Asymmetric Reduction of 1-Acetoxy-2-alkanones with Bakers' Yeast: Purification and Characterization of α-Acetoxy Ketone Reductase

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An α-acetoxy ketone reducing enzyme has been purified and characterized from the cell-free extract of bakers' yeast (*Saccharomyces cerevisiae*). Only one NADPH-dependent dehydrogenase that catalyzed the reduction of α-acetoxy ketone was found in bakers' yeast. The molecular weight of the enzyme was estimated to be 36 kDa by SDS-polyacrylamide gel electrophoresis. The enzyme was composed of a single polypeptide chain. The enzyme had reducing activity for both aliphatic and aromatic α-acetoxy ketones, although no reducing activity toward α-chloro ketones and α-hydroxy ketones was found. The enzyme catalyzed the reduction of not only α-acetoxy ketones, but also β-keto esters. Studies on the chromatographic behavior and stereospecificity indicated that the enzyme was identical with one of the β-keto ester reductases purified from bakers' yeast.

Optically active aliphatic diols and their derivatives are of interest as pharmaceuticals, pharmaceutical intermediates[1,2] and chiral ligands.[3—5] To synthesize chiral diols, chemical methods are generally investigated. For example, Fuji et al.[6] and Sharpless et al.[7—11] reported that the asymmetric dihydroxylation of substituted allyl aryl ethers by use of AD-mix-β gave the corresponding chiral diols with high stereoselectivity. Biological methods are also effective; it is known that a bakers' yeast reduction of 1-hydroxy-2-alkanones gives (*R*)-1,2-alkanediols[12—16] in high optical purities. The problem remaining in the reduction with biocatalysts is the development of a new method to obtain optically pure (*S*)-diols. To control the stereochemistry of a bakers' yeast reduction for the preparation of (*S*)-diols, we previously investigated the reduction of 1-acetoxy-2-alkanones by a bakers' yeast cell-free extract with glucose as a hydride source for the coenzyme regeneration. We found that the reduction gave antipodal (*S*)-1-acetoxy-2-alkanols in high enantiomeric excesses (>95%e.e.) with high chemical yields (59—88%) for all of the substrates having short to long alkyl groups.[17]

To gain in insight into the mechanistic interpretation of the bakers' yeast reduction, we have tried to isolate the α-acetoxy ketone reductase from the cells of pressed bakers' yeast. Herein, we would like to report the purification and characterization of the enzyme.

Results and Discussion

Purification of α-Acetoxy Ketone Reductase. The α-acetoxy ketone reductase (AcKR) has been isolated from a bakers' yeast cell-free extract by hydrophobic interaction chromatography. The enzyme was further purified in short steps by chromatographic methods, which included anion-exchange column chromatography and gel filtration chromatography. The enzyme activities of the enzyme purification steps are summarized in Table 1. In each of the purification stages, no other enzyme proteins having the acetoxy ketone reducing activity were found. The enzyme utilized NADPH as a sole coenzyme. The enzyme was purified 3061-fold over a crude extract; the specific activity of the purified AcKR was 150 units/mg-protein. The molecular weight of the enzyme was estimated to be 36 kDa by SDS-PAGE (Fig. 1) and was 39 kDa by Superdex 75 gel filtration. The results showed that the enzyme protein was composed of a single polypeptide chain.

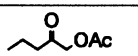
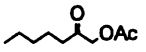
Table 1. Purification Steps of the Enzyme

Steps	Total activity	Total proteins	Specific activity	Yield
	U	mg	U mg^{-1}	%
Cell-free extract	458	9340	0.0490	100
50% $(NH_4)_2SO_4$	429	7750	0.0533	94
Butyl-Toyopearl	272	726	0.375	59
Phenyl-Superose	189	68.6	2.76	41
Mono Q	82.1	16.3	5.03	18
Superdex 75	70.1	0.467	150	15

Stereospecificity of AcKR. To clarify the stereoselectivity of the product alcohol reduced by AcKR, α-acetoxy ketones (1-acetoxy-2-pentanone and 1-acetoxy-2-heptanone) were reduced by purified AcKR. The yields of the products were 71 and 79% (1-acetoxy-2-pentanol and 1-acetoxy-2-heptanol, respectively). The ee's of both products were more than 99%, which is identical with that obtained by use of bakers' yeast whole cells[18] (Table 2). This result supports that α-acetoxy ketones are reduced by a single enzyme (α-acetoxy ketone reductase) in bakers' yeast.

