Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Yuri SARATANI, Eiji UHEDA, Hiroaki YAMAMOTO, Atsuo NISHIMURA & Fumiki YOSHIZAKO (2001) Stereoselective Reduction of Ethyl 4-chloro-3-oxobutanoate by Fungi, Bioscience, Biotechnology, and Biochemistry, 65:7, 1676-1679, DOI: 10.1271/bbb.65.1676

To link to this article: http://dx.doi.org/10.1271/bbb.65.1676

Biosci. Biotechnol. Biochem., **65** (7), 1676-1679, 2001

Note

Stereoselective Reduction of Ethyl 4-chloro-3-oxobutanoate by Fungi

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Received January 11, 2001; Accepted February 24, 2001

The enantioselectivity of ECAA to ECHB by eight fungi of four genus was evaluated. All strains showed (*S*)-selectivity, and *Cylindrocarpon sclerotigenum* IFO 31855 gave the highest yield and good optical purity (*e.e.*; >99%). Cell-free extract and acetone-dried cells of *C. sclerotigenum* IFO 31855 reduced ECAA to (*S*)-ECHB in the presence of NADPH (*e.e.*; >99%) and the *e.e.* was not decreased by heat treatment of the cell-free extract or the acetone-dried cells. The active fractions shown by two peaks on a DEAE-Toyopearl 650 M column gave preferentially (*S*)-ECHB (*e.e.*; >99%).

Key words: stereoselective reduction; ethyl 4-chloro-3-oxobutanoate; (*S*)-ethyl 4-chloro-3-hydroxybutanoate; *Cylindrocarpon sclerotigenum* IFO 31855

It has been proposed that both enantiomers of ethyl 4-chloro-3-hydroxybutanoate (ECHB) could be used for the synthesis of pharmaceuticals such as the HMG-CoA reductase inhibitor from the (*S*)-enantiomer[1] and the L-carnitine from the (*R*)-enantiomer.[2] Biochemical approaches involving microorganisms and enzymes have played a major role in the practical synthesis of chiral ECHB.

Various kinds of yeasts have been found to catalyze the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (ECAA) to (*S*)-or (*R*)-ECHB.[3-5] Some studies have succeeded in the isolation and characterization of the enzymes from yeasts capable of reducing ECAA: baker's yeast,[6,7] *Sporobolomyces salmonicolor*,[8-10] *Candida magnoliae*,[11-13] and *Kluyveromyces lactis*.[14] The stereoselective reduction of ECAA by an *Escherichia coli* transfomant coexpressing the aldehyde reductase and glucose dehydrogenase genes, and by an *E. coli* transformant overexpressing the carbonyl reductase were also reported.[15-17]

On the other hand, although it has been known that several strains of fungi had good ECAA reducing ability,[4,18,19] very little has been known so far on the physicochemical and enzymatic characterization of the ECAA- reducing enzyme in those fungi : Patel *et al.*, reported the preparation of (*S*)-ECHB from ECAA using whole cells of *Geotrichum candidum* (yield 95%, 96% enantiomer excess; *e.e.*) and the characterization of NADPH-dependent oxidoreductase responsible for the reduction.[18] However, for the practical use of those fungi and their enzymes for the synthesis of chiral ECHB, it is important to obtain more detailed information on the properties of the enzymes involved in the reductions.

We investigated the distribution of ECAA-reducing abilities of various fungi, and found that *Cylindrocarpon sclerotigenum* IFO 31855 produces (*S*)-ECHB in high *e.e.* and high yields. In this paper, we describe the high stereoselective reduction of ECAA to (*S*)-ECHB by *C. sclerotigenum* IFO 31855. Eight strains of fungi, which were selected as a result of a screening test for high ECAA-reducing ability, were used in this work: *Botrytis fabae* IFO 7171, *B. allii* IFO 9430, *C. sclerotigenum* IFO 31855, *C. olidum* IFO 32527, *Trichoderma longibrachiatum* IFO 4847, *T. polysporum* IFO 9322, *Penicillium oxalicum* IFO5748, and *P. purpurogenum*. The medium used was 0.5% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.025% $CaCl_2 \cdot 2H_2O$, 0.025% $MgSO_4 \cdot 7H_2O$, yeast extract 0.1%, and 5.0% sucrose. Stock cultures of fungi were kept on potato-sucrose medium solidified with 1.2% agar.

Biotransformation of ECAA using whole cells was done as follows: Each fungus was cultured as described previously.[20] After 3 d of culture, 0.74 mmol of ECAA was added to 50 ml of culture medium and incubated for 24 h at 25°C with shaking (in the case of Table 1 and Fig. 1).

