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ARTICLE

A carbonyl reductase from *Candida parapsilosis* ATCC 7330: Substrate selectivity and enantiospecificity

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Candida parapsilosis ATCC 7330, a rich source of highly stereospecific oxidoreductases, catalyzes oxidation-reduction of a plethora of compounds yielding industrially important intermediates. An (*S*)-specific carbonyl reductase (*S*RED) purified and characterized from this yeast is reported here. (*R*)-specific carbonyl reductase (*Cp*CR) was reported by us earlier. *S*RED asymmetrically reduces ketones with excellent enantiospecificity (ee > 99%) and α-ketoesters with higher catalytic activity but moderate enantiospecificity (ee 70%) in the presence of NADPH. Minimal activity is shown towards reduction of aldehydes. While the reduction of α-ketoesters with *S*RED can occur with either NADPH or NADH, for ketone reduction *S*RED requires NADPH specifically. *S*RED with subunit molecular weight of 30 kDa shows optimal activity at pH 5.0 and 25 ^{o}C, and its activity is affected by Cu^{2+}. Taken together, *S*RED and *Cp*CR offer substrates which on asymmetric reduction give products of opposite absolute configurations.

Introduction

Candida parapsilosis ATCC 7330 (Cp) is an efficient biocatalyst to prepare industrially important chiral building blocks as shown by us.[1] The reactions involved are mainly oxidation and reduction which occur by deracemization, asymmetric reduction and enantioselective oxidation. Deracemization of racemic ethyl mandalate[2] and ethyl-3-hydroxy-3-phenylpropionate[3] in whole cells follows a stereoinversion mechanism with the aid of stereospecific oxidoreductases. We have also shown that asymmetric reduction can result in either (*R*)- or (*S*)-alcohols depending on a variety of factors.[4] In order to understand the basis for their stereospecificities and their substrate selectivity, the next logical progression was to purify these carbonyl reductases and reconstitute deracemization reactions *in vitro.* The present study reports the isolation and purification of an (*S*)-specific carbonyl reductase (*S*RED) which is quite different from its (*R*)-specific counterpart, *Cp*CR which was isolated from this yeast, crystallized (1.86 Å resolution) and characterized.[5] The (*R*)-specific, Zn-dependent enzyme reduces aldehydes and α-ketoesters with high catalytic activity but shows minimal activity towards ketones.[6]

Y. Nie *et al*., reported purification of multiple (*S*)-specific carbonyl reductases from *Candida parapsilosis* such as SCR, SCR1, SCR2, SCR3, SCRII and an (*R*)-specific carbonyl reductase (RCR).[7-10] Many carbonyl reductases from *Candida* sp. have been purified and characterized. The detailed study of the enzyme with its biochemical characterization and substrate scope builds its way to a valuable biocatalyst in synthesizing important chiral molecules. For example, carbonyl reductase S1 purified from *Candida magnoliae* AKU4643 was co-expressed with glucose dehydrogenase (GDH) in *E. coli* HB101 produced 208 g L^{-1} of optically pure (*S*)-4-chloro-3-hydroxybutanoate (*S*-CHBE), a precursor for HMG-CoA reductase inhibitors.[11,12] (*R*)-carbonyl reductase (RCR) and (*S*)-carbonyl reductase (SCR) isolated and characterized from *Candida parapsilosis* CCTCC M203011 were used in an efficient one-step production of (*S*)-1-phenyl-1,2-ethanediol (*S*-PED) from (*R*)-1-phenyl-1,2-ethanediol (*R*-PED).[7,10,13-15] A bacterial β-ketoacyl-ACP reductase (FabG) from *Bacillus* sp. ECU0013 co-expressed with GDH in *E. coli* reduced 620 g L^{-1} of ethyl-2-oxo-4-phenylbutanoate (EOPB) to (*S*)-ethyl-2-hydroxy-4-phenylbutanoate (*S*-EHPB).[16-18] Optically pure PED and EHPB are used in the synthesis of Propiconazole (fungicide) and Benazepril (anti-hypertensive) respectively. Also an (*R*)-specific carbonyl reductase (CprCR) from *Candida parapsilosis* co-expressed with GDH in *E. coli* Rosetta (DE3) cells asymmetrically reduced 100 g L^{-1} of *N*-Boc-3-piperidone to (*S*)-*N*-Boc-3-hydroxypiperidine, a chiral intermediate for the synthesis of Ibrutinib used for the treatment of lymphoma.[19] Such applications of biocatalysts in industries make the process greener.

