Purification and cDNA Cloning of NADPH-Dependent Aldoketoreductase, Involved in Asymmetric Reduction of Methyl 4-Bromo-3-Oxobutyrate, from *Penicillium citrinum* IFO4631

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***Penicillium citrinum* was found to catalyze the reduction of methyl 4-bromo-3-oxobutyrate to methyl (S)-4-bromo-3-hydroxybutyrate [(S)-BHBM] with high optical purity. From the strain, a cDNA clone encoding a novel NADPH-dependent alkyl 4-halo-3-oxobutyrate reductase (KER) was isolated. *Escherichia coli* cells over-expressing KER produced (S)-BHBM in the presence of an NADPH-regeneration system.**

Recently, many studies have reported the synthesis of β -hydroxyesters from the corresponding ketones (8, 11). According to these studies, enzymatic reduction processes are the most efficient methods to obtain β -hydroxyesters with high optical purity. β -Hydroxyesters are useful for key pharmaceutical intermediates: L-carnitine from the (R)-enantiomer (19) and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor from the (S)-enantiomer (13). Until now, some kinds of yeasts, fungi, and bacteria (1, 3, 13, 14, 15, 17, 18, 19) have been found to catalyze the asymmetric reduction of ethyl 4-chloro-3-oxobutyrate to ethyl (S)- or (R)-4-chloro-3-hydroxybutyrate. However, there has been little information about the enzyme which could reduce methyl 4-bromo-3-oxobutyrate (BAM) to methyl (S)-4-bromo-3-hydroxybutyrate [(S)-BHBM]. Because of its high chemical reactivity, BHBM would be easier to convert to an intermediate of a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor than ethyl 4-chloro-3-hydroxybutyrate. Patel et al. reported the preparation of (S)-BHBM from BAM with whole cells of *Geotrichum candidum* (13). However, *G. candidum* catalyzed the reduction of BAM to (S)-BHBM with a low enantiomeric excess (EE; 80%). Therefore, we have screened various kinds of microorganisms for the reduction of BAM to BHBM.

From 522 microorganisms (bacteria, 435 strains; yeast, 49 strains; fungi, 38 strains), 28 microorganisms (bacteria, 7 strains; yeast, 4 strains; fungi, 17 strains) which produced BHBM with a high EE (>80%) were selected in the first screening, and then five microorganisms were chosen in the second and third screenings (Table 1). The first screening was performed in an aqueous system, but the second and third

screenings were performed in a two-phase system of water-*n*-butyl acetate, because BAM was unstable in water but stable in some organic solvents. In the third screening, acetone-dried cells were adopted to select the organic solvent tolerant biocatalyst. From the screening, we found that *Penicillium citrinum* IFO4631 and *Bacillus alvei* IFO3343 could catalyze the reduction of BAM to optically active BHBM with more than 90% EE. *P. citrinum* IFO4631 showed (S)-selectivity for BAM. With regard to the coenzyme requirement, *P. citrinum* IFO4631 depended on NADPH, while NADH did not serve as a cofactor.

The NADPH-dependent alkyl 4-halo-3-oxobutyrate reductase (KER) was purified about 100-fold from the cell extract of *P. citrinum* by three steps of column chromatography with a yield of 0.1% (Table 2). The purified KER showed a single band, with a molecular mass of about 39 kDa, on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The molecular mass of the native enzyme was 33 kDa by gel filtration. These data showed that KER was a monomeric protein.

The purified KER was digested with trypsin, and the digested peptides were separated by high-pressure liquid chromatography. Five peptides (S1 to S5) were isolated, and the amino acid sequences were determined by a protein sequencer. These peptides showed the following amino acid sequences: S1, NIMPVAYSPLGSQNQVP; S2, IPGVFGTFAS; S3, SIELSDADFEAINAVAK; S4, MIGVANYTIADLEK; and S5, YEDVLXXIDDSLKR. Comparison of the S1 sequence with proteins in databases revealed that it showed a strong similarity to the putative oxidoreductase gene of *Gibberella zeae* (6). As the N-terminal amino acid sequence of native KER was not determined, the N-terminal amino acid of KER seemed to be blocked.

