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Chiral alcohol synthesis with yeast carbonyl reductases

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Abstract

Synthesis of chiral alcohols, (*R*)- and (*S*)-4-chloro-3-hydroxybutanoate ethyl esters (CHBE), was performed through the enzymatic asymmetric reduction of 4-chloroacetoacetate ethyl ester (CAAE). The enzymes reducing CAAE to (*R*)- and (*S*)-CHBE were found to be produced by *Sporobolomyces salmonicolor* and *Candida magnoliae*, respectively. The enzyme of *S. salmonicolor* is a novel NADPH-dependent aldehyde reductase (AR) belonging to the aldo-keto reductase superfamily. The *C. magnoliae* enzyme also seems to be a novel NADPH-dependent carbonyl reductase. When AR-overproducing *Escherichia coli* transformant cells or *C. magnoliae* cells were incubated in an organic solvent–water two-phase system, 300 or 90 mg/ml of CAAE was almost stoichiometrically converted to (*R*)- or (*S*)-CHBE ($>$ 92% e.e.), respectively. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Carbonyl reductase; Aldehyde reductase; 4-Chloro-3-hydroxybutanoate esters; *Sporobolomyces salmonicolor*; *Candida magnoliae*

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1. Introduction

Chiral alcohols with additional functional groups are promising building blocks for the synthesis of enantiomeric pure pharmaceuticals and other chemicals. We have reported that microorganisms are good sources of carbonyl reductases and alcohol dehydrogenases with different substrate specificity and stereospecificity, and shown that these enzymes are potential catalysts for the conversion of prochiral carbonyl compounds to optically active alcohols [1–3].

(*R*)- or (*S*)-4-chloro-3-hydroxybutanoate ethyl ester (CHBE) is one of the important chiral building blocks [4–8]. 4-Chloroacetoacetate ethyl ester (CAAE), i.e., prochiral carbonyl compound of CHBE, might be a promising starting material for the synthesis of chiral CHBE. Practical production methods for chiral CHBE through microbial asymmetric reduction of CAAE are described.

2. Diversity of microbial reducing ability of CAAE

Various microorganisms have been assayed with regard to their reducing ability of CAAE.

Abbreviations: CHBE, 4-Chloro-3-hydroxybutanoate ethyl ester; CAAE, 4-Chloroacetoacetate ethyl ester; e.e., Enantiomeric excess; AR, Aldehyde reductase; CR, Carbonyl reductase; GDH, Glucose dehydrogenase; AKR, Aldo-keto reductase

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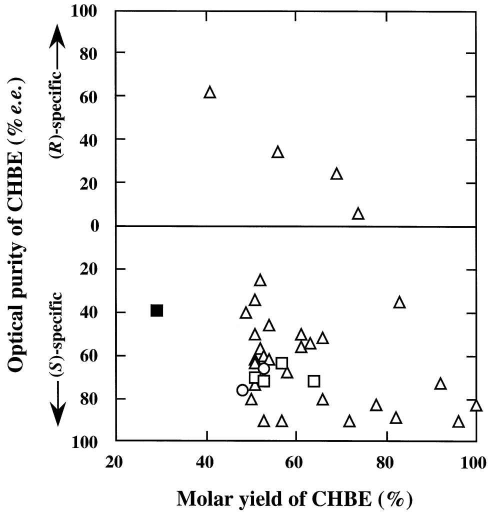


Fig. 1. Diversity of microbial reduction of CAAE. Symbols: △, yeasts; ○, molds; □, bacteria; ■, actinomycetes. See [9] for details.

Air-dried cells of each strain, from bacteria, actinomycetes, yeasts and molds, were incubated in the reaction mixture containing NAD^+, $NADP^+$, glucose dehydrogenase (GDH, *Bacillus megaterium*), glucose and CAAE (10 mg/ml). NAD^+, $NADP^+$, GDH and glucose were added for the regeneration of cofactors, i.e., NADH and NADPH, required for the reduction reactions. CHBE formed and CAAE remaining were extracted with ethyl acetate, and then the extract was analyzed as to the conversion yield and optical purity of CHBE [9].

Microbial reduction of CAAE usually proceeds stereospecifically. This activity is widely distributed in yeasts, molds, bacteria and actinomycetes, most of which give (*S*)-enantiomer (Fig. 1). *S. salmonicolor*, *Torulopsis nagoyaensis* and *Debaryomyces castellii* have been found to produce the (*R*)-enantiomer predominantly (24–62% e.e.) in high molar conversions. On the other hand, several *Candida* yeasts, such as *C. magnoliae*, *C. lipolytica* and *C. apis*, formed (*S*)-CHBE with high optical purity ($> 90\%$ e.e.) [9].

