by the SCR11 and its variants.

Types	Specific activity (U/mg)	Transformants ^a		Enzyme and NADPH ^b	
		Optical purity (%)	Yield (%)	Optical purity (%)	Yield (%)
SCR	2.58 ± 0.03	96.1 ± 0.11	83.6 ± 0.07	97.3 ± 0.14	85.4 ± 0.05
SCR11	6.12 ± 0.09	100 ± 0.16	96.5 ± 0.16	100 ± 0.12	98.1 ± 0.09
N142S	5.50 ± 0.02	100 ± 0.08	95.8 ± 0.09	100 ± 0.17	97.7 ± 0.13
N142G	5.56 ± 0.05	100 ± 0.07	96.3 ± 0.09	100 ± 0.14	97.8 ± 0.13
A220D	0.32 ± 0.01	98.2 ± 0.20	3.4 ± 0.02	99.1 ± 0.12	5.9 ± 0.12
A220R	0.28 ± 0.01	100 ± 0.12	1.3 ± 0.02	100 ± 0.17	5.4 ± 0.15
A220G	0.37 ± 0.02	98.5 ± 0.15	3.9 ± 0.04	100 ± 0.13	7.6 ± 0.07
E228S	0.34 ± 0.01	91.4 ± 0.09	1.9 ± 0.02	95.3 ± 0.12	3.5 ± 0.04
E228R	0.35 ± 0.02	100 ± 0.13	3.7 ± 0.04	100 ± 0.19	5.6 ± 0.02
E228A	0.25 ± 0.02	100 ± 0.16	0.8 ± 0.01	100 ± 0.23	2.7 ± 0.01

^a Recombinant *E. coli* BL21 (DE3) was cultured until the turbidity of culture at 600 nm reached 0.6–0.8, and cells were cultured for another 8 h after the addition of 1 mM IPTG at 30 °C.

^b The purified enzyme was used as catalyst by the addition of 2 mM NADPH.

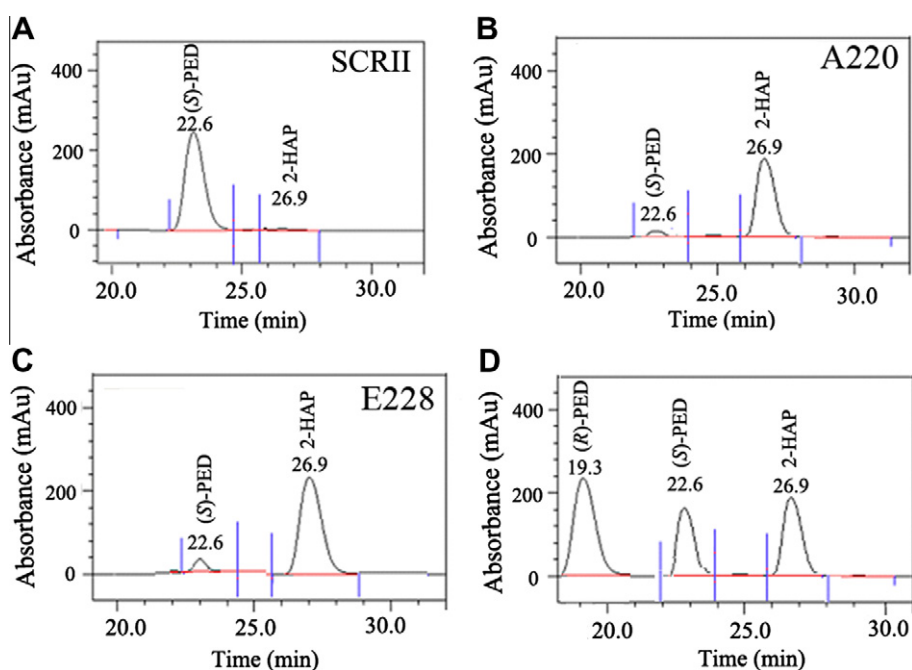


Fig. 3. Asymmetric reduction of 2-hydroxyacetophenone using purified proteins in the presence of NADPH. Reaction products catalyzed by the SCR11 (A), the mutants of A220 (B), and the mutants of E228 (C). (D) Retention times of standard samples are as follows: (R)-PED, 19.3 min; (S)-PED, 22.6 min; 2-hydroxyacetophenone, 26.9 min. AU, arbitrary unit.