With the intention of understanding the mechanism of the enantiospecific preference of these enzymes, the (*S*)-specific carbonyl reductase was purified and characterized. This enzyme had opposite enantio-preference compared to *Cp*CR and reduced ketones unlike *Cp*CR[6] and both the enzymes show activity towards α-ketoesters.

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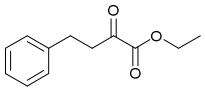
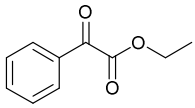
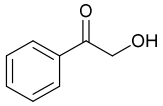
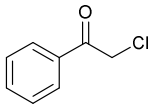
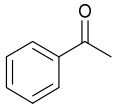
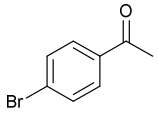
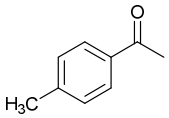
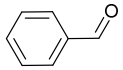
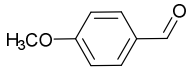
Results and discussion

Substrate selectivity and enantiospecificity

The activity of purified *S*RED, a member of short-chain dehydrogenase/reductase (SDR) family (see protein overexpression and purification section) as determined for α-ketoesters, ethyl-2-oxo-4-phenylbutanoate (EOPB) and ethyl-2-oxo-2-phenylacetate (EOPA), ketones such as 2-hydroxyacetophenone (2HAP), 2-chloroacetophenone (2ClACP), acetophenone (ACP), *p*-bromoacetophenone, *p*-methylacetophenone and aldehydes, benzaldehyde and anisaldehyde is presented in Table 1. Aldehydes are poor substrates as compared to ketones as is the case with most of the reported SDRs.[7,8] On the contrary, *Cp*CR which is a medium-chain dehydrogenase/reductase (MDR) displayed higher activity with aldehydes than ketones.[6] For ketones, *S*RED showed highest activity with 2HAP followed by 2ClACP. There was 6-fold decrease in the activity with ACP as compared to 2HAP. It is known that the substrate binding site in alcohol dehydrogenase consists of two hydrophobic substrate binding pockets which differ from one another in volume and affinity towards the R groups of the substrate.[20] In substrates such as 2HAP and 2ClACP, the presence of an electronegative atom like oxygen and chlorine respectively at C2 position showed much better activity than ACP with methyl group at C2 position. There could be a possibility for an electrostatic interaction in the small group binding pocket that facilitates better binding and hence better activity of 2HAP and 2ClACP. *S*RED showed no activity with *ortho*- and minimal activity with *meta*- substituents of ACP (results not shown). This could be due to the steric effect of the substitution. But *para*-bromoacetophenone showed ~ 3 fold increase in activity and *para*-methylacetophenone showed 0.6 fold decrease in activity with respect to ACP. The electron withdrawing and electron donating nature of bromo- and methyl- respectively at *para* position of ACP can explain this. For aldehyde and ketone substrates, identical assay conditions were followed. However, for α-ketoesters, EOPB and EOPA, *S*RED had high catalytic activity and the assay conditions were modified *i.e.* decrease in concentrations of substrate, NADPH and the amount of enzyme to increase the reaction time in order to monitor it experimentally. Between EOPB and EOPA, EOPA showed greater activity implying less the number of carbons between the phenyl and carbonyl group, better was the activity which was in contrast to *Cp*CR (MDR)[6] and an aldo-keto reductase (AKR) from *Neurospora crassa* (NcCR).[21] In summary, for *S*RED, the relative activities are as follows: α-ketoesters > ketones > aldehydes.