3. Enzymatic synthesis of (*R*)-CHBE using microbial aldehyde reductase

The enzyme giving (*R*)-CHBE was isolated in crystalline form from *S. salmonicolor* cells, and was characterized in some detail [10–12]. It is a novel NADPH-dependent enzyme 'aldehyde reductase (AR)' belonging to the aldo-keto reductase (AKR) superfamily enzymes [13] (Table 1). The enzyme is thermostable and resistant to organic solvents. It catalyzes the asymmetric reduction of 4-haloacetoacetic es-

Table 1
Properties of the aldehyde reductase (AR) of *S. salmonicolor* and carbonyl reductase (CR) of *C. magnoliae*[a]

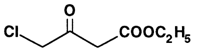
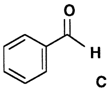
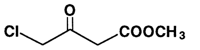
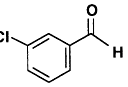
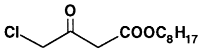
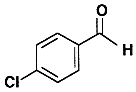
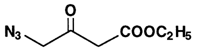
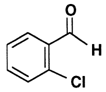
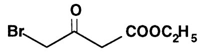
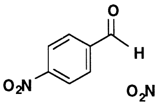
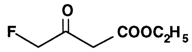
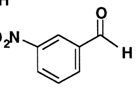
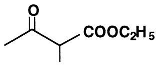
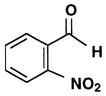
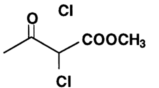
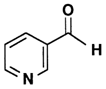
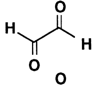
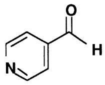
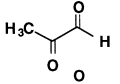
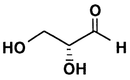
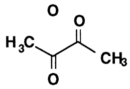
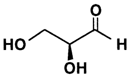
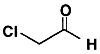
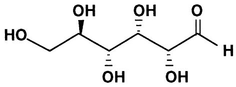
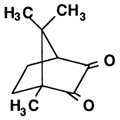
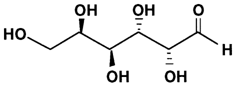
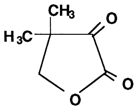
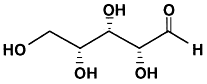
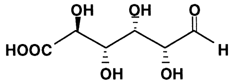
	AR	CR
Native molecular mass	37,000	76,000
Subunit molecular mass	37,000	32,000
Number of subunit	1	2
K_m for CAAE (mM)	0.36	4.6
V_{max} for CAAE (μmol min^{-1} mg^{-1})	144	273
Stereoselectivity	(*R*)-CHBE	(*S*)-CHBE
Cofactor (K_m)	NADPH (37.5 μM)	NADPH (16.7 μM)
Optimum pH	7.0	5.5
Optimum temperature	60	55
Thermal stability	80% (60°C, 10 min)	90% (45°C, 30 min)
Inhibitor	Dicoumarol, quercetin	Quercetin
Reaction mechanism	Ordered Bi–Bi	n.d.
Enzyme formation	Constitutive	Constitutive

[a] See [9–12] for details.
n.d.: not determined.

ters to the corresponding (*R*)-4-halo-3-hydroxybutanoate esters, in addition to common substrates of AKR superfamily enzymes, such as *p*-nitrobenzaldehyde and pyridine-3-aldehyde (Table 2). We cloned and sequenced cDNA coding for AR from *S. salmonicolor* [14], and the enzyme has shown to have similarity to the AKR superfamily enzymes in primary protein structure.

Practical preparation is carried out in an organic solvent–water two-phase system as shown in Fig. 2, because both substrate and product strongly inhibit enzyme reactions. Crude extract from *S. salmonicolor* after heat- and acetone-

Table 2
Substrate specificity of aldehyde reductase (AR) of *S. salmonicolor* and carbonyl reductase (CR) of *C. magnoliae*[a]

Substrate	Relative activity (%)		Substrate	Relative activity (%)	
	AR	CR		AR	CR
	100	100		14	0
	25	11		56	0
	240	36		52	0
	65	n.d.		58	0
	75	n.d.		468	0
	153	n.d.		63	0
	330	90		14	0
	74	11		228	0
	74	0		54	0
	219	0		64	37
	75	19		n.d.	65
	17	0		81	0
	16	0		24	0
	0	78		472	0
				173	0

[a] See [9–12] for details.
n.d., not determined.