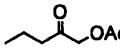
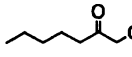
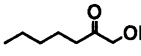
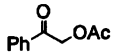
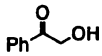
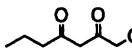
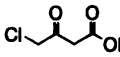
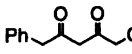
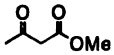
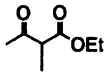
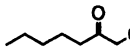
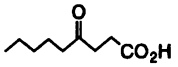
Substrate Specificity of AcKR. The relative activities of the enzyme towards various α-hydroxy ketones, α-acyloxy ketones, α-chloro ketones, β-keto esters, and a keto acid were examined with a view of gaining some information concerning the identity of its substrate specificity. 1-Acetoxy-2-propanone was used as a standard (Tables 3 and 4).

Table 2. Enzymatic and Microbial Reductions of 1-Acetoxy-2-alkanones

Substrate	Purified AcKR[a]			Bakers' yeast[b]		
	Yield/%	e.e./%	*R/S*	Yield/%	e.e./%	*R/S*
	71	>99	*S*	55	99	*S*
	79	>99	*S*	51	99	*S*

a) AcKR refers to α-acetoxy ketone reductase. b) Data taken from Ref. 18.

Table 3. Relative Activity of AcKR Catalyzed Reduction of Various Ketones

Compound	Rel. rate[a]	Compound	Rel. rate
	100		0
	79		0
	56		0
	2		0
	20		85
	12		5
	0		1
	0		0

a) Relative rates were determined as described in the Experimental section.

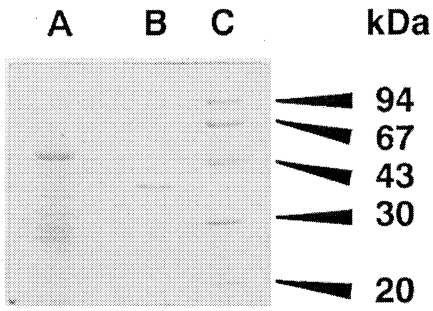
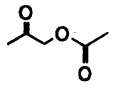
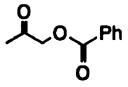
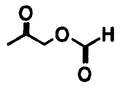
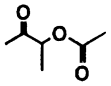
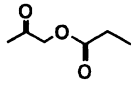
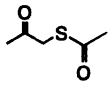
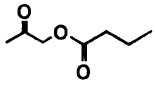


Fig. 1. SDS-PAGE of the purified enzyme. SDS-gel electrophoresis using 12.5% polyacrylamide was performed by the method of Laemmli[31] in the presence of 0.1% SDS; A: 10 μg of the cell-free extract; B: 10 μg of the purified enzyme (AcKR); C: 10 μg of molecular weight standards; phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000).

Table 4. Relative Activity of AcKR Catalyzed Reduction of α-Acyloxy Ketones and Derivatives

Compound	Rel. rate[a]	Compound	Rel. rate
	100		107
	31		15
	110		167
	124		27

a) Relative rates were determined as described in the Experimental section.

AcKR showed reducing activity for both aliphatic and aromatic α-acetoxy ketones. It is probable that AcKR is related to the highly enantioselective reduction of phenacyl acetate by bakers' yeast as reported by Santaniello et al.[19]

AcKR also indicated reducing activity toward various β-keto esters such as ethyl 4-chloro-3-oxobutanoate,

methyl 3-oxobutanoate, and ethyl 2-methyl-3-oxobutanoate. Thus AcKR catalyzes the reduction not only of α-acetoxy ketones, but also of β-keto esters. However, AcKR had no reducing activity toward α-chloro ketones (aliphatic and aromatic), α-hydroxy ketones (aliphatic and aromatic), and 4-oxononanoic acid.