In case of SDRs such as NADPH-dependent carbonyl reductases from *Candida parapsilosis* CCTCC M203011 (SCRs),[8] *Candida magnoliae* AKU 4643 (S1 & S4)[11,22] and NADH-dependent carbonyl reductase from *Kluyveromyces aestuarii* (KaCR1),[23] higher activity was shown towards ketoester substrates and very minimal activity was shown towards aryl substituted benzaldehyde and ACP. The trend of higher activity for ketoesters followed by 2ClACP and aryl substituted ACP was also followed by carbonyl reductase from *Candida krusei* SW 2026[24] and *Sporobolomyces salmonicolor* (SSCR).[25] Interestingly, SSCR showed higher activity with *o*- and *m*- than *p*- substituted ACP.

Table 1 Specific activity of *S*RED determined for carbonyl substrates

S No	Substrate	Substrate (mM)	NADPH (mM)	Specific activity (Umg^{-1})
	α-Ketoesters			
1	Ethyl-2-oxo-4-phenylbutanoate 	1	0.05	1.17 ± 0.19
2	Ethyl-2-oxo-2-phenylacetate 	1	0.05	2.31 ± 0.31
	Ketones			
3	2-Hydroxyacetophenone 	4	0.20	3.68 ± 0.32
4	2-Chloroacetophenone 	4	0.20	3.15 ± 0.10
5	Acetophenone 	4	0.20	0.59 ± 0.10
6	*p*-bromoacetophenone 	4	0.20	1.66 ± 0.26
7	*p*-methylacetophenone 	4	0.20	0.34 ± 0.05
	Aldehydes			
8	Benzaldehyde 	4	0.20	0.57 ± 0.07
9	Anisaldehyde 	4	0.20	0.47 ± 0.13

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Comparing the two carbonyl reductases *S*RED and *Cp*CR from the same yeast Cp, the substrate specificity trend was very different. *S*RED preferred EOPA, EOPB (ketoesters), then 2HAP, 2ClACP, *p*-bromoactophenone, ACP, *p*-methylacetophenone (ketones) and benzaldehyde, anisaldehyde (aldehydes). But highest activity of *Cp*CR was observed with aldehydes followed by α-ketoesters.[6] Minimal activity of *Cp*CR was found with ketones unlike *S*RED. These reductases belong to different families. Taken together, these have a reasonable substrate bank which on asymmetric reduction gives products of opposite absolute configurations.

The whole cells of Cp are established excellent catalysts for carrying out wide range of chemical transformations.[1] The 14 h culture of Cp asymmetrically reduced phenyl glyoxal (PG) to (*S*)-PED completely in 3 h at 25 ^{o}C with 99% ee and 2HAP is the intermediate. The whole cells reduced the aldehyde function first to the intermediate in 10 min and then the keto group was reduced in 170 min.[26] When the keto intermediate, 2HAP was given as a substrate to the isolated *S*RED, incomplete conversion was observed in 3 h with > 99% ee of the formed (*S*)-alcohol. Thus to ensure complete conversion the reaction time was extended. In addition to this, the reaction conditions were varied such as pH (5, 7.5) and co-solvents (DMSO, IPA). Complete conversion of 2HAP to (*S*)-PED was observed in 10 h with > 99% ee in 0.1 M HEPES buffer, pH 7.5 and co-solvent IPA. This is because at pH 7.5, cofactor regeneration occurs by oxidation of IPA (co-substrate) to acetone which is not possible at pH 5 and with DMSO as co-solvent. The extended reaction time could be due to the shortage of the cofactor unlike the case with the whole cells. Thus, the purified *S*RED reinforced the mechanism of asymmetric reduction of PG to (*S*)-PED in the host Cp. Formation of (*S*)-PED has also been reported by enzymes SCR and SCRII (> 99% ee), SCR 1 – 3 (ee not mentioned).[7-9] The same reaction conditions were followed and 2ClACP was reduced by *S*RED to its (*S*)-alcohol with ee > 99%.

