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Stereochemical Control in Microbial Reduction. XXIX. Mechanism of Stereochemical Control with an Additive in the Diastereoselective Reduction by *Geotrichum candidum*

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The reduction of ethyl 2-methyl-3-oxobutanoate with *Geotrichum candidum* yields approximately equal amounts of the corresponding *syn*-(2*R*,3*S*)- and *anti*-(2*S*,3*S*)-3-hydroxy esters. When the microbe is incubated with methyl vinyl ketone (MVK) or chloroacetone before it is subjected to the reduction, the corresponding *anti*-(2*S*,3*S*)-hydroxy ester is obtained with exclusive enantio- as well as diastereoselectivity. In order to obtain more insight into the mechanism of this stereochemical control in microbial reduction, β-keto ester reductases were isolated and purified from the cells of *G. candidum*. Three dominantly competing enzymes were purified. One of them affords the *anti*-(2*S*,3*S*)-hydroxy ester selectively, whereas the others give the *syn*-(2*R*,3*S*)-hydroxy ester selectively. The rates of enzymatic reductions were measured in the presence and absence of an additive. Chloroacetone and MVK are competitive and suicide substrates, respectively, for the *syn*-enzymes, whereas they hardly affect the activity of the *anti*-enzyme. In the presence of an additive, the reduction of ethyl 2-methyl-3-oxobutanoate with *G. candidum* is catalyzed by the *anti*-enzyme only.

Microbial reduction of carbonyl compounds has been widely applied to the syntheses of optically active compounds.[1] However, the stereoselectivity associated with the reduction of an artificial substrate by a microbe is not always satisfactory, and a particular microbe does not necessarily afford a product of the desired configuration. In such a case, it is necessary to control and improve the stereoselectivity. Recently, we developed a new and valuable method to improve enantio- as well as diastereoselectivity of the reduction of ethyl 2-alkyl-3-oxobutanoates with a microbe.[2—4] The method includes the addition of an additive such as methyl vinyl ketone (MVK) or chloroacetone. Thus, the reduction by a mold with an appropriate additive affords the corresponding *anti*-(2*S*,3*S*)-hydroxy ester selectively, whereas the reduction by a yeast with an additive gives the corresponding *syn*-(2*R*,3*S*)-hydroxy ester selectively.[5]

In order to elucidate the mechanism of controlling the stereoselectivity of microbial reduction by an achiral additive, several β-keto ester reductases from the microbe have been isolated and partially purified. This approach makes a significant contribution to shed light on the black box of the chemisitry in a microbe. We recently reported the isolation and characterization of α-[6,7] and β[3,8—11] keto ester reductases from bakers' yeast; the difference in inhibition constant of an additive among the enzymes plays a crucial role for the stereochemical control.

Now we report the isolation and purification of β-keto ester reductases from the cells of *Geotrichum candidum* to study the effects of the additive on the reactivity of these enzymes.

Experimental

Instruments. NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$ solutions with tetramethylsilane (TMS) as an internal reference. UV spectra were obtained on a Hitachi U-3210 spectrophotometer. Gas chromatograms were recorded on a Shimadzu GC-14A (PEG-20M Bonded, 25 m) and GC-9A (Chiraldex G-TA, 30 m) gas chromatographs.

Materials. Organic reagents and solvents were purchased from Nacalai Tesque, Inc. unless otherwise indicated. Ethyl 2-methyl-3-oxobutanoate (**1**) was purchased from Tokyo Kasei Kogyo Co. DEAE-Toyopearl 650 and Butyl-Toyopearl 650 were purchased from Tosoh Co. CentriCell ultrafilters (10000 NMWL) were purchased from Polysciences, Inc. NADH and NADPH were purchased from Kohjin Co., Ltd.

The basic buffer consisted of 20 mM Tris-HCl (pH 7.0), 1.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, and 3.0% ethanol.

