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Enzymatic ketone reduction: mapping the substrate profile of a short-chain alcohol dehydrogenase (YMR226c) from *Saccharomyces cerevisiae*

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Abstract—A short-chain alcohol dehydrogenase (YMR226c) from *Saccharomyces cerevisiae* was cloned and expressed in *Escherichia coli*, and the encoded protein was purified. The activity and enantioselectivity of this recombinant enzyme were evaluated with a series of ketones. The alcohol dehydrogenase (YMR226c) was found to effectively catalyze the enantioselective reductions of aryl-substituted acetophenones, α-chloroacetophenones, aliphatic ketones, and α- and β-ketoesters. While the enantioselectivity for the reduction of β-ketoesters was moderate, the acetophenone derivatives, aromatic α-ketoesters, some substituted α-chloroacetophenones, and aliphatic ketones were reduced to the corresponding chiral alcohols with excellent enantioselectivity. The enantiopreference of this enzyme generally followed Prelog's rule for the simple ketones. The ester functionality played some role in determining the enzyme's enantiopreference for the reduction of α- and β-ketoesters. The present study serves as a valuable guidance for the future applications of this versatile biocatalyst.

1. Introduction

Chiral alcohols are important and valuable intermediates in the synthesis of pharmaceuticals and other fine chemicals. The asymmetric reduction of prochiral ketones is one of the straightforward approaches used to access enantiomerically pure alcohols. In this regard, a variety of chiral metal complexes have been developed as catalysts for asymmetric ketone reductions.[1–4] An alternative to the chemical asymmetric reduction process is biocatalytic transformation using isolated enzymes or whole-cell microorganisms, which offers advantages such as mild and environmentally benign reaction conditions, high chemo-, regio-, and stereoselectivity, and is void of residual metals in the products.[5–7] Therefore, biocatalytic ketone reduction has attracted the attention of both academic and industrial communities, and many examples with isolated alcohol dehydrogenases have been described.[8–17] However, the substrate profile evaluation of these enzymes has usually been limited to one or two categories of ketones, although the information about the substrate specificity and enantioselectivity of enzymes is very valuable and instructive in guiding the selection of appropriate biocatalysts for a specific transformation. Furthermore, correlating substrate profiles of the alcohol dehydrogenases with their sequential and structural information will provide valuable insight into our understanding of how enzymes control activity and enantioselectivity, and thus facilitate our efforts to (semi)rationally design novel enzymes with desired substrate specificity and enantioselectivity. With this perspective in mind, we have studied substrate specificity and enantioselectivity for the carbonyl reductases from red yeast *Sporobolomyces salmonicolor* AKU 4429 and *Candida magnoliae* with various ketones of diverse structures, and have found that these carbonyl reductases catalyze reductions of aryl, alkyl ketones, and α- and β-ketoesters to the corresponding chiral alcohols with excellent enantiomeric purity.[18–20] Stewart et al. also systematically investigated the catalytic properties of 18 alcohol dehydrogenases from baker's yeast *Saccharomyces cerevisiae* with α- and β-ketoesters as the substrates, and these studies provided valuable information for the future application of these enzymes.[21] However, one interesting alcohol dehydrogenase encoded by gene YMR226c, which belongs to the short-chain alcohol dehydrogenase family,[22] is not covered in their elegant studies. Recently, it has been reported that