To isolate a cDNA clone encoding KER on the basis of its partial amino acid sequences, primer P1 (5'-TANGCNACNGGCATAATGTT-3') for the internal amino acid sequence (S1) and primer SK (Stratagene, La Jolla, Calif.) for λ ZapII were

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KER	1:MSNGKTF--T-----LSNGVKIPGVGFGTFASEGSKGETYTAVTALKT	42
GCY1	1:MPATLHD--STKI-L-----S----LNTGAQIPQIGLGTWQSKEN--DAYKAVLTALKD	45
ALDX_SPOSA	1:MVGTTT-----LNTGASLELVGYGTWQAAP--GEVGGQGVKVAIET	38
YPR1	1:MPATLKN--SSAT-L-----K----LNTGASIPVLGFGTWRSDVN--NGYHSVIAALKA	45
ARA1	1:MSSSVASTENIVENMLHPKTTEIYFSLNNGVRI PALGLGTANPHEKLAETKQAVKAAIKA	60
ALD1	1:MATFVE-----LSTKAKMPLVGLGTWKSSPG--QVKEAVKAAIDA	38
hADR	1:ASRLI-----LNNGAKMPIGLGTWKSPPG--QVTEAVKVAIDV	37
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KER	43:GYRHLDCAWYYLNEGEVGEGRDFLKENPSVKREDIFVCTKVWNHLHRYEDVLWSIDDSL	102
GCY1	46:GYRHIDTAAYRNEDQVQAIDKSGVP----REEIFVTTKLWCTQHHEPEV--ALDQSL	98
ALDX_SPOSA	39:GYRHLDLAKVYSNQPEVGAAIK---EAG-VKREDLFITSKLWNNSHRPEQVEPALDDTL	93
YPR1	46:GYRHIDAAAIYLNNEEVGRAIKDSGVP----REEIFITTKLWGTEQRDPEA--ALNKS	98
ARA1	61:GYRHIDTAWAYETEPFVGEAIKELLEDSIKR-EDLFIITTKVMPVLWDE--VDRSLNESL	117
ALD1	39:GYRHIDCAYVYHNENEVGEAIQEKI KENAV-KREDLFIVSKLWATFFEKSLVKKAFQNTL	97
hADR	38:GYRHIDCAHVYQNEVGVAIQEKLRQV--KREELFIVSKLWCTYHEKGLVKGACQKTL	96
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KER	103:KRLGLDYVDMFLVHWPAAEKNQGQEPKI-G--PDG--KYVILKDLTENPEPTWRAMEKI	157
GCY1	99:KRLGLDYVDLYLMHWPARDPAYIKNEDI-LSVPTKKDGSRAVDITNWNFIKTWELMQEL	157
ALDX_SPOSA	94:KELGLELYDLYLHWPVAFPPGEDITQNL-F--PKANDKEVKL-DLEVSLVDTWKAMVKL	149
YPR1	99:KRLGLDYVDLYLMHWPVPLKTDRTVDGNV-LCIPTLEDGTVDIDTKENFIKTWELMQEL	157
ARA1	118:KALGLELYDLYLQHWPLCFEIKDPKGISGLVKTVPVDDSGKTMAYADGDYLETYQLEKI	177
ALD1	98:SDLKLDYLDLYLVHWPQGFQAGNALLPKD-NKGKVLSSKSTFLDA-----WEAMEEL	148
hADR	97:SDLKLDYLDLYLHWPFGFKPGKEFFPLD-ESGNVVPSDTNILDT-----WAAMEEL	147
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KER	158:YEDRKA---RSIGVSNWTIADLEKMSKFAKVM--PHANQIEIHPFLPNEELVQYCFSKNI	212
GCY1	158:PKTGKT---KAVGVSNFSINNLDLLASQGNKLTAAANQVEIHPLLPQDELINFCCKSKI	214
ALDX_SPOSA	150:LDTGKV---KAIGVSNFDKMDVDAIEATGVT--PSVNQIERHPLLLQPELIAHAKKNI	204
YPR1	158:PKTGKT---KAVGVSNFSINNLIKELLESNNKVPATNQIEIHPLLPQDELIAFCKEKG	214
ARA1	178:YLDPNDRHVRVRAIGVSNFSIEYLERLIKECRVKP--TVNQVETHPLPQEMELRKFCFMHDI	235
ALD1	149:VDQGLV---KALGISNPNHFQIERLLNKPGLKHKPVTNQIESHPYLTQEKLIQYQCSKI	205
hADR	148:VDEGLV---KAIGISNPNHLQVEMILNKPGLKYPVAVNQIECHPYLTQEKLIQYQCSKI	204
	 * * * * *	
KER	213:MPVAYSPLGSQNVPTTGERVSENK-TLNEIAE-KGNTLAQVLIWGLRRGYVVLPKSS	270
GCY1	215:VVEAYSPLGSTDAPLLK-----EPVILEI-AKKNVQPGHVVISWHVQRGYVVLPKSV	266
ALDX_SPOSA	205:HITAYSPLGNN---TVGAPLLVQHPEIKRIAE-KNGCTPAQVLIAWAIVGGHVSIPKSV	259
YPR1	215:VVEAYSPLGSANAPLLK-----EQAIIDM-AKKHGVPAQLIISWSIQRGYVVLAKSV	266
ARA1	236:LLTAYSPLGSHGAPNLKI-----PLVKLAEKYVNTGNDLLISYHIRQGTIVIPRSL	287
ALD1	206:AVTAYSPLGSPDRPYAKPEDPVVMEIPKIEIAAKHKKTVAQVLIHFVQRNVVPIPKSV	265
hADR	205:VVTAYSPLGSPDRPWAKPEDPSLLEDPRKIAIAAKHNKTTAQVLIHFVQRNVVPIPKSV	264
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KER	271:NPKRIESNFKSI-ELSDADFEAINAVAKGRHFR-FVNMKDTF---GYDVWPEETAK---	321
GCY1	267:NPDRIKTRNKIF-TLSTEDFEAINNIS----KEKG-----EKRNVHPNWS-PFEV	310
ALDX_SPOSA	260:TPSRIGENFKQV-SLSQEDVDVAVSKLGEGRRRRYN-IPCTYSPKWDINVFGEDEKSKC	317
YPR1	267:NPERIVSNFKIF-TLPEDDFKTISNLS----KVHG-----TKRVVDMKWG-SFPI	310
ARA1	288:NPVRISSIE-FASLTQDELQELNDFGEKYPV--RFIDE----P-FAA-ILPEFTGNPG-	337
ALD1	266:TPSRIQENLQVF-DFQLSEEDMAAILL----FNRN-----WRACDLLDAR-TEED	309
hADR	265:TPERIAENFKVF-DFELSSQDMTTLIS----YNRN-----WRVCALLSCT-SHKD	308
	. * * * * * *	
KER	322:N-LSA----	325
GCY1	311:--FK----	312
ALDX_SPOSA	318:NAVKIK---	323
YPR1	311:--F-----	311
ARA1	338:N-L-DNLKY	344
ALD1	310:YPFHEEY--	316
hADR	309:YPFHEEF--	315