The cell-free extract was prepared as follows: the 3 d precultured cells were harvested by centrifugation (22,200 × *g* for 15 min at 4°C), and washed twice with

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Abbreviations: ECHB, ethyl 4-chloro-3-hydroxybutanoate; ECAA, ethyl 4-chloro-3-oxobutanoate; *e.e.*, enantiomer excess

0.85% NaCl and once with 50 mM potassium phosphate buffer (KPB, pH 7.0) containing 20% glycerol and 0.5 mM dithiothreitol. The washed cells were suspended in the same KPB, disrupted with a Bead-Beater cell disrupter (Biospec Products, Bartlesville, Oklahoma, USA), and centrifuged at 15,200 × *g* for 20 min at 4°C. The resulting supernatant was used as the cell-free extract. Acetone-dried cells were prepared by the method of Yasohara *et al.*[3] Biotransformation of ECAA with cell-free extract, acetone-dried cells, and two active fractions on Toyopearl 650 M column was done by incubation without shaking at 30°C for 3 h using 15 ml of a mixture containing 1.5 mmol of KPB (pH 6.5), 0.1 mmol of ECAA, 10 μmol of NADPH, and appropriate amounts of cell mass or protein (in the case of Table 2).

The reductive metabolites were extracted with ethyl acetate and dissolved in methanol (3 ml) as described previously.[20] A sample of the methanol solution (0.3 ml) was used for the analyses of both keto ester and hydroxy ester by a gas chromatograph (Shimadzu GC-14A, Japan) with a Supelco wax 10 fused silica capillary column (φ 0.53 mm by 30 m, 0.5 μm film thickness, Supelco). The conditions for gas liquid chromatography (GLC) were as follows: oven temperature, 80°C (5 min) to 220°C at 10°C min^{-1}; injection temperature and flame ionization detector temperature, 200°C; and a carrier gas, He at 20 ml min^{-1}. The retention times for ECAA and ECHB were 11.3 min and 13.0 min, respectively. To the remaining methanol solution (2.7 ml) were added 5 ml of distilled water and 5 ml of a 20% water solution of semicarbazide hydrochloride. The mixture stood for 12 h at room temperature and then was extracted with ethyl acetate (3 ml). The ethyl acetate was passed through a Wakogel C-200 column and the effluent was dried under a stream of nitrogen. The oily residue was dissolved in a mobile phase solution and the solution was used for analysis of the optical purity of ECHB. The optical purity of ECHB was measured with high pressure liquid chromatography (HPLC) with a Chiralpak AS column (φ 46 mm by 25 cm, Daicel Chemical Industries Ltd., Japan). The HPLC conditions were a *n*-hexane/2-propanol/ethanol/cyclohexanol (92/2.5/1.25/0.25, v/v) as mobile phase, a flow rate of 0.5 ml min^{-1}, and detection at 220 nm. The retention time was 25.4 min for the (*S*)-ECBH and 26.8 min for the (*R*)-ECBH.

The assay mixture for ECAA reducing activity contained 1.8 ml of 50 mM KPB (pH 6.5) containing 0.9 μmol of dithiothreitol, 1 μmol of NADPH, 0.1 mmol of ECAA, and 0.1 ml of the enzyme solution. After 3 min of incubation at 35°C the reaction was started by an addition of ECAA and then the decrease in A_{340} was monitored spectrophotometrically.

Protein was measured by the method of Bradford,[21] using bovine serum albumin as the standard.

ECAA and (*S*)-ECHB were donated by Daicel Chemical Industries Ltd., (Tokyo, Japan). (*R*)-ECHB was also kindly donated by Daiso Co. Ltd., (Amagasaki, Japan). NAD(P) and NAD(P)H were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). Semicarbazide hydrochloride was purchased from Katayama Chemical (Osaka, Japan). All other chemicals were of analytical grade and are commercially available.

We examined the reduction of ECAA by 8 fungi which were selected as a result of screening test for high ECAA reducing ability (Table 1). These strains had high ECAA-reducing ability comparable to those of *G. candidum*,[18] *Fusarium merismoides*,[19] *Gibberella fujikuroi*,[19] *Cephalrosporium acremonium*,[4] and *Paecilomyces marquandii*,[4] and reduced ECAA predominantly to the corresponding (*S*)-ECHB, similar to the results with most of the yeasts and fungi.[4,5,18,22] Among them, *C. sclerotigenum* IFO 31855 catalyzed the reduction in the highest optical purity (>99% *e.e.*) and 1.58 g of ECHB per liter culture medium was accumulated. Thus *C. sclerotigenum* IFO 31855 was used for the further study.

The continuous biotransformation of ECAA by whole cells of *C. sclerotigenum* was started with an addition of 0.74 mmol of ECAA to 50 ml of culture medium (Fig. 1). In the control culture without cells, 11.2 mM ECAA was recovered. Figure 1 shows that

Table 1. Biotransformation of ECAA by 8 Fungi[a]

Strain	Consumed ECAA (%)	ECHB		
		Molar Yield (%)	*e.e.* (%)	(*R*/*S*)
Botrytis fabae IFO 7171	>99	58	92.7	*S*
B. allii IFO 9430	100	62	95.1	*S*
Cylindrocarpon olidum IFO 32527	89	42	93.3	*S*
C. sclerotigenum IFO 31855	100	64	>99	*S*
Penicillium purpurogenum	100	55	83.4	*S*
P. oxalicum IFO 5748	51	23	82.6	*S*
Trichoderma polysporum IFO 9322	64	39	80.3	*S*
T. longibrachiatum IFO 4847	100	63	74.5	*S*

[a] To 50 ml of culture medium was added 0.74 mmol of ECAA.