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bioreduction of bicyclo[2.2.2]octane-2,5-dione and bicyclo-[2.2.2]oct-7-ene-2,5-dione by genetically engineered yeast, which over-expressed YMR226c gene, resulted in (1*R*,4*R*,5*S*)-5-hydroxybicyclo[2.2.2]octan-2-one (>99% ee) and (1*S*,4*S*,5*S*)-5-hydroxybicyclo[2.2.2]octan-2-one (98% ee), (1*R*,4*R*,5*S*)-5-hydroxybicyclo[2.2.2]oct-7-en-2-one (>99% ee), and (1*S*,4*S*,5*S*)-5-hydroxybicyclo[2.2.2]oct-7-en-2-one (93% ee), respectively.[23,24] This indicates that the alcohol dehydrogenase encoded by gene YMR226c may have great potential in the synthesis of chiral building blocks. Therefore, the gene YMR226c was cloned and expressed in *E. coli* by following a modified literature procedure.[22] The recombinant protein was purified and assayed against a variety of ketones. Herein, we present the activity and enantioselectivity of the recombinant alcohol dehydrogenase YMR226c toward the reduction of aryl-substituted acetophenones, α-chloroacetophenones, aliphatic ketones, and α- and β-ketoesters, which will serve as a guideline for the future application of this enzyme in the synthesis of useful chiral intermediates.

2. Results and discussion

An open reading frame YMR226c of the yeast genome encoded an enzyme, which is a member of the short-chain alcohol dehydrogenase family. This gene was amplified from the genomic DNA of *S. cerevisiae*, and the amplified DNA fragment was cloned and expressed in *E. coli*. The encoded protein was purified and lyophilized as a white powder, and the SDS-polyacrylamide gel electrophoresis is shown in Figure 1. The enzyme could be stored at 4 °C for months without significant loss of activity.

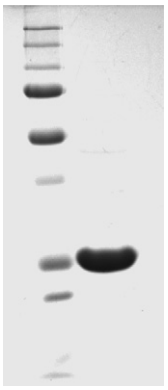
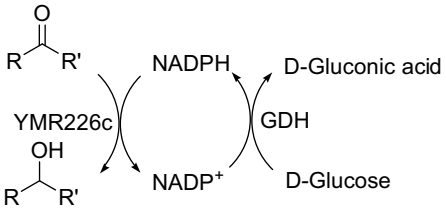


Figure 1. The SDS-polyacrylamide gel electrophoresis of the purified enzyme. Left lane, protein marker; right lane, purified protein.

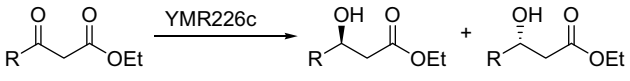
The recombinant alcohol dehydrogenase was evaluated toward the reductions of various ketones with diverse structures. The specific activity of the enzyme was determined spectrophotometrically by measuring the oxidation of NADPH at 340 nm at room temperature. Activity assay with the control cell-free extract, which was obtained by expression of pTxB1 vector without YMR226c gene in *E. coli* Rosetta2(DE3) strain, did not show any activity toward all the substrates tested. The enantioselectivity of the enzyme toward the reduction of ketones was studied using a NADPH regeneration system consisting of D-glucose dehydrogenase (**GDH**) and D-glucose (Scheme 1).[25] The enantiomeric excess (ee) values of the product alcohols were determined by chiral GC or HPLC analysis. The absolute configurations of product alcohols were assigned by comparing the retention time with a standard sample.[18,19]



Scheme 1. Ketone reduction catalyzed by a yeast alcohol dehydrogenase YMR226c using a NADPH regeneration system consisting of D-glucose dehydrogenase (**GDH**) and D-glucose.

As shown in Table 1, the yeast alcohol dehydrogenase YMR226c catalyzed the reduction of various β-ketoesters. Activities were not greatly affected by the structures of R groups. Compared with the other *S. cerevisiae* alcohol dehydrogenases, enzyme YMR226c shows lower enantioselectivity for the reduction of aliphatic β-ketoesters.[21] The R groups exerted a relatively significant effect on enantioselectivity, which decreased as the chain length of R groups became longer. This alkyl chain dependence has not been observed for the other yeast alcohol dehydrogenases,[21] and might be due to the longer alkyl chain destabilizing the lower-energy substrate orientation in the active site, leading to the formation of an L-enantiomer (Fig. 2, **A**). When the R group was changed to *iso*-propyl, D-enantiomer was obtained in 87% ee, indicating that the *iso*-propyl group further destabilized conformation **A** in Figure 2 and that orientation **B** was preferred. A trifluoromethyl group also inverted the lower-energy substrate conformation in the enzyme active site as the D-enantiomer was the major product. The similar alkyl-induced reversal of enantiopreference has also been observed for three other