Cultivation of *Geotrichum candidum*. In 4 L of 0.1 M potassium phosphate buffer at pH 6.2, 120 g of glycerol, 40 g of yeast extract, and 20 g of polypeptone were dissolved. The solution was sterilized for 20 min at 121 °C in an autoclave; then *Geotrichum candidum* (IFO 4597) was cultivated at 27 °C for 2 d. The mixture was filtered on a filter paper.

Purification of β-Keto Ester Reductase from Cells of *Geotrichum candidum*. The enzymes were purified and stored at 4 °C. For prolonged storage, the enzymes were frozen in 10% aqueous glucose. Microbe material (160 g) was suspended in 400 mL of the basic buffer. The suspended microbes were disrupted with a Dyno–Mill (0.25—0.50 mm glass-beads) at 0 °C for 20 min. The disrupted mixture was centrifuged at 10000 g for 30 min. The supernatant liquid (750 mL) was dialyzed for 12 h against the basic buffer (3 L), and the dialysis was repeated three times. The

dialyzate was concentrated by dialysis against polyethylene glycol (MW 20000) for 2 d. The concentrated dialyzate was centrifuged at 10000 g for 30 min. Finally, 300 mL of crude enzyme extract was obtained.

A solution of crude enzyme was applied to a column (5.2 cm×18 cm) packed with DEAE-Toyopearl 650 equilibrated with the basic buffer. The column was eluted with the basic buffer (0.7 L). A nonadsorbed enzyme (GKER-I) was eluted. Then, elution was continued with a 0—0.2 M linear gradient concentration of KCl dissolved in the basic buffer (1.2 L). Twelve-mL fractions were collected. Two enzymes were eluted: at 0.05 M (GKER-II) and 0.08 M (GKER-III) KCl concentrations. Each of the active fractions was concentrated, and then dialyzed for 1 d against the basic buffer.

Each of the three enzyme solutions was then applied to a column (2.6 cm×10 cm) packed with Butyl-Toyopearl 650 equilibrated with the basic buffer, which also contained 20% ammonium sulfate. The column was eluted with the basic buffer, which also contained 20% ammonium sulfate, and then eluted with a 20—0% linear gradient concentration of ammonium sulfate dissolved in basic buffer (600 mL). Ten-mL fractions were collected. The active fractions were combined, then concentrated and desalted by ultrafiltration with a CentriCell centrifugal ultrafilter (10000 NMWL).

Enzyme Assay. A 100 μL aliquot of chromatographic fraction was added to 3.15 mL of a solution of 50 mM (1 M = 1 mol dm^{-3}) Tris-HCl buffer (pH 7.0) that contained keto ester **1** (3.15 mM) and NAD(P)H (95.3 μM). The rate of reduction was determined spectrophotometrically at 30 °C by following the decrease in absorbance of NAD(P)H at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NAD(P)H per minute at 30 °C under the conditions employed.

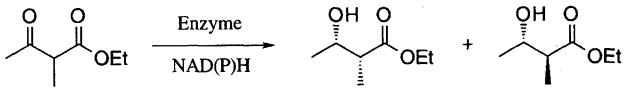
Enzymatic Reduction of Ethyl 2-Methyl-3-oxobutanoate (1). In a glass reaction vessel were placed 1 mL of 100 mM Tris-HCl buffer (pH 7.0) containing 45 mU of enzyme, 15 mM of keto ester **1**, and 18 mM of NAD(P)H. The reaction vessel was shielded from light. The mixture was stirred magnetically for 24 h at 30 °C. The mixture was purified by column chromatography on silica gel with hexane/ethyl acetate (5/1) used as an eluent, giving ethyl 3-hydroxy-2-methylbutanoate (**2**).