Further, the substrate specificity with ketones having other acyloxy groups were investigated (Table 4). The enzyme has wide substrate specificities at the acyloxy groups; a formyloxy, an acetoxy, an propionyloxy, a butyryloxy, a benzoyloxy, an acetylthio, and a methylthio groups served as substrates. The carbonyl group in the acyloxy groups is necessary for the high reducing activity of AcKR, since the enzyme had no reducing activity toward α-hydroxy ketones. It is suggested that the high reducing activity of 1-acetylthio-2-propanone can be attributed to steric and electronic effects of the sulfur atom. The AcKR had a low activity toward 1-methylthio-2-propanone, apparently because of the lack of a second carbonyl group in the substituent.

The kinetic parameters, K_m (Michaelis constant) and k_{cat}, of purified AcKR on 1-acetoxy-2-propanone and ethyl 4-chloro-3-oxobutanoate were measured in order to gain an insight into the velocity of enzymatic reductions of the substrates (Table 5). The K_m and k_{cat} values of 1-acetoxy-2-propanone were measured to be 0.21 mM and 5.04 s^{-1}, respectively. Their values of ethyl 4-chloro-3-oxobutanoate were 0.22 mM and 5.01 s^{-1}. The k_{cat}/K_m ratios of both substrates (the former : the later=100 : 90) reflect their relative activity (100 : 85).

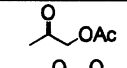
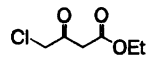
N-Terminal Amino Acid Sequence and Homology of AcKR. To investigate the protein structure, the N-terminal amino acid sequence of the purified AcKR was analyzed by a protein sequencer.[20]

The N-terminal amino acid sequence of the first 16 residues of the enzyme was ^{1}TAPLVVLGNPLLDFQA---. The homology of the amino acid sequences in the N-terminal region of the enzyme and other enzymes was investigated by a computer search of the NBRF protein sequence data bank.[21] No enzyme proteins had a significant sequence similarity to AcKR.

Relationship between α-Acetoxy Ketone Reductase and β-Keto Ester Reductases. Seven kinds of the β-keto ester reductases have been found and purified from bakers' yeast.[22—26] Nakamura et al.[22] have isolated four β-keto ester reductases (L-enzyme-1, L-enzyme-2, D-enzyme-1, D-enzyme-2) from bakers' yeast (Oriental Yeast). Sih et al.[23,24] also isolated three enzymes of β-keto ester reductases (L-enzyme 1, L-enzyme 2, D-enzyme) from bakers' yeast (Red star). Our isolated enzyme might be identical with the L-enzyme-2,[22] considering the enzymological properties, including the molecular weight, stereoselectivity substrate specificity, kinetic parameters, and chromatographic behavior as described above.

The exact chromatographic separation of enzymes in the cell-free extract by a Mono Q ion-exchange column on a SMART™ System (Pharmacia)[27] was investigated in order to judge whether AcKR is the same protein as L-enzyme-2 or not, because only the two L-selective enzyme proteins have been known in the extract. As shown in Figs. 2 and 3, AcKR and L-enzyme-2 were eluted into the same fractions. The molecular weight (36—39 kDa) of the purified AcKR was the same as that of L-enzyme-2 reported by Nakamura et al.[22] α-