Table 1. Reduction of β-ketoesters catalyzed by yeast alcohol dehydrogenase YMR226c



D L

R	Specific activity[a]	ee[b] (%)
CH_3	26	84 (L)
C_2H_5	27	38 (L)
n-C_3H_7	23	16 (L)
iso-C_3H_7	23	87 (D)
CH_2Cl	73	43 (L)
CF_3	19	58 (D)
C_6H_5	45	85 (L)

[a] The specific activity was defined as nmol of NADPH oxidized in 1 min by 1 mg of enzyme (nmmol min^{-1} mg^{-1}).

[b] The enantiomeric excess (ee) values of the product alcohols were determined by chiral GC analysis.

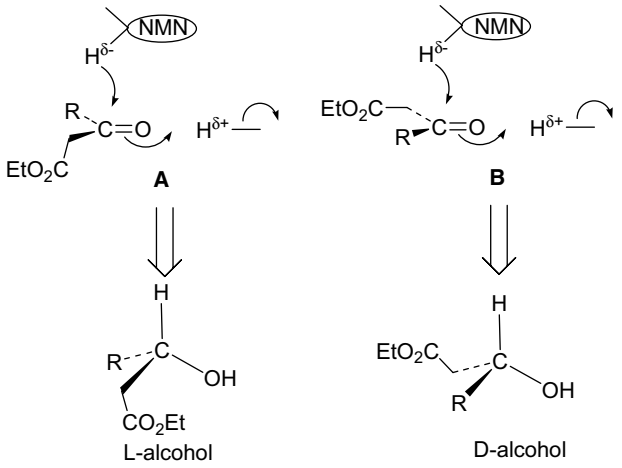
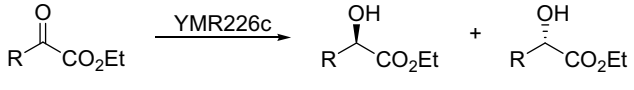


Figure 2. A catalytic site model for alcohol dehydrogenase.

alcohol dehydrogenases from baker's yeast *S. cerevisiae*.[21] However, when the R group was a planar benzene ring, the reduction gave an L-enantiomer with 85% ee.

The reductions of aromatic and aliphatic α-ketoesters catalyzed by this enzyme were also examined and the results are summarized in Table 2. The substituents on the phenyl ring exerted some effect on enzyme activity, and it seemed that the electron-withdrawing substituents increased the activity while the electron-donating groups demolished the activity. Compared with the unsubstituted ethyl 2-phenyl-2-oxo-acetate, the activity for substrates with F, Cl, Br, and CN substituents increased, while the substrate with a methyl group shows lower activity. For aliphatic α-ketoesters, this enzyme was surprisingly more active toward the reduction of ethyl 3,3-dimethyl-2-oxo-butyrate than less sterically crowded ethyl 3-methyl-2-oxo-butyrate.

Table 2. Reduction of α-ketoesters catalyzed by the yeast alcohol dehydrogenase YMR226c



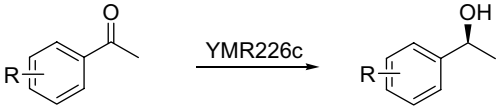
R	Specific activity[a]	ee[b] (%)
C_6H_5	101	99 (*S*)
4′-FC_6H_4	173	94 (*R*)
4′-ClC_6H_4	263	99 (*R*)
4′-BrC_6H_4	193	98 (*R*)
4′-$CH_3C_6H_4$	70	99 (*R*)
4′-CNC_6H_4	472	91 (*R*)
3′,5′-$F_2C_6H_3$	126	98 (*R*)
iso-C_3H_7	25	28 (*R*)[c]
tert-C_4H_9	130	59 (*R*)[c]

[a] The specific activity was defined as nmol of NADPH oxidized in 1 min by 1 mg of enzyme (nmmol min^{-1} mg^{-1}).
[b] The enantiomeric excess (ee) values of the product alcohols were determined by chiral HPLC analysis unless indicated otherwise.
[c] The ee values were measured by chiral GC analysis.