Determination of Enantiomeric and Diastereomeric Excesses. Diastereomeric excesses (d.e.) in hydroxy ester **2** were determined with GLC equipped with a capillary column PEG 20M (Bonded, 0.25 mm×25 m, 110 °C).[12] Enantiomeric excesses (e.e.) in hydroxy ester **2** were determined with GLC equipped with a capillary column Chiraldex G-TA (0.25 mm×20 m, 70 °C). The absolute configuration of the isomer corresponding to each peak of hydroxy ester **2** obtained from the reduction by $NaBH_4$ (composed of all four isomers) was determined by comparing its retention time with those of the authentic samples prepared by methylation of racemic and ethyl (*S*)-3-hydroxybutanoate, and by the reduction of keto ester **1** with bakers' yeast.[13]

Results and Discussion

Purification of β-keto Ester Reductase from *Geotrichum candidum*. Three β-keto ester reductases were purified from a cell-free solution of *Geotrichum candidum* by anion-exchange column chromatography (DEAE-Toyopearl). The enzymes have been named as GKER-I, II, and III (*Geotrichum candidum* Keto Ester Reductase). A typical chromatogram is shown in Fig. 1. Each enzyme was purified further by hydrophobic chromatography (Butyl-Toyopearl). Among these, two enzymes utilize NADH as the coenzyme preferentially, and the other is NADPH-dependent. The stereochemistry of reaction catalyzed by each enzyme was determined by employing ethyl 2-methyl-

Table 1. Enzymatic Reduction of Ethyl 2-Methyl-3-oxobutanoate



1 *syn*-(2*R*,3*S*)-**2** *anti*-(2*S*,3*S*)-**2**

Enzyme	Coenzyme	Total activity/U	Config.	E.e./%	D.e./%
GKER-I	NADH	42	2*R*,3*S*	>99	88
GKER-II	NADH	40	2*R*,3*S*	>99	84
GKER-III	NADPH	36	2*S*,3*S*	95	>99

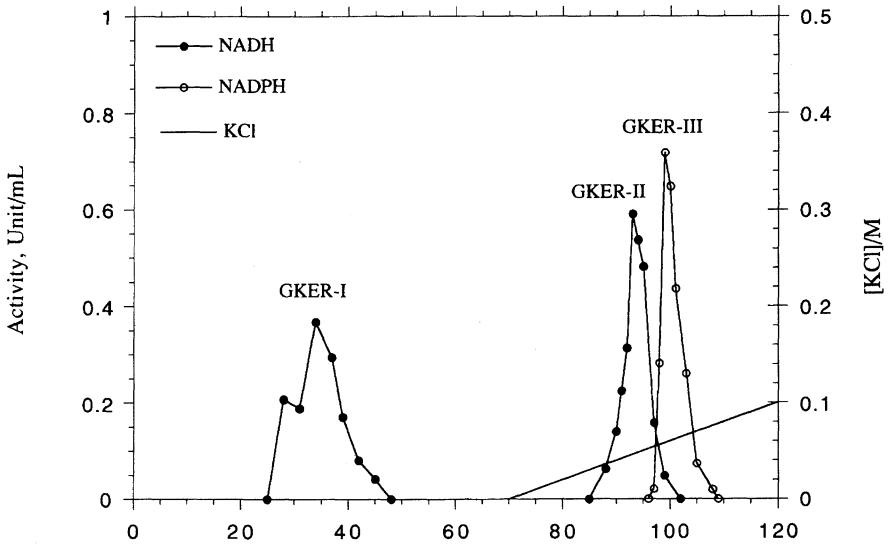


Fig. 1. Elution chromatogram of *G. candidum* keto ester reductases obtained with DEAE-Toyopearl.

3-oxobutanoate (**1**) as a substrate (Table 1). The data listed in Table 1 show that all three enzymes reduce keto ester **1** enantioselectively, and diastereoselectivities of GKER-I and II are *syn* predominant, whereas GKER-III affords the *anti*-isomer selectively. The total activities of the enzymes calculated from the chromatogram are also summarized in Table 1.

To elucidate kinetic parameters of GKERs for keto ester **1**, initial rates of the reductions were measured at various concentrations of the substrate. Michaelis constants, K_m, were determined by [S]/v–[S] plots. At least five concentration points of the substrate and three concentration points of the enzymes were used for obtaining a plot. The calculated K_ms are listed in Table 2. It should be noted that the K_m of *anti*-enzyme, GKER-III, is 50 times larger than those of *syn*-enzymes, GKER-I and -II; this difference proposes that the diastereoselectivity of the reduction depends on the substrate concentration. The dependency of diastereoselectivity on the concentration of **1** in the reduction with *Geotrichum candidum* was tested; the results are shown in Fig. 2. Although it has been confirmed that the substrate concentration influences the diastereoselectivity, the selectivity observed is still unsatisfactory for practical purposes.