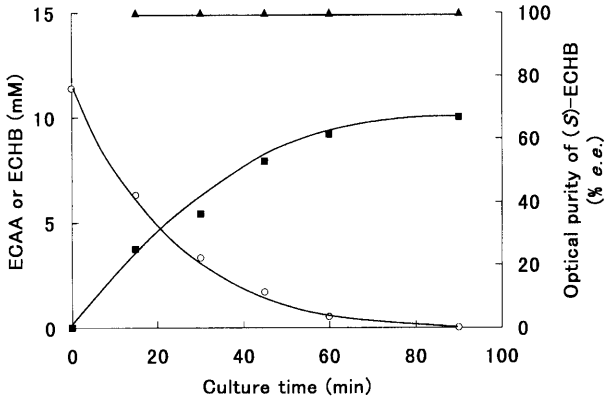

Fig. 1. Biotransformation of ECAA by *Cylindrocarpon sclerotigenum* IFO 31855.

Symbols: ○, Ethyl 4-chloro-3-oxobutanoate; ■, (*S*)-Ethyl 4-chloro-3-hydroxybutanoate; and ▲, *e.e.*%.

C. sclerotigenum reduced ECAA to (*S*)-ECHB rapidly. ECAA added at the beginning of the reaction, was completely consumed at 90 min to give 10 mM (*S*)-ECHB (1.66 g/l). The *e.e.* of (*S*)-ECHB showed over 99% all through the experiment. This strain also converted ethyl 3-oxobutanoate and ethyl 3-oxopentanoate to the corresponding alcohols with the relatively high (*R*)-selectivity (*e.e.* of 52% and 83% respectively).

Most ECAA- reducing enzymes in microorganisms require NADPH as a coenzyme.[6-13,18] The effects of redox cofactors on the ECAA-reducing enzyme activity in *C. sclerotigenum* were examined. Both cell-free extract and acetone-dried cells of *C. sclerotigenum* showed ECAA-reducing activity only when NADPH was used as a cofactor. Optimal pH and temperature of ECAA-reducing activity in cell-free extract were pH 6.0 and 45°C respectively.

Multiple enzymes with opposite stereochemical preferences for ECAA biotransformation have been isolated.[6-13] The incomplete stereoselectivty of the product in biotransformation of ECAA can be often improved by the disruption of cells, or the heat treatment of whole cells or acetone-dried cells because of the inactivation of the opposite enzymes.[3,4,18] Thus, the high purity of (*S*)-selectivity of ECAA reduction in *C. sclerotigenum* should be affected by the heat treatment of acetone-dried cells or cell-free extract before biotransformation. However, as shown in Table 2, the amount of ECHB formed was decreased by the heat treatment of the cell-free extract at the higher temperature (0.71 g/l at 30°C, 0.66 g/l at 40°C and 0.33 g/l at 50°C respectively), but no decrease of the *e.e.* was observed in any case. Similar results were also obtained in the preparation of acetone-dried cells (the concentration of ECHB formed was 0.13 g/l at 30°C and 0.1 g/l at 40°C respectively). Furthermore, the ECAA- reducing activities eluted with a gradient of 0–0.6 M NaCl were separated into two active fractions on a column of DEAE-Toyopearl 650 M. Both of the fractions gave preferentially (*S*)-ECHB with *e.e.* of more than 99% and the amounts of ECHB formed was 0.11 g/l in peak I and 0.07 g/l in peak II respectively (Table 2). These facts strongly suggest that, at least, *C. sclerotigenum* IFO 31855 has two or more NADPH-dependent oxidoreductases responsible for the high (*S*)-selective reduction of ECAA and very few if any (*R*)-selective enzymes operating in the reduction of ECAA.

This work is thus the first to demonstrate the highly stereoselective reduction of ECAA to (*S*)-ECHB by the genus of *Cylindrocarpon*. More detailed study on the purification and characterization of ECAA reducing enzymes in *C. sclerotigenum* are now underway.

Table 2. Reduction of ECAA to ECHB with Various Treated Cells[a]

Preparation	Molar Yield of ECHB(%)	Stereo-selectivity	*e.e.* (%)
Cell-free extract			
(26.3 mg[c], 30°C[d])	64	*S*	>99
(26.3 mg[c], 40°C[d])	61	*S*	>99
(26.3 mg[c], 50°C[d])	30	*S*	>99
AcDC[b]			
(50 mg[c], 30°C[d])	5.3	*S*	>99
(50 mg[c], 40°C[d])	4	*S*	>99
Toyopearl 650 M 1st[e]			
peak I (8.6 mg[c])	11	*S*	>99
peak II(21.7 mg[c])	6	*S*	>99

[a] To 15 ml reaction mixture was added 0.1 mmol of ECAA.
[b] AcDC; Acetone-dried cells.
[c] Amounts of cell mass or protein.
[d] Cell-free extract and AcDC were treated by heating at 30°C, 40°C or 50°C for 20 min before biotransformation and incubated at 30°C for 3 h without shaking.
[e] Biotransformation was done at 30°C for 3 h without shaking.

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