EOPB and EOPA (α-ketoesters) were asymmetrically reduced 100% and 40% by *S*RED using the optimized reaction conditions respectively in 6h with 70% ee for both. The moderate enantiospecificity could be due to the presence of an ethyl ester group (hydrophobic) in EOPB against oxygen of hydroxyl (polar) in 2HAP as the small group. In EOPB, a competition is involved between phenyl and ester moiety, both being hydrophobic, in binding in the substrate binding pockets leading to lower enantiospecificity, as explained by T. Ema *et al*.[27] However, 40 h culture of Cp asymmetrically reduced EOPB to (*S*)-EHPB in 4 h at 25 ^{o}C with 99% ee.[28] Deracemization of (*RS*)-EHPB to (*S*)-EHPB and (*RS*)-EHPA to (*S*)-EHPA was carried out by 24 h culture of Cp in 1 h at 25 ^{o}C.[29] Evidently, in the whole cells, different reductases are induced at different time intervals that catalyze these transformations. On the other hand, purified *Cp*CR reduced EOPB to (*R*)-EHPB with > 99% ee.

In essence, *S*RED showed higher catalytic activity and moderate specificity with α-ketoesters as compared to moderate catalytic activity and excellent specificity with ketones.

Cofactor selectivity

*S*RED reduced 2HAP only in presence of NADPH. Carbonyl reductases SCR and SCR1-3 from *Candida parapsilosis* also preferred NADPH for the asymmetric reduction of prochiral carbonyl compounds including 2HAP and EOPB. The authors related the presence of the positively charged residue His (His70 in SCR1, His68 in SCR2, SCR3, SCR) to play a role in the affinity of the enzyme for NADPH, rather than NADH, by forming a salt bridge with the phosphate moiety at the 2'-position of AMP.[7,8] His 68 is present in *S*RED but showed activity in presence of both NADPH and NADH with respect to the substrate, EOPB. This difference in selectivity of cofactor depending on the type of substrate may indicate that not only the presence of specific amino acid in the enzyme that interacts with the cofactor is important but the nature of substrate and its interaction with the cofactor also influences cofactor selectivity. Similar observation was made with *Cp*CR from Cp which preferred NADPH for the reduction of aldehydes but showed dual cofactor specificity for reduction of α-ketoesters.[6]

Protein overexpression and purification

*S*RED was overexpressed in *E. coli* BL21 (DE3) cells using genomic DNA of Cp as the template. The translated protein sequence was identical to that of the carbonyl reductase (SCR) from *Candida parapsilosis* CCTCC M203011 which belongs to the SDR. This implied *S*RED to be an SDR with identical conserved motifs.[7] *S*RED was purified by affinity chromatography with N-terminal GST tag (cleaved using PreScission protease). The purified fraction (25-fold purity) contained *S*RED in ~20% yield (2 – 4 mg from 1 L culture). The subunit molecular weight as observed by 12% SDS PAGE was ~ 30 kDa (Fig. 1A), in agreement with its theoretical value (279 amino acid residues) similar to the carbonyl reductases (SDRs) from *Candida parapsilosis* CCTCC M203011 (SCR),[7] *Candida magnoliae* AKU 4643 (S1 & S4),[11,22] *Kluyveromyces aestuarii* (KaCR1),[23] *Pichia stipitis* (PsCR I & PsCR II).[30,31] *S*RED is smaller than *Cp*CR which is 40 kDa and a dimer.[5] Most purified SDRs are reported in either dimeric or tetrameric forms.[8,11,22] *S*RED had an apparent molecular weight of ~ 58 kDa when eluted through gel filtration column indicating that *S*RED could be a homodimer. However, *S*RED on native PAGE showed a major band comparable to that of a BSA dimer (132 kDa) indicating that it could be a tetramer (Fig. 1B). In 2007, Y. Nie *et al*., reported that purified CPADH (renamed as SCR later) existed as a monomer.[7] But in 2008, R. Zhang *et al*., described SCR as a homotetramer in its crystal structure (PDB 3CTM) and in solution. However the functional unit was shown to be a dimer (A-C type). The need for dimer-dimer interface, formed due to overlapping of N-terminal peptide, the helix αG and the strand βG with no hydrogen bond formation, is unclear.[14] Thus, the tetramer formation could be concentration dependent with no obvious role in protein function. That *S*RED is a dimer as per gel filtration could be due to the dilution of protein in the column which is not the case with native PAGE. (*S*)-1-

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Phenylethanol dehydrogenase from denitrifying bacterium strain EbN1 showed a similar discrepancy in the apparent molecular weight which was 59 kDa by gel filtration and 93 kDa by native PAGE. This behavior was attributed to the interaction of the enzyme with the column material which led to its retardation.[32]