Characterization of GKER-III. GKER-III is the most useful for synthetic purpose, because it produces a hydroxy ester with exclusive enantio- as well as diastereoselectivity. In a previous paper, we reported the reduction of keto ester **1** by this enzyme, in which glucose and glucose dehydrogenase were employed to regenerate NADPH.[14] Since it would be valuable to regenerate a coenzyme without any other enzyme, activities of some alcohols for regeneration of NADPH with this enzyme were measured. The results are summarized in Table 3. Hydrophobic 2-alkanols are the substrates that are oxidized with this enzyme. Among the alcohols tested, 2-hexanol was found to be most effective. However, the rate of oxidation of 2-hexanol is four-fold slower than that of the reduction of **1**, which means that the regeneration of NADPH is rate-limiting in the coupled redox system.

Effect of Additives on Enzyme Activity. As reported in a previous paper,[4] the addition of an additive to a system of microbial reduction can improve the stereoselectivity to afford the *anti*-isomer in the reduction of keto ester **1** with *Geotrichum candidum*. To obtain the information on the

Table 2. K_ms and K_i of GKERs

Enzyme	K_m/mM			K_i/mM
	1	MVK	Chloroacetone	MVK
GKER-I	0.24	0.22	0.17	—
GKER-II	0.22	0.22	0.045	—
GKER-III	11.0	—	61	180

Table 3. Oxidation of Alcohol with GKER-III

Alcohol	Relative activity[a]
2-Propanol	0
2-Butanol	2
2-Pentanol	26
4-Methyl-2-pentanol	36
2-Hexanol	100
2-Octanol	75[b]
3-Pentanol	29
3-Hexanol	27
1-Phenylethanol	55
2-Phenylethanol	0
1-Phenyl-2-propanol	3
4-Phenyl-2-butanol	53[c]
Cyclopentanol	0
Cyclohexanol	0
Methyl (*S*)-3-hydroxybutanoate	6
D-Glucose	0
Glycerol	0

a) [S] = 32 mM. b) [S] = 13 mM. c) [S] = 25 mM.

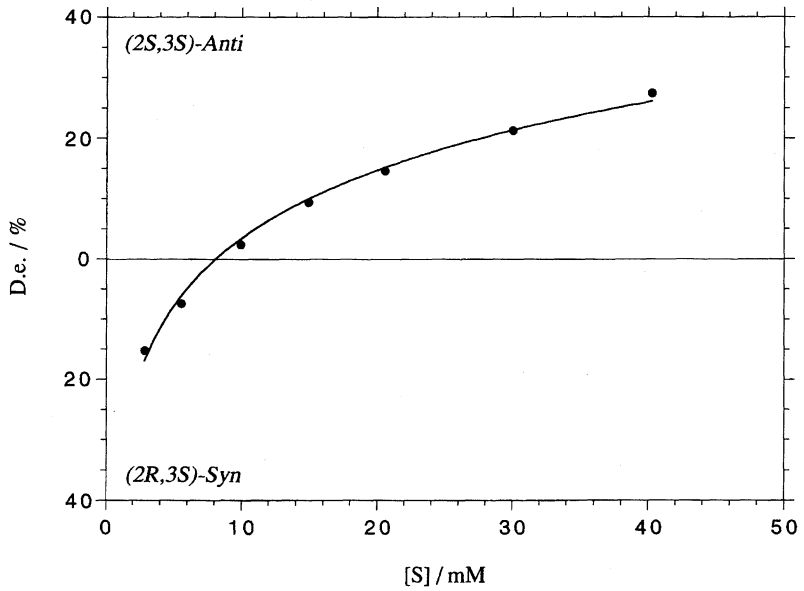


Fig. 2. Effect of substrate concentration on diastereoselectivity of the reduction of ethyl 2-methyl-3-oxobutanoate (**1**) with *G. candidum*.

