Purification and Properties of an Asymmetric Reduction of Diethyl 2-Methyl-3-Oxosuccinate in *Saccharomyces fermentati*[†]

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Diethyl 2-methyl-3-oxosuccinate reductase was purified from cells of *Saccharomyces fermentati* by streptomycin treatment, Sephadex G-50 gel filtration, a first DEAE-cellulose chromatography, a first Toyopearl HW-60F gel filtration, a second DEAE-cellulose chromatography and a second Toyopearl HW-60F gel filtration. The enzyme was purified 169-fold and gave one band in sodium dodecyl sulfate(SDS)/polyacrylamide-gel electrophoresis. The molecular weight of the purified enzyme was 61,000 to 63,000. The enzyme was NADPH-dependent and had maximum activity at pH 6.0 and 50°C. It was stable at pH 7 to 8. The *K_m* values at pH 7.0 were 1.25 mM for diethyl 2-methyl-3-oxosuccinate and 111 mM for the α -methyl β -hydroxy esters *syn*-(2) and *anti*-(3). The enzyme reduced methyl 2-methyl-3-oxosuccinate, but did not act on other synthetic substrates.

The optical yields of the products *syn*-(2) and *anti*-(3) reduced by the purified enzyme were more than 99% enantiomer excess (*e.e.*) by measurement of the corresponding (+)-MTPA esters[*syn*-(10) and *anti*-(11)] on a 400 MHz NMR instrument.

α -Methyl β -keto esters^{1~8)} and β -methyl α -keto ester⁹⁾ have been reduced asymmetrically by yeasts. Both kinds of keto esters are reduced by a variety of yeasts to give optically active hydroxy esters with two chiral centers. For example, benzyl 2-methyl-3-oxobutyrate (5) is reduced by baker's yeast, *Saccharomyces cerevisiae*, to give a mixture of the (2*R*,3*S*)-*syn*- β -hydroxy ester and the (2*S*,3*S*)-*anti*- β -hydroxy ester with high optical purity (>99% *e.e.* and 84% *e.e.*, respectively).

The enzyme involved in this reaction has been purified from cells of baker's yeast. It seems to be benzyl 2-methyl-3-hydroxybutyrate dehydrogenase. The enzyme is constitutive and it does not have subunits.¹⁰⁾

Diethyl 2-methyl-3-oxosuccinate (1) is reduced by various yeasts to give a mixture of the (2*R*,3*R*)-*syn*- β -hydroxy ester (2) and the

(2*S*,3*R*)-*anti*- β -hydroxy ester (3).¹¹⁾

This paper reports the purification of the enzyme from *Saccharomyces fermentati* and its enzymatic properties.

MATERIALS AND METHODS

Microorganisms. *S. fermentati* KSY-16 was cultured in 800 ml of liquid medium in a 2-liter Erlenmeyer flask at 30°C for 48 hr on a rotary shaker. The composition of the medium was 5% glucose, 0.5% KH₂PO₄, 0.1% (NH₄)₂SO₄, 0.05% urea, 0.05% MgSO₄·7H₂O, 0.05% CaCl₂·2H₂O, 0.1% yeast extract, and a trace mineral solution (0.00002% FeSO₄·7H₂O, 0.00002% MnCl₂·4H₂O, and 0.00002% ZnSO₄·7H₂O). The cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and washed with distilled water. The washed cells were washed once with 5 mM phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 2 μ g/ml of phenylmethylsulfonylfluoride (PMSF). The harvested cells were stored at 4°C until use.

[†] Enzymes Producing Two Chiral Centers. Part II.

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Enzyme assay. Reduction of diethyl 2-methyl-3-oxosuccinate (**1**) was measured spectrophotometrically through the decrease of absorbance at 340 nm with a Hitachi spectrophotometer (model 200-10) at 30°C. The assay system contained 0.4 ml of 100 mM phosphate buffer (pH 7.0), 0.2 ml of 1 mM NADPH, 0.2 ml of 1 mM diethyl 2-methyl-3-oxosuccinate, and the enzyme solution to make a total volume of 1 ml in a cuvette with a light path of 1 cm. Under these conditions, the relationship between enzyme activity and concentration was linear. One unit of this enzyme activity is defined as the amount of enzyme that oxidizes 1 μ mol of NADPH per minute at 30°C under the assay conditions.

Analytical methods. Measurement of the protein concentration, estimation of the molecular weight of the enzyme, disc electrophoresis, isoelectrofocusing, and SDS electrophoresis were done by as described elsewhere.¹⁰⁾ Protein was stained with Coomassie Brilliant Blue R 250.

NMR spectra were measured on a JEOL GX-400 instrument. Spectra were taken in CDCl_3 with Me_4Si as the internal reference.