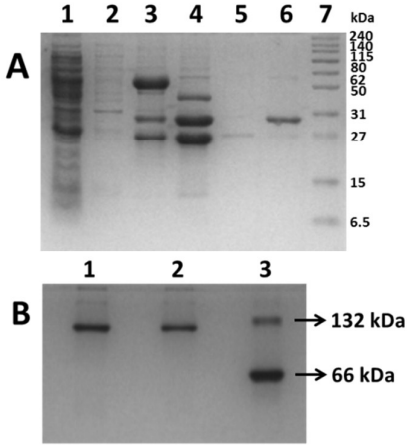
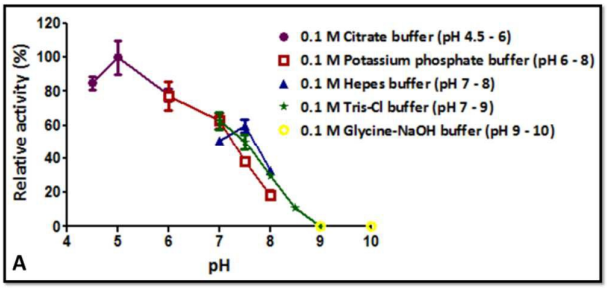


Fig. 1 Analysis by electrophoresis. (A) SDS-PAGE analysis. Lane 1, cell-free extract; Lane 2, flow through; Lane 3, eluate containing *S*RED with GST tag (56 kDa); Lane 4, *S*RED and GST after cleavage; Lane 5, GST (26 kDa); Lane 6, purified *S*RED (30 kDa); Lane 7, protein ladder. (B) Native PAGE analysis. Lane 1, 10 µg of SRED; Lane 2, 5 µg of SRED; Lane 3, 20 µg of BSA.

pH and temperature for optimal enzyme activity

2HAP was used as a standard substrate for measuring the activity of *S*RED unless mentioned otherwise. For the optimal activity of the purified *S*RED, the effect of pH and temperature on its activity for the reduction of 2HAP was monitored in the range of pH 4.5 - 10 and 20 – 50 ^{o}C respectively. 0.1 M of different buffers were used with overlapping buffer range for determining the activity. Maximal activity was observed at pH 5.5 (Fig. 2A). The activity decreased by 50% with pH 7 – 7.5 and no activity was observed beyond pH 9. Given the reported mechanism of these carbonyl reductases, it is reasonable to expect the activity in the acidic range. In acidic pH, Tyr is protonated allowing the proton abstraction by oxygen of the carbonyl group from the substrate. Other reported SDRs S1,[11] S4,[22] KaCR1[23] have their optimal pH between 5.5 – 6.5. The activity of *S*RED was optimum between 25 – 30 ^{o}C (Fig. 2B). However, reductases S1, S4, KaCR1 have reported their optimal temperature in the higher range between 45 – 55 ^{o}C.[11,22,23] Beyond 50 ^{o}C, *S*RED was found to be inactive but was stable at the ambient temperatures.



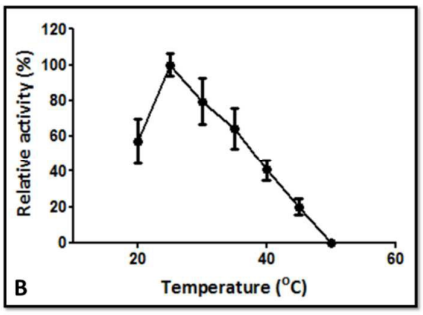


Fig. 2 Effect of pH and temperature on *S*RED. (A) Activity-pH profile. (B) Activity-temperature profile. Relative activity was expressed as a percentage of the maximum activity under experimental conditions.