Table 5. Kinetic Constants of Reduction Catalyzed by AcKR

Substrate	K_m/mM	k_{cat}/s^{-1}
	0.21	5.04
	0.22	5.01

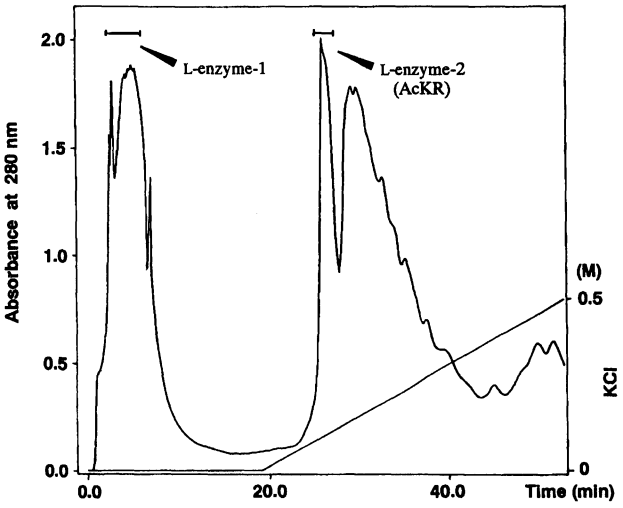


Fig. 2. Chromatographic separation of reductases from the cell-free extract of *S. cervisiae*.

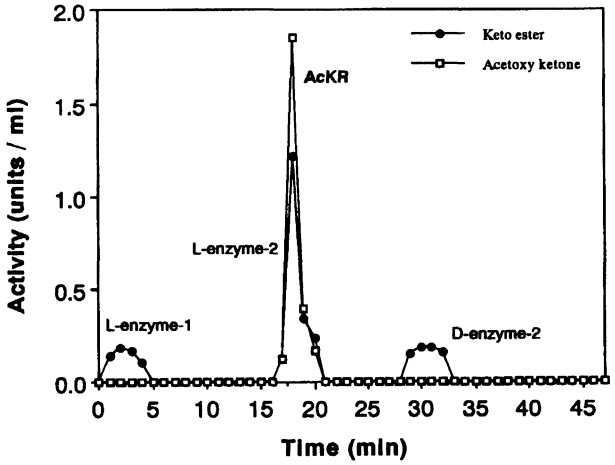
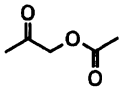
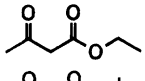
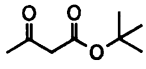


Fig. 3. Reducing activity toward β-keto ester and α-acetoxy ketone on Mono Q chromatography. α-Acetoxy ketone : 1-acetoxy-2-propanone, β-keto ester : ethyl 4-chloro-3-oxobutanoate.

Table 6. Relative Rates of AcKR Catalyzed Reduction of Various Ketones

Compound	Rel. rate[a)]
	100
	8
	10
Acetyl-CoA	0
Acetoacetyl-CoA	5

a) Relative rates were determined by arbitrarily setting the rate of reduction for 1-acetoxy-2-propanone to be 100.

Acetoxy ketones did not serve as substrates for L-enzyme-1. Considering these results, the protein of AcKR is identical with that of L-enzyme-2.

Further details concerning the protein structure and reaction mechanism are currently under investigation.

Role of AcKR in Whole Yeast Cells. So far, several enzymes that catalyzed the stereoselective reduction of β-keto esters were purified from *Saccharomyces cerevisiae*.[22—26] However, the natural substrates of these enzymes have not yet been elucidated. Here we would like to consider the role of α-acetoxy ketone reductase (AcKR) in a microbial cell. AcKR has high activity toward substrates having hydrophobic and bulky groups in the acyl moiety as indicated by 1-benzoyloxy-2-propanone (Table 4). Further screening of active substrates revealed that AcKR showed activity toward *t*-butyl 3-oxobutanoate and acetoacetyl-CoA, but no activity toward acetyl-CoA (Table 6). Previously, Sih et al.[23] reported that mitocondrial fractions of bakers' yeast reduced acetoacetyl-CoA, although only a trace of reductase activity was detectable using a β-keto ester as a substrate. However, they didn't describe the natural substrate of isolated enzyme from yeast and the role of the enzyme in the yeast cell. We found that AcKR has a low, but distinct, reducing activity toward acetoacetyl-CoA. It is suggested that the natural substrate of AcKR is acetoacetyl-CoA.[28]

Experimental

Instruments. UV spectra were obtained on a Beckmann DU-64 spectrophotometer. Preparation of a bakers' yeast cell-free extract was performed by using a Vibrogen Vi4 cell mill and a Hitachi HIMAC SCR20B centrifuge. Preparative chromatography for enzyme purification was performed on a Pharmacia FPLC system.