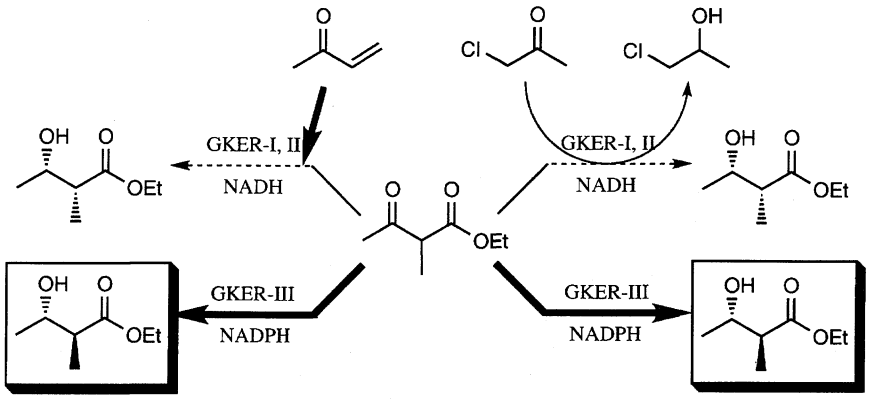


Fig. 3. Mechanism of stereochemical control with an additive in the diastereoselective reduction by *G. candidum*.

effect of additives on the stereoselectivity of the reduction, activities of each enzyme were measured in the presence of varying amounts of such additives as MVK and chloroacetone. Chloroacetone is a substrate for these enzymes, while MVK is a substrate only for GKER-I and -II. Thus, Michaelis constants, K_ms, of MVK and chloroacetone for these enzymes were determined by the same manner as that for **1** described above, and they are listed in Table 2. The results shown that these additives, rather than keto ester **1**, are good substrates for GKER-I and -II. On the other hand, K_m value, 61 mM, of chloroacetone for GKER-III is too large to be a practical substrate for the enzyme. GKER-III does not exhibit detectable activity toward MVK. Thus, the inhibition constant, K_i, of MVK for the enzyme was determined by a Dixon plot (plot of $1/v$ vs. [I]), from which K_i of 180 mM has been calculated. The value reveals that MVK hardly affects GKER-III activity.

In order to clarify the effect of an additive on the enzymes, GKER-I and -II were preincubated at 30 °C in the presence of an additive or both an additive and NADH. The concentration of each additive and NADH employed here was 1 mM, which is 5—20 times as large as K_m of each enzyme for the additive. After 5 min, the solution was diluted to 40 times, NADH and keto ester **1** were added to the mixture, then the mixture was subjected to kinetic measurements immediately. When the enzymes were pretreated by MVK only, the enzyme retained the original activities. In contrast, the activity was reduced to one-fourth of their original value when coenzyme coexisted during the preincubation. Since MVK exerts an inhibitory effect only when it is incorporated into the reduction system, it has been elucidated that MVK is a suicide substrate for GKER-I and -II. Similar experiments were performed with respect to chloroacetone. About 20% deactivation was observed when the enzymes were incubated in the presence of chloroacetone and NADH. It seems likely that chloroacetone is a competitive substrate rather than a suicide substrate.

Thus, we conclude that the increase in the *anti*-selectivity in the reduction of keto ester **1** in the presence of MVK is caused by the selective deactivation of *syn*-enzymes by MVK, because MVK is a suicide substrate for the *syn*-enzymes and hardly affects the *anti*-enzyme (Fig. 3). We also conclude that the *anti*-selective reduction of **1** in the presence of chloroacetone is caused by the difference in K_m for chloroacetone and **1**. The *syn*-enzymes catalyzes the reduction of chloroacetone in preference to **1**, whereas the *anti*-enzyme has opposite substrate selectivity; that is, keto ester **1** is reduced in preference to chloroacetone (Fig. 3).

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