Effect of various metal ions on the enzyme activity

It is well known that zinc forms an integral part of structure and function of alcohol dehydrogenases. Zinc coordinates with the oxygen of the substrate, acts as a Lewis acid facilitating the proton relay *via* side chain of Ser and His (acts as a base).[37] We have noticed decrease in activity of Zn-dependent *Cp*CR by addition of chelators which was reversed by addition of zinc.[6] Consequently the effect of metal ions on *S*RED activity for reduction of 2HAP was investigated. Divalent cations such as Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} did not have any effect on the *S*RED activity. However, Cu^{2+} and Fe^{3+} reduced the activity of *S*RED by around 25% and 40% respectively (Table 2). Also, the metal chelator, EDTA (1 mM) reduced the *S*RED activity by 15% (result not shown).

Furthermore, the effect of metal ions on the secondary structure of *S*RED was studied by circular dichroism (CD) to correlate with its activity. Examining the molar ellipticities (θ) at 217 nm and 222 nm of *S*RED in presence of these metal salts (Fig. 3) revealed information about the effect of metal ions on the secondary structural components such as α-helix (at 222 nm) and β-sheet (at 217 nm). After addition of the cofactor NADPH, the negative value of θ increased at both 217 and 222 nm indicating that there was increase in orderliness in β-sheet and α-helix respectively. This was as expected.[11] Similarly, most of the metal salts showed increase in the negative value of θ with respect to the control (only *S*RED) except in case of $CuSO_4$ as reported for SCR enzyme.[7]

Reduction of 2HAP was also studied in presence of 1 mM of Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and EDTA with the optimized reaction conditions. Fe^{2+}, Fe^{3+}, Zn^{2+} and EDTA had no effect on the reduction of 2HAP (> 99% conversion) when compared with the control (without metal salt). However, in presence of Cu^{2+} only 27% conversion was observed. Cu^{2+} is one of the thiol specific metal ion, Ag^{+} and Hg^{+} being the others.[24] It is interesting to know that OR2T11, a human olfactory thiol receptor had a strong copper effect for the compound *t*-butyl mercaptan (TBM) and copper was found to coordinate with sulfur atoms of TBM, Met, Cys and nitrogen atom of His residues.[38] Since *S*RED has 2 Met and 5 Cys residues in its primary sequence, there is a possibility of copper coordinating with the sulfur atoms and altering the structure (Fig. 3)

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thereby reducing the activity of this enzyme. Similar inhibitory effect of copper was found in carbonyl reductases from *Candida parapsilosis* CCTCC M203011,[7] *Kluyveromyces aestuarii*,[23] *Candida krusei* SW 2026,[24] *Kluyveromyces lactis* NRIC 1329,[39] *Candida viswanathii* MTCC 5158.[40]
Presence of EDTA made no difference to *S*RED activity indicating the enzyme could be metal independent similar to reductases from *Candida parapsilosis* CCTCC M203011[7] and *Candida viswanathii* MTCC 5158.[40]

Table 2 Effect of metal salts on *S*RED activity

Entry	Metal salt	Relative specific activity(%)
1	Control	100.0 ± 3.7
2	$CaCl_2$	89.8 ± 1.2
3	$CoCl_2$	91.4 ± 8.0
4	$CuSO_4$	74.8 ± 3.1
5	$FeCl_3$	61.1 ± 7.6
6	$FeSO_4$	ND[a]
7	$MgSO_4$	98.6 ± 5.3
8	$MnSO_4$	96.1 ± 2.7
10	$NiCl_2$	90.0 ± 1.9
11	$ZnSO_4$	99.2 ± 5.5

[a]ND: not detectable

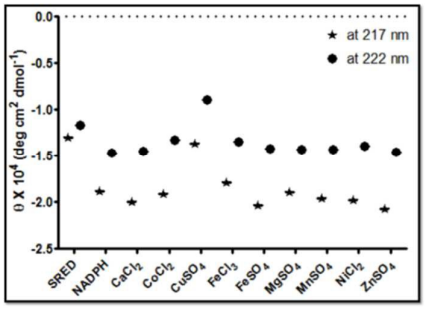

Fig. 3 The plot of molar ellipticities at 217 nm and 222 nm against *S*RED with different metal salts.

Experimental

Materials

All the carbonyl substrate molecules were procured and some alcohol products were synthesized by the reported methodology (as given in the supplementary data file). Coenzymes (NADH, NADPH) were purchased from SRL (India). GSTrap FF column was purchased from GE Healthcare Life Sciences (India). BLUltra pre-stained protein ladder for SDS-gel electrophoresis was purchased from GeneDireX, Inc. (India). All other reagents were of analytical grade.