Chemicals. The substrates were synthesized as described previously.^{5~7,9)} NAD, NADP, NADH, and NADPH were purchased from the Kyowa Hakko Kogyo Co., Ltd. PMSF, AMP, ADP, ATP, α -ketoglutaric acid, D-glucuronic acid, and dihydroxyacetone phosphate lithium salt were purchased from the Sigma Chemical Co. Oxalacetic acid, cysteine, NaF, KCN, thiourea, moniodoacetate, and $\alpha\alpha'$ -dipyridyl were purchased from the Wako Pure Chemical Industries, Ltd. and (+)- α -methoxy α -trifluoromethylphenylacetic acid ((+)-MTPA) was purchased from the Aldrich Chemical Co. EDTA sodium salt was purchased from the Junsei Pure Chemicals Co., Ltd. 2-Mercaptoethanol, *o*-phenanthroline, and sodium PCMB were purchased from the Tokyo Kasei Kogyo Co., Ltd. Pyruvate was obtained from the Daiichi Pure Chemicals Co., Ltd. NaN_3 was purchased from the Kishida Chemicals Co., Ltd. Streptomycin sulfate, blue dextran 2000, cytochrome *c*, ovalbumin, and bovine serum albumin were obtained from the Boehringer GmbH. Toyopearl HW-60F and HW-55SF were purchased from the Toyo Soda M. Co., Ltd. An isoelectric focusing calibration kit for measurement of the isoelectric point of proteins in the pH range of 3~10, Sephadex G-50, and SP-Sephadex C-50 were obtained from the Pharmacia Fine Chemicals. Servalyt Precotes pH 3~10 for isoelectric focusing were obtained from the SERVA Feinbiochemica. All other chemicals were of reagent grade.

RESULTS

Purification

All purification procedures were at 4°C and the enzyme samples were stored at 4°C.

Centrifugation was done at $10,000 \times g$ for 20 min. The cells were harvested by centrifugation and washed once with 5 mM phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 2 $\mu\text{g}/\text{ml}$ of PMSF.

Step 1. Extraction. The washed cells (60 g, wet weight) in 5 mM phosphate buffer (200 ml) were disrupted twice by a French pressure cell press at $1,300 \text{ kg}/\text{cm}^2$. The resulting cell homogenate was spun down in an automatic preparative ultracentrifuge at $225,000 \times g$ for 30 min. The supernatant solution was used for further purification of the enzyme.

Step 2. Streptomycin-sulfate treatment. Streptomycin-sulfate (362 mg) was added to the cell-free extract to remove nucleic acids. The suspension was left for 30 min. The precipitate was removed by centrifugation ($17,000 \times g$ for 20 min), and the supernatant solution (153 ml) was obtained.

Step 3. Desalting by Sephadex G-50. The supernatant solution was concentrated on an Amicon membrane PM-10 and passed through a column of Sephadex G-50 equilibrated with 5 mM phosphate buffer (pH 6.0). The fractions (169 ml) with enzyme activity were collected.

Step 4. First column chromatography on DEAE-cellulose. The above solution was put on a column ($3 \times 20 \text{ cm}$) of DEAE-cellulose equilibrated with 5 mM phosphate buffer (pH 6.0). The column was washed with the same buffer and chromatography was done with a linear gradient concentration of NaCl (0~0.5 M) in 5 mM phosphate buffer (pH 6.0). The enzyme was eluted at 0.1 M NaCl. The active fractions (173 ml) were pooled and concentrated by ultrafiltration on an Amicon membrane PM-10.

Step 5. First gel filtration on Toyopearl HW-60F. The concentrated enzyme solution was put on a column ($2.5 \times 100 \text{ cm}$) of Toyopearl HW-60F equilibrated with 5 mM phosphate buffer (pH 6.0), and eluted with the same buffer. The active fractions were pooled and concentrated by ultrafiltration on an Amicon membrane PM-10.

Step 6. Second chromatography on DEAE-cellulose and second gel filtration on Toyopearl

TABLE I. SUMMARY OF THE ENZYME PURIFICATION

	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (μ /mg prot.)	Yield (%)	Purification (-fold)
Crude extract	196	19.1	4980	0.004	100	1
Ultracentrifugation	181	15.9	1730	0.009	83.2	2.4
Streptomycin-sulfate	153	13.5	861	0.016	70.7	4.1
Sephadex G-50	169	10.7	565	0.019	59.7	4.9
1st DEAE-cellulose	173	6.81	74.1	0.092	35.7	23.9
1st Toyopearl HW-60 _F	42	5.03	19.9	0.253	26.3	65.9
2nd DEAE-cellulose	51	3.49	8.75	0.399	18.3	104
2nd Toyopearl HW-60 _F	41	2.31	3.56	0.649	12.1	169

FIG. 1. Disc Electrophoresis and SDS Gel Electrophoresis of Purified Enzyme.

a, disc electrophoresis; b, SDS gel electrophoresis; A, RNA polymerase β' -subunit (165,000); B, RNA polymerase β -subunit (155,000); C, bovine serum albumin (68,000); D, RNA polymerase α -subunit (39,000); E, sample.