Table 3Kinetic parameters for 2-hydroxyacetophenone reduction by the WT and mutant enzymes.^a

Proteins	Value for the parameter with NADH as cofactor				Value for the parameter with NADPH as cofactor			
	K_m (μ M)	k_{cat} (S^{-1})	k_{cat}/K_m ($\times 10^6 s^{-1} M^{-1}$)	Relative k_{cat}/K_m ^b	K_m (μ M)	k_{cat} (S^{-1})	k_{cat}/K_m ($\times 10^6 s^{-1} M^{-1}$)	Relative k_{cat}/K_m ^b
SCR	7.89 ± 0.19	1.32 ± 0.05	0.17	–	0.59 ± 0.01	1.48 ± 0.06	2.51	–
SCR11	13.08 ± 0.34	1.52 ± 0.05	0.12	100	4.52 ± 0.12	15.54 ± 0.23	3.44	100
N142S	13.75 ± 0.27	1.39 ± 0.13	0.10	87.0	4.67 ± 0.19	14.76 ± 0.11	3.16	91.9
N142G	13.56 ± 0.12	1.31 ± 0.07	0.10	83.1	4.55 ± 0.24	14.81 ± 0.12	3.25	94.7
A220D	17.41 ± 0.15	0.68 ± 0.03	0.04	33.6	9.69 ± 0.11	1.26 ± 0.07	0.13	3.8
A220R	17.33 ± 0.09	0.72 ± 0.14	0.04	35.8	9.54 ± 0.04	1.32 ± 0.06	0.14	4.0
A220G	17.56 ± 0.21	0.63 ± 0.08	0.04	30.9	9.87 ± 0.13	1.20 ± 0.12	0.12	3.5
E228S	16.84 ± 0.09	0.84 ± 0.03	0.05	42.9	9.03 ± 0.07	1.99 ± 0.05	0.22	6.4
E228R	16.52 ± 0.03	0.91 ± 0.02	0.06	47.4	8.89 ± 0.09	2.12 ± 0.14	0.24	6.9
E228A	17.08 ± 0.16	0.72 ± 0.07	0.04	36.3	9.37 ± 0.21	1.80 ± 0.05	0.19	5.6

All experiments were repeated three times.

^a All reactions involved in the calculation of kinetic constants were assayed at 100 mM acetate buffer (pH 6.0) and 35 °C.

^b Relative values were compared to the WT value for the same cofactor.

trends were observed. Upon mutations of A220 and E228, the K_m values showed approximate 1.3-fold increase and the k_{cat} values

exhibited about twofold decrease, resulting in decrease of 2–4 times in their k_{cat}/K_m ratios than that of WT form in the NADH-

linked reaction. Based on these results, it was suggested that the substituted Asp220 or Ser228 is deleterious to the substrate binding, which reflects the loss of enantioselectivity efficiency, as with other carbonyl reductases (Dey et al., 2007; Heiss et al., 2001; Igarashia et al., 2004).

4. Conclusion

This work reports an excellent (*S*)-specific carbonyl reductases (SCR11) of anti-Prelog type from *C. parapsilosis*, which catalyzes the reduction of 2-hydroxyacetophenone to (*S*)-PED with absolute stereochemical selectivity for valuable industrial applications. Our mutational analysis of this stereospecific carbonyl reductase basing on the sequence-structural alignment confirms the important roles of A220 and D228 in substrate-binding and/or catalysis. The missense mutations associated with the deficient stereoselectivity also provide a framework for our further research aimed at structure–function correlations in SDR family.

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