Materials. Bakers' yeast and MW-Maker (a mixture of cytochrome c, 12.4 kDa; adenylate kinase, 32 kDa; enolase, 67 kDa; lactate dehydrogenase, 142 kDa; glutamate dehydrogenase, 290 kDa) were purchased from Oriental Yeast Co. Butyl-Toyopearl was purchased from Tosoh Co. Glucose 6-phosphate dehydrogenase, acetyl-CoA, and acetoacetyl-CoA were purchased from SIGMA. The Phenyl-Superose column, Mono Q column, and Superdex 75 column were purchased from Pharmacia Fine Chemicals. CentriCell centrifugal ultrafilters (10000 NMWL) were purchased from Polysciences, Inc. Centricon-3 microconcentrators (3000 MW cutoff) were purchased from Amicon Grace Co. 2-Morpholinoethanesulfonic acid (MES) and 2-hydroxy-3-morpholinopropanesulfonic acid (MOPSO) were purchased from Dojin Chemical Laboratory Co. Diisopropyl fluorophosphate (DFP) was purchased from Wako Pure Chemical Industries, Ltd. 1-Chloro-2-propanone, phenacyl chloride, and acetol (hydroxyacetone) were purchased from Nacalai Tesque Co. Ethyl 4-chloro-3-oxobutanoate, methyl 3-oxobutanoate, and ethyl 2-methyl-3-oxobutanoate were purchased from Tokyo Kasei Organic Chemicals. Other reagents used were of analytical grade.

1-Acetoxy-2-alkanones were prepared by the reaction of the corresponding 1-chloro-2-alkanones with potassium acetate in benzene–water (20 : 1) in the presence of tetrabutylammonium bromide under reflux for 6 h in a similar way to that described in our previous paper.[29] Other substrates were prepared by our laboratory. The methods of preparation will be reported in additional papers in due course.

Enzyme Assay. A 10 μl aliquot of chromatographic fraction (vide infra) was added to 890 μl of a solution of 0.1 M MES buffer (pH 6.0) that also contained 1-acetoxy-2-propanone (0.25 mM) and NADPH (0.3 mM) (1 M=1 mol dm^{-3}). The rate of reaction was determined spectrophotometrically at 37 °C by following the decrease in the absorbance of NADPH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute at 37 °C. Specific activity was expressed as units per milligram of protein. The relative activity was determined by arbitrarily setting the rate of reduction for 1-acetoxy-2-propanone to be 100.

Protein Determination. Protein was measured by the method of Bradford (Bio-Rad Co.)[30] with crystalline bovine serum albumin as a standard.

Purification of the α-Acetoxy Ketone Reductase. The enzyme was isolated, purified, and stored at 4 °C. For prolonged storage, the enzyme was frozen in 10% aqueous glycerol. Thus, raw pressed bakers' yeast (350 g) was suspended in 700 ml of 0.1 M MES buffer (pH 6.0) containing 0.5 mM DFP and 1 mM 2-mercaptoethanol. The suspended cells were homogenized with 700 ml of glass beads (0.5 mm in diameter) using a Vibrogen cell mill at 4 °C. The homogenate was centrifuged at 10000×*g* for 30 min. To a supernatant liquid (725 ml) was added $(NH_4)_2SO_4$ solid (211 g, 50% saturation) and stirred for 10 h at 4 °C, and then centrifuged at 10000×*g* for 60 min. The supernatant solution (796 ml) was applied to a 44 mm×110 mm column packed with Butyl-Toyopearl 650 equilibrated with 50 mM MES buffer (pH 6.0) containing 1 M $(NH_4)_2SO_4$. The protein was eluted with 1 to 0 M linear gradient of $(NH_4)_2SO_4$ dissolved in 50 mM MES buffer (500 ml). The active fractions were collected and concentrated to 7.2 ml by a ultrafiltration membrane (CentriCell). The enzyme solution was applied to a Phenyl-Superose column equilibrated with 50 mM MES buffer (pH 6.0) containing 1 M $(NH_4)_2SO_4$. The column was eluted with a linear gradient concentration of 1 to 0 M $(NH_4)_2SO_4$ dissolved in the same buffer. The fractions that