Similar to ethyl 3-phenyl-3-oxo-propionate, ethyl 2-phenyl-2-oxo-acetate adopted orientation **A** in the active site as shown in Figure 1, and ethyl (*S*)-2-phenyl-2-hydroxyacetate was obtained in enantiomerically pure form. It was interesting to note that the reductions of aromatic α-ketoesters with substituents on the phenyl ring produced (*R*)-configured α-hydroxyesters as the major products in excellent enantiomeric purity, implying that the substituents on the phenyl ring inverted the substrate conformation in the enzyme active site from **A** to **B**. This might be due to the bulkiness of the substituted phenyl ring, which failed to be accommodated into the hydrophobic binding pocket of the active site. For ethyl 3,3-dimethyl-2-oxo-butyrate and ethyl 3-methyl-2-oxo-butyrate, (*R*)-enantiomeric α-hydroxyesters were the major products. This indicated that substrate conformation **B** was preferred for these α-ketoesters and it was consistent with the observation for ethyl 4-methyl-3-oxo-pentanionate.

The yeast alcohol dehydrogenase YMR226c also effectively catalyzed the reductions of aryl-substituted acetophenone derivatives. The results are presented in Table 3. From the table it can be seen that the enzyme activity was dependent on the substituents on the benzene ring of acetophenone derivatives, although no apparent trend was observed. The position of the substituent affected the enzyme activity. For example, the activity decreased in the order of 4′-chloroacetophenone > 3′-chloroacetophenone > 2′-chloroacetophenone. For the methyl substituent, the *meta*-substituted substrate shows higher activity than the *para*-substituted counterpart. For all the aryl ketones tested, the product alcohols were obtained in excellent enantiomeric excess and the enantiomeric preference followed Prelog's rule.

Table 3. Reduction of aryl ketones catalyzed by the yeast alcohol dehydrogenase YMR226c



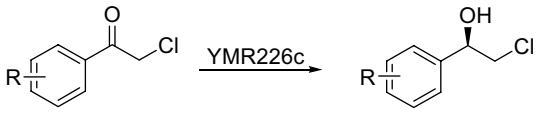
R	Specific activity[a]	ee[b] (%)
4′-H	20	99 (*S*)
4′-Cl	504	99 (*S*)
4′-Br	78	99 (*S*)
4′-CH_3	31	99 (*S*)
4′-CF_3	36	98 (*S*)
4′-CH_3O	54	99 (*S*)
2′-Cl	19	99 (*S*)
3′-Cl	100	99 (*S*)
3′-CH_3	90	98 (*S*)

[a] The specific activity was defined as nmol of NADPH oxidized in 1 min by 1 mg of enzyme (nmmol min^{-1} mg^{-1}).
[b] The enantiomeric excess (ee) values of the product alcohols were determined by chiral GC analysis.

Optically active chlorohydrins are versatile intermediates for the syntheses of biologically active compounds of pharmaceutical and agricultural interest. For example, (*R*)-2-chloro-1-(4′-fluorophenyl)ethanol has been used as a building block in the preparation of optically pure *N*-(*p*-fluorophenyl)hydroxyethyl-4-(*p*-fluorophenyl)hydroxy-

methyl-piperidine, a novel nonnarcotic analgesics.[26] Recently, Chung et al. have used optically active 2-chloro-1-(2′,4′-difluorophenyl)ethanol to construct (3*S*,4*R*)-1-*tert*-butyl-4-(2,4-difluorophenyl)pyrrolidine-3-carboxylic acid, a drug candidate for treatment of melanocortin receptor mediated diseases such as obesity and diabetes.[27,28] Therefore, the enzymatic reductions of a series of α-chloroacetophenone derivatives were investigated with the yeast alcohol dehydrogenase YMR226c. The specific activity and ee values are presented in Table 4. The results show that the substituent on the phenyl ring exerted effects on enzyme activity and enantioselectivity. In all cases, (*R*)-configured chlorohydrins were obtained, which followed the Prelog's rule as the chlorine changed the CIP priority although CH_2Cl is smaller than phenyl group.