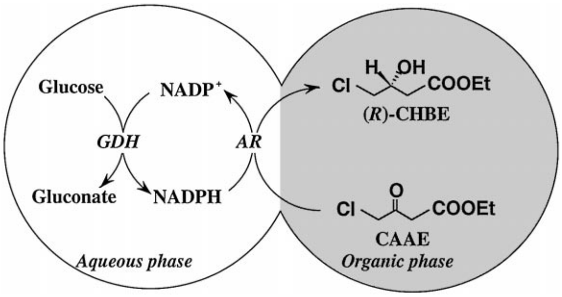


Fig. 2. Outline of the stereospecific reduction of CAAE by aldehyde reductase (AR) with glucose dehydrogenase (GDH) as a cofactor regenerator in an organic solvent–water two-phase system. See [16] for details.

treatments (these treatments remove (*S*)-CHBE-forming enzyme(s) from cell extract) and commercially available GDH are used as a source of AR and a cofactor regenerator, respectively. *n*-Butyl acetate is selected as the most suitable organic solvent for two-phase system, because it shows high partition efficiencies toward both the substrate and product, and both of the enzymes are stable in the presence of this organic solvent. In a 1.6 l–1.6 l bench-scale two-phase reaction, 83.8 mg/ml of (*R*)-CHBE (86% e.e.) was produced from CAAE in a molar yield of 95.4% [15,16].

Recently, the AR gene from *S. salmonicolor* [14] and the GDH gene from *B. megaterium* [17] were transformed into a same *Escherichia coli*, and consequently the transformant overproducing both AR and GDH, *E. coli* JM109 [pKAR, pKKGDH], was obtained [18–20]. The reduction reaction is carried out in an organic solvent–water two-phase system containing CAAE, glucose, NADP$^+$ and *E. coli* JM109 [pKAR, pKKGDH] cells as the catalyst. When the *E. coli* cells were incubated in the water-*n*-butyl acetate two-phase system, 300 mg/ml of CAAE was almost stoichiometrically converted to (*R*)-CHBE (92% e.e.) in 16 h (Fig. 3).

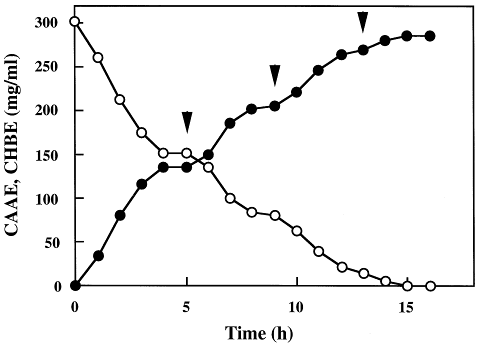


Fig. 3. Conversion of CAAE to (*R*)-CHBE in two-phase reaction by *E. coli* JM109 [pKAR, pKKGDH] cells. Glucose (100 mg/ml) was added to the aqueous phase periodically (▼). Symbols: ○, CAAE; ●, CHBE. See [18–20] for details.

4. Enzymatic synthesis of (*S*)-CHBE using microbial carbonyl reductase

The enzyme catalyzing the asymmetric reduction of CAAE to (*S*)-CHBE was isolated from *C. magnoliae* cells and was well characterized (Table 1) [9]. It is also a novel NADPH-dependent enzyme, and is resistant to organic solvents. The enzyme catalyzes the reduction of some carbonyl compounds such as diacetyl, glyceraldehyde and ketopantoyl lactone, other than 4-haloacetoacetate esters, whereas typical substrates of AR, such as benzaldehyde derivatives and pyridine aldehydes, did not serve as substrate (Table 2). NH_2–terminal amino acid sequence shows no significant homology with the AKR superfamily enzymes and other oxidoreductases. These results suggest that the *C. magnoliae* enzyme seems to be a novel carbonyl reductase (CR).

When washed cells of *C. magnoliae* were incubated with *E. coli* cells expressing GDH gene, *E. coli* JM109 [pGDA2] [17], in a similar two-phase system described in the previous section, 90 mg/ml of CAAE was stoichiometrically converted to (*S*)-CHBE (96% e.e.) in 50 h.

5. Conclusion

Since the use of these microbial cells as the catalyst is simple and requires no isolation of enzymes, it is highly advantageous for practical synthesis of chiral CHBE and other related chiral alcohols.

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