HW-60F. The concentrated enzyme solution was put on a column (2.5×14 cm) of DEAE-cellulose equilibrated with the phosphate buffer and the enzyme was eluted with an increasing linear gradient of NaCl ($0 \sim 0.2$ M) in 400 ml of the same buffer. The active fractions were pooled and concentrated by ultrafiltration on an Amicon PM-10 membrane. Gel filtration on a column (2.5×100 cm) of Toyopearl HW-60F equilibrated with the same buffer followed. The active fractions were combined and concentrated. This preparation was used for the following experiments. The purification is summarized in Table I.

Homogeneity

The homogeneity of the purified enzyme was

checked by disc electrophoresis and SDS-electrophoresis. The purified enzyme was a single polypeptide (Fig. 1).

Physical properties

The molecular weight of the enzyme was estimated by gel filtration on a Toyopearl HW-55 superfine column by the method of Andrews.¹²⁾ The apparent molecular weight was 61,000. SDS-polyacrylamide gel electrophoresis gave a single protein band with a molecular weight of 63,000. The results suggested that the enzyme was a single polypeptide chain. The isoelectric point was calculated with the Servalyt Precotes (pH 3~10). The enzyme activity was as a single band located at pH 4.9.

FIG. 2(a). Effects of pH on Activity of Enzyme.

The optimum pH of the enzyme activity was studied in 100 mM tartaric acid for pH 4~5, 100 mM phosphate buffer for pH 6~8, and 100 mM glycine-NaOH buffer for pH 9~11.

FIG. 2(b). Effects of pH on Stability of Enzyme.

Purified enzyme was treated in each buffer for 30 min at 30°C. After the pH was adjusted to 7.0 with 200 mM phosphate buffer at pH 7, the residual activity was measured with the standard assay conditions.

Effect of pH

The optimum pH of the purified enzyme was 6.0 in 100 mM phosphate buffer (Fig. 2a). The stability of the enzyme at various pH values was studied by incubation of the enzyme solution at each pH indicated in Fig. 2b for 30 min at 30°C. The remaining activity was assayed under the standard assay conditions. The enzyme was most stable at pH 6~9.

Effect of temperature

Changes in the enzyme activity with temperature were investigated. The optimum temperature was about 50°C (Fig. 3a). The thermal stability of the enzyme was also examined. The enzyme solution was incubated at each

FIG. 3(a). Effects of Temperature on Activity of Enzyme.

Purified enzyme activity was assayed by the standard assay method at various temperatures.

FIG. 3(b). Thermal Stability of Enzyme.

Purified enzyme was treated at each temperature for 10 min in 100 mM phosphate buffer (pH 7) and the residual activity was measured by the standard assay method.

temperature indicated in Fig. 3b for 10 min and the remaining activities were assayed under the standard assay conditions. The enzyme was stable up to 30°C, and it readily lost its activity at above 60°C.

K_m values

The velocity of reduction by the enzyme was measured at pH 7. The assay mixture contained 0.8 ml of a diethyl 2-methyl-3-oxosuccinate (1) solution and 0.5 ml of enzyme solution. The decrease in absorbance at 340 nm was measured at 40°C. The *K_m* value for the reduction was 1.25 mM. The velocity of the oxidation was also measured at pH 7. The assay mixture contained 0.8 ml of 100 mM phosphate buffer (pH 7), 1.2 ml of various concentrations of α -methyl β -hydroxy esters

[*syn*-(2) and *anti*-(3)] solutions, 0.5 ml of 2 mM NADP, and 0.5 ml of enzyme solution. The increase in absorbance at 340 nm was measured at 40°C. The *K_m* value for the oxidation was 111 mM.

Substrate specificity

The ability of the enzyme to catalyze the reduction of various compounds was examined by the standard assay method. Synthetic substrates such as benzyl 2-methyl-3-oxobutyrate (5), ethyl 2-chloro-3-oxobutyrate (6), methyl 3-phenyl-2-oxobutyrate (7), ethyl 2-methyl-3-oxobutyrate (8), and methyl 3-(2'-furyl)-2-methyl-3-oxopropionate (9) were not reduced. Dimethyl 2-methyl-3-oxosuccinate (4) was reduced slightly. Little activity was observed with the other compounds tested.