Protein expression and purification

The genomic DNA of *Candida parapsilosis* ATCC 7330 was used as a template for amplifying *S*RED gene using the primers F- 5'-ATCGGATCCGATGGGCGAAATCGAATCTTATTG-3' (BamHI) and R-5'-TGACTCTCGAGTGGACACGTGTATCCACCGTC-3'(XhoI).[7] The amplified product was digested with BamHI and XhoI, and was ligated in to the expression vector, pGEX-6-P-1 by directional cloning. The clone was verified by DNA sequencing from Shrimpex (India) and was transformed in *E. coli* BL21 (DE3). The successfully transformed BL21 cells containing *S*RED gene were grown in Luria Bertani media until the OD_{600} reached 0.6 – 0.8 at 37 °C. Upon standardization of protein over-expression using different induction parameters such as IPTG (isopropyl β-D-1-thiogalactopyranoside) concentration, incubation temperature and time, the soluble fraction of protein gave better yield at 0.3 mM IPTG at 23 °C incubated for 18 h. Protein purification was performed using a fast protein liquid chromatography (FPLC) system (ÄKTA purifier, GE Biosciences). All the purification steps were carried out at 4 °C using phosphate buffer saline with 10% glycerol. The cleared lysate after ultra-sonication and centrifugation was loaded onto a GSTrap FF column. The recombinant protein was eluted with 50 mM Tris (pH 8.0) containing 25 mM glutathione. *S*RED with GST tag at N-terminal was digested with PreScission protease for 12–16 h at 4 °C. The PreScission protease digested samples were loaded again onto GSTrap FF column and the flow-through was collected that contained 30 kDa *S*RED. This protein solution was concentrated and quantified (Bradford method).[41] The purified protein samples were analyzed by SDS–PAGE followed by Coomassie Brilliant Blue R250 staining.

Determination of enzyme activity

The reductase activity of the enzyme was determined by monitoring the decrease in the absorbance of NADPH at 340 nm spectrophotometrically at 25 °C.[6] The standard assay mixture consisted of 0.1 M citrate buffer (pH 5), appropriate amount of the protein, 4 mM of the aldehyde/ketone substrate and 0.2 mM of NADPH. For, α-ketoester substrates, the assay mixture constituted 0.1 M citrate buffer (pH 5), appropriate amount of the protein, 1 mM of the substrate and 0.05 mM of NADPH. One unit of the enzyme activity was defined as the amount of enzyme that oxidizes 1 µmol of NADPH per minute at the specified assay condition.

Effect of pH and temperature on the enzyme activity

The enzyme activity was calculated by performing the standard assay (as mentioned in the previous section) with 2HAP as the substrate. To determine the pH for the optimum activity the assay was performed in 0.1 M buffer, pH ranging from 4.5 to 10, using citrate for pH 4.5 – 6, phosphate for pH 6 – 8, HEPES for pH 7 – 8, Tris-Cl for pH 7 – 9 and Glycine - NaOH for pH 9 – 10. The optimal enzyme activity was also determined by varying temperature from 20 – 50 °C.

Effect of various metal ions on the enzyme activity

The enzyme activity in presence of 1 mM of various metal salts was estimated by the standard assay (as mentioned in determination of enzyme activity section) with 2HAP as the substrate and 0.18 mM of *S*RED. The metal salts that were

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used for the assay were $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $NiCl_2$, $ZnSO_4$. The enzyme activity was also determined in presence of chelating agent EDTA (1 mM). The effect of metal salts and EDTA on the secondary structure of *S*RED, indirectly relating to its activity, was examined by Jasco J-815 CD spectrometer. The experiment was performed with 2.6 mM of *S*RED in 20 mM of citrate buffer (pH 5) with 0.5 mM of NADPH. Different metal salts of final concentration 15 mM were added separately to the solution and the measurement was taken from 200 – 250 nm.