showed activity were combined and concentrated to 4.2 ml and the buffer was exchanged with 20 mM MOPSO (pH 7.0) on CentriCell. The concentrated solution of enzyme was applied to Mono Q column equilibrated with 20 mM MOPSO buffer (pH 7.0). The protein was eluted with a linear gradient concentration of 0 to 0.5 M KCl dissolved in the same buffer. The active fractions were combined and concentrated to 0.9 ml by a ultrafiltration membrane (Centricon-3).

Estimation of the Molecular Weight of the Enzyme. A solution of enzyme of MW-Marker was applied to a Superdex 75 gel filtration column equilibrated with 20 mM MOPSO buffer (pH 6.0) containing 0.1 M KCl. After elution with the same buffer (flow rate of 0.8 ml min^{-1}), the activity of each fraction was assayed as described previously. A portion of the purified enzyme was subjected to SDS-polyacrylamide gel (12.5%) electrophoresis.[31]

Determination of K_m and k_{cat} Values of Enzyme. The K_m and k_{cat} values of the enzyme were calculated from the initial rates of the reaction in an appropriate range of the substrate concentration using at least five points by a [S]/v-[S] plot.

Enzymatic Reduction of 1-Acetoxy-2-alkanone. In a glass reaction vessel were placed a concentrated solution of the enzyme (5 units), NADPH (10 mg), G6PDH (glucose 6-phosphate dehydrogenase, 25 units, 0.15 mg), G6P (glucose 6-phosphate, 300 mg), 1-acetoxy-2-alkanone (0.5 mmol), and 0.1 M-MES buffer (pH 6.0, 10 ml). The reaction vessel was shielded from light. The mixture was magnetically stirred for 12 h at 35 °C. Hyflo-Super-Cel and ether were added. The mixture was filtrated. The solid collected was washed with ether. The combined washings and filtrate were washed (brine), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by preparative silica-gel chromatography to give the corresponding alcohols. The enantiomeric excesses of the products were determined by using 200 MHz ^1H NMR spectra of MTPA esters.

N-Terminal Sequence Analysis. The purified enzyme (500 pmol) was used directly for automated Edman degradation with an Applied Biosystems 476A gas–liquid phase protein sequencer.[20]

Computer Analysis for Protein Sequence Homology. A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank for sequence homology was carried out using an NEC PC-9801RS computer with DNASIS software.[21]

Chromatographic Separation of the Reductases from Bakers' Yeast. The bakers' yeast cell-free extract (20 ml, corresponding to 10 g yeast) of *S. cerevisiae* was analyzed by a SMART™ system equipped on a Mono Q column.[27] Anion-exchange chromatography was performed on a Mono Q PC 1.6/5 (1.6 mm×5 cm) column with a linear gradient of 0 to 0.5 M KCl in MOPSO buffer (pH 7.0) at a flow rate of 0.2 ml min^{-1} at 4 °C.

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28) It is probable that the low activity for acetoacetyl-CoA depends on a low value of Vmax. In fact, although

L-3-hydroxybutyryl-CoA dehydrogenase from *Clostridium kluyveri* has a low K_m value (50 μM) against acetoacetyl-CoA,[32] fatty acid synthease from yeast has a low specific activity (0.775 U mg^{-1}).[33] Thus L-3-hydroxyacyl-CoA dehydrogenase may have a low reducing activity for acetoacetyl-CoA in the cell of yeast. Considering these results, it is suggested that AcKR with NADPH in the yeast whole cell takes acetoacetyl-CoA as a natural substrate, being defined as 3-hydroxyacyl-CoA dehydrogenase.

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