Reduction of aldehydes

The activity of the enzyme on various aldehydes was examined. Acetaldehyde, formaldehyde, isobutyraldehyde, *n*-valeraldehyde, *n*-hexylaldehyde, *n*-heptaldehyde, *p*-hydroxy benzaldehyde, *p*-dimethylamino benzaldehyde, benzaldehyde, and furfural were not reduced.

Substrate specificity with various alcohols and other substrates

For millimolar solutions of methanol, ethanol, *n*-propyl alcohol, isopropyl alcohol, *n*-butyl alcohol, isobutyl alcohol, *n*-amyl alcohol, isoamyl alcohol, benzyl alcohol, glycerin, shikimic acid, 2-deoxy-D-glucose, and glucose-6-phosphate in buffer, the solutions were not oxidized at all in the presence of NAD or NADP.

Effects of metal ions on the enzyme activity

The effects of metal salts on the enzyme activity were investigated. Results are shown in Table II. The enzyme activity was strongly inhibited by Cu²⁺, Fe³⁺, and Ag⁺.

Effects of various reagents on the enzyme activity

The enzyme activity was measured in the

TABLE II. EFFECTS OF METAL IONS ON ENZYME ACTIVITY

The assay was done by the standard assay method with NADPH as a cofactor.

Reagents (1 mM)	Activity (%)
None	100
CaCl ₂ · 2H ₂ O	100
ZnSO ₄ · 7H ₂ O	69
MgCl ₂ · 6H ₂ O	100
CuSO ₄ · 5H ₂ O	0
HgCl ₂	100
MnCl ₂ · 4H ₂ O	100
NiCl ₂ · 6H ₂ O	93
BaCl ₂ · 2H ₂ O	107
FeCl ₃ · 6H ₂ O	0
AgNO ₃	0
CoCl ₃ · 6H ₂ O	105

TABLE III. EFFECTS OF VARIOUS REAGENTS ON THE ENZYME ACTIVITY

Reagents	Concentration (mM)	Relative activity (%)
None		100
NaCl	1000	0
KCl	1000	17
NAD	0.2	100
NADH	0.2	97
NADP	0.2	86
FMN	0.2	62
FAD	0.2	93
AMP	1	100
ADP	1	100
ATP	1	100
Cysteine	10	0
α,α'-Dipyridyl	10	69
EDTA	10	100
PCMB	10	100
<i>o</i> -Phenanthroline	10	28
NaF	10	100
NaN ₃	10	100
Thiourea	10	100
KCN	10	0
Monoiodoacetate	10	0

presence of various salts, nucleotides, and inhibitors. The results are summarized in Table III. The enzyme activity was inhibited by 10 mM cysteine, 10 mM KCN, and monoiodoacetate.

The first systematic study of the stereochemical course of reduction of acyclic ketones by *S. cerevisiae* was reported by MacLeod *et al.*¹⁴⁾ They examined the reduction of a series of ketones with all combinations of substituents: methyl, ethyl, *n*-propyl, *n*-butyl, and phenyl. The sterically hindered ketones, including *n*-butyl *n*-propyl ketone, *t*-butyl methyl ketone, isobutyl isopropyl ketone, and *n*-amyl phenyl ketone, were not reduced by yeast. Similar results were obtained by other groups.^{15~18)} There are many oxidoreductases in baker's yeast that might catalyze the reduction of these β -keto-carboxylic-acid derivatives. However, crystalline yeast alcohol dehydrogenase I (EC 1.1.1.1) and purified yeast L-lactate dehydrogenase (EC 1.1.1.27) do not reduce ethyl or octyl 4-chloroacetoacetate.¹⁹⁾ On the other hand, β -hydroxyacetyl-CoA dehydrogenase from porcine heart²⁰⁾ (EC 1.1.1.35), an enzyme involved in the degradation of fatty acids, reduces both keto esters in the presence of NADPH to their respective (*R*)-4-chloro-3-hydroxybutyrates.¹⁹⁾ The purified enzyme reported here differs from the enzyme reported elsewhere¹⁰⁾ in that its oxidation activity was very weak, its molecular weight was 61,000 to 63,000, and the substrate specificity was different.

We concluded that the enzyme from *S. fermentati* is an NADPH-dependent dehydrogenase and that it produces two chiral centers by asymmetric reduction of diethyl 2-methyl-3-oxosuccinate (**1**) or dimethyl 2-methyl-3-oxosuccinate (**4**). Its enzymatic activity was inhibited by Cu^{2+} , Fe^{3+} , and Ag^{+} . Of the chemical reagents tested, the enzymatic activity was inhibited by cysteine, KCN, and monoiodoacetate. When the purified enzyme was used, the optical purity of the reduction products *syn*-**2** and *anti*-**3** was almost 100% *e.e.* in comparison with the use of the native microorganism.

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