Stereospecificity and cofactor selectivity

The enantiospecificity and cofactor selectivity of the enzyme was determined by the reduction of the carbonyl substrates in presence of NAD(P)H. The reaction mixture (1 ml) consisted of 0.1 M HEPES buffer (pH 7.5), 5 mM of the carbonyl substrate in isopropanol (10% v/v), 0.3 U of the purified *S*RED and 2 mM of NAD(P)H. The reaction mixture was incubated at 25 ^{o}C for 10 h with shaking followed by extraction with ethyl acetate (2 times) and analysis.

Biotransformation and substrate selectivity

Bioreduction of different carbonyl substrates were carried out in 1 ml reaction mixture with 0.1 M HEPES buffer (pH 7.5), 5 mM of the carbonyl substrate in 10% v/v isopropanol (IPA), 0.3 U of the purified *S*RED and 2 mM of NADPH incubated at 25 ^{o}C with shaking. After the reaction time, the unreacted substrate and the formed product were extracted using ethyl acetate (2 times). The extract was dried over anhydrous sodium sulphate, concentrated using rotavapor and analyzed.

Conversion was determined by GC-MS (Shimadzu GC-2010 Plus) using Supelco Omegawax^{TM} 320 capillary column (30 m X 0.32 mm X 0.25 µm film thickness) with injector / ion source temperature of 250 ^{o}C / 250 ^{o}C and temperature profile of 50 ^{o}C for 2 min, increased to 210 ^{o}C at the rate of 4 ^{o}C min^{-1} and then held for 6 min. The retention time of ketones 2HAP, 2ClACP and the corresponding alcohols were 30.9 min, 30.4 min and 40.2 min, 33.0 min respectively. α-ketoesters EOPB, EOPA and the corresponding α-hydroxyesters had retention times of 35.0 min, 30.0 min and 36.8 min, 32.6 min respectively. All the carbonyl substrates and the alcohol products were characterized by MS (refer supplementary data file).

The enantiomeric excess of the alcohols PED (*S*- 9.5 min; *R*- 7.4 min) and 2-chloro-1-phenylethanol (*S*- 9.7 min; *R*- 7.5 min) were determined by HPLC (Jasco PU-1580 with PDA detector) using Chiralcel OB-H column (Daicel, 0.46 cm X 25 cm), mobile phase of hexane:isopropanol = 90:10 at 1 ml min^{-1}. For α-hydroxyesters ethyl-2-hydroxy-4-phenylbutanoate (*S*- 11.2 min; *R*- 17.7 min) and ethyl-2-hydroxy-2-phenylacetate (*S*- 10.7 min; *R*- 19.3 min), enantiomeric excess was calculated using Chiralcel OD-H column (Daicel, 0.46 cm X 25 cm), mobile phase of hexane:isopropanol = 98:02 at 1 ml min^{-1}. The chiral products were characterized by matching their retention time with the corresponding standard racemic alcohols (refer supplementary data file).

Optimization of pH, co solvent and reaction time for the reduction of 2-hydroxyacetophenone (2HAP) and ethyl-2-oxo-4-phenylbutanoate (EOPB)

4 mM of a model ketone substrate, 2-hydroxyacetophenone and an α-ketoester, ethyl-2-oxo-4-phenylbuanoate were reduced separately by 0.6 U of *S*RED, 2 mM NADPH in 0.1 M buffer. The conversion of the substrate was maximized by varying the buffer pH, co-solvent and reaction time. 0.1 M citrate buffer, pH 5 and 0.1 M HEPES buffer, pH 7.5 with co-solvent IPA and DMSO were used by examining conversion up to 10 h. After the reaction, the mixture was extracted and analyzed for conversion and enantiomeric excess as explained in the previous section.

Conclusion

The substrate selectivity and enantiospecificty of the purified *S*RED from *Candida parapsilosis* ATCC 7330 revealed that *S*RED preferred α-ketoesters > ketones > aldehydes. This reductase asymmetrically reduced ketones with excellent ee > 99% and α-ketoesters with moderate ee 70% in the presence of NADPH to their (*S*)-alcohols. *S*RED, together with *Cp*CR, from the same source, which shows opposite enantiospecificity, offers the possibility of synthesis of numerous optically pure industrially important chiral building blocks.

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