Table 4. Reduction of α-chloroacetophenone derivatives catalyzed by the yeast alcohol dehydrogenase YMR226c

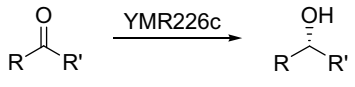


R	Specific activity[a]	ee[b] (%)
4′-H	75	98 (*R*)
4′-F	68	99 (*R*)[c]
4′-Cl	48	99 (*R*)
4′-NO_2	35	43 (*R*)
4′-CH_3CONH	26	56 (*R*)
3′-NO_2–4′-CH_3CONH	31	99 (*R*)
2′,4′-F_2	15	96 (*R*)[c]
3′-Cl	36	99 (*R*)
3′,4′-Cl_2	40	84 (*R*)
3′,4′-$(OH)_2$	18	97 (*R*)

[a] The specific activity was defined as nmol of NADPH oxidized in 1 min by 1 mg of enzyme (nmmol min^{-1} mg^{-1}).
[b] The enantiomeric excess (ee) values of the product alcohols were determined by chiral HPLC analysis unless indicated otherwise.
[c] The ee values were measured by chiral GC analysis.

Asymmetric reduction of simple aliphatic ketones is usually more of a challenge for both chemical and biocatalytic approaches. The yeast alcohol dehydrogenase YMR226c was also examined with several aliphatic ketones as the substrates. The results are summarized in Table 5. It can be seen that the chain length did not significantly affect the enzyme activity. In all cases, (*S*)-configured alcohols were produced as the major products, as predicted by Prelog's rule. The methyl ketones were reduced with high enantioselectivity (>84% ee), while the enantioselectivity for the reduction of 3-octanone was moderate.

Table 5. Reduction of aliphatic ketones catalyzed by the yeast alcohol dehydrogenase YMR226c



R	R′	Specific activity[a]	ee[b] (%)
CH_3	*n*-C_5H_{11}	14	98 (*S*)
CH_3	*n*-C_6H_{13}	16	98 (*S*)
CH_3	*n*-C_7H_{15}	20	84 (*S*)
C_2H_5	*n*-C_5H_{11}	17	48 (*S*)

[a] The specific activity was defined as nmol of NADPH oxidized in 1 min by 1 mg of enzyme (nmmol min^{-1} mg^{-1}).
[b] The enantiomeric excess (ee) values of the product alcohols were determined by chiral GC analysis.

3. Conclusion

The alcohol dehydrogenase (YMR226c) from *S. cerevisiae* has been cloned and the encoded protein has been purified. The substrate profile (activity and enantioselectivity) of this recombinant enzyme has been evaluated with various structurally diverse ketones. The alcohol dehydrogenase (YMR226c) shows a broad substrate preference and effectively catalyzes enantioselective reductions of aryl-substituted acetophenones, α-chloroacetophenone derivatives, aliphatic ketones, and α- and β-ketoesters. Especially, the acetophenone derivatives, aromatic α-ketoesters, and some of α-chloroacetophenone derivatives and aliphatic ketones are reduced to the corresponding chiral alcohols with excellent enantioselectivity. The enantiopreference of this enzyme generally follows Prelog's rule for the aliphatic and aromatic ketones, while both the ester functionality and steric factor are important in determining the enzyme's enantiopreference for the reduction of α- and β-ketoesters. This may be due to the possible hydrogen bonding of ester group with the enzyme. Therefore, the alcohol dehydrogenase (YMR226c) is a versatile biocatalyst for asymmetric reductions of a variety of ketones, and the present study will provide valuable guidance for further application of this enzyme in the synthesis of important pharmaceutical and agricultural intermediates.

4. Experimental

4.1. General remarks

Chiral HPLC analysis was performed on an Agilent 1100 series high-performance liquid chromatography system with (S,S)-Whelk-O 1 column (25 cm × 4.6 mm, Regis Technologies Inc.). Chiral GC analysis was performed on a Hewlett Packard 5890 series II plus gas chromatograph equipped with autosampler, EPC, split/splitless injector, FID detector, and CP-Chirasil-Dex CB chiral capillary column (25 m × 0.25 mm). Ketones and alcohol standard samples were purchased from Sigma–Aldrich or prepared as previously reported.[19,29]

4.2. Gene expression and purification of YMR226c

The alcohol dehydrogenase gene YMR226c was cloned from *S. cerevisiae* by following the literature procedure.[22] The gene YMR226c was cloned into pTXB1 expression vector at the Nde I/BamH I restriction sites to yield plasmid pYY23.0, which was transformed into *E. coli* Rosetta2(DE3) strain for expression. Overnight culture was diluted into fresh LB medium containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL), and incubated at 37 °C until the optical density reached 1.0 at

595 nm. The expression was induced by 0.1 mM IPTG and the culture was incubated at 18 °C for another 10 h. Cells were harvested by centrifugation at 4100 rpm at 4 °C for 30 min. The cell pellet was resuspended in potassium phosphate buffer (100 mM, pH 7.4, 0.01% 2-mercaptoethanol) and the cells were disrupted by EmulsiFlex®-C5 High Pressure Homogenizer (Avestin, Inc.). The cell-free extract was mixed with an equal volume of PEI solution (0.25% polyethyleneimine, NW 40–60K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids. The supernatant was precipitated with 55% ammonium sulfate, and the protein pellet was resuspended in potassium phosphate buffer (10 mM, pH 7.4, 0.01% of 2-mercaptoethanol). The resulting enzyme solution was desalted by gel filtration into potassium phosphate buffer (10 mM, pH 7.4, 0.01% of 2-mercaptoethanol). Lyophilization of the enzyme solution afforded the YMR226c enzyme as a white powder.

4.3. Activity assay of the alcohol dehydrogenase from *S. cerevisiae* (YMR226c)

The enzyme activity toward the reduction of ketones was determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm ($\varepsilon = 6.22\ \mathrm{mM^{-1}\,cm^{-1}}$) in the presence of an excess amount of ketones. The activity was measured at room temperature in 96-well plate, in which each well contained ketone (6.25 mM) and NADPH (0.25 mM) in potassium phosphate buffer (100 mM, pH 6.5, 180 μL). The reaction was initiated by the addition of the enzyme (20 μL solution containing 10–40 μg of enzyme). The specific activity was defined as the number of nmol of NADPH converted in 1 min by 1 mg of enzyme ($\mathrm{nmol\ min^{-1}\ mg^{-1}}$).

4.4. The enantioselectivity of reduction of ketones catalyzed by the alcohol dehydrogenase from *S. cerevisiae* (YMR226c)

The enantioselectivity of the enzymatic reduction of ketones was studied using an NADPH recycle system.[25] The general procedure was as follows: D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADPH (0.5 mg), the alcohol dehydrogenase (0.5 mg), and ketone solution in DMSO (50 μL, 0.25 M) were mixed in a potassium phosphate buffer (1 mL, 100 mM, pH 6.5) and the mixture was shaken overnight at room temperature. The mixture was extracted with methyl *tert*-butyl ether (1 mL). The organic extract was dried over anhydrous sodium sulfate and was subjected to chiral GC or HPLC analysis to determine the conversion and enantiomeric excess.[30] The absolute configuration of product alcohols was identified by comparing the chiral GC or HPLC data with the standard samples.[18,19]

Acknowledgements

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