FIG. 1. Sequence alignment of KER from *P. citrinum* with a closely related AKR family. From top to bottom in each set, the proteins are KER from *P. citrinum*, GCY1 protein from *Saccharomyces cerevisiae* (GCY1) (accession number P14065) (9), aldehyde reductase from *Sporoboromyces salmonicolor* (ALDX_SPOSA) (accession number P27800) (7), aldoketoreductase from *S. cerevisiae* (YPR1) (accession number Q12458) (10), D-arabinose dehydrogenase from *S. cerevisiae* (ARA1) (accession number P38115) (5), aldose reductase-related protein from *Mus musculus* (ALD1) (accession number P21300) (12), and human aldose reductase (hADR) (accession number P15121) (16). Dashed lines indicate gaps introduced for better alignment. Asterisks denote amino acids perfectly conserved in all seven proteins, and dots denote well-conserved amino acids. The similarities between KER and *S. cerevisiae* GCY1 protein, aldehyde reductase from *S. salmonicolor*, aldoketoreductase YPR1 from *S. cerevisiae*, D-arabinose dehydrogenase from *S. cerevisiae*, aldose reductase-related protein from *Mus musculus*, and human aldose reductase were 42, 38, 39, 39, 38, and 39%, respectively.

TABLE 1. Biotransformation of BAM by acetone-dried microorganisms

Strain	Coenzyme availability ^a					
	NADPH-dependent			NADH-dependent		
	Molar yield (%)	Stereoselectivity	EE (%)	Molar yield (%)	Stereoselectivity	EE (%)
<i>Arthrobacter paraffineus</i> ATCC21003	4.2	<i>S</i>	54.7	4.1	<i>S</i>	27.7
<i>B. alvei</i> IFO3343	44.1	<i>R</i>	71.6	30.0	<i>R</i>	95.5
<i>Rhodotorula minuta</i> IFO879	10.1	<i>S</i>	82.1	1.0	NT	NT
<i>Cryptococcus humicola</i> IFO1527	12.9	<i>S</i>	88.0	0.4	NT	NT
<i>P. citrinum</i> IFO4631	36.0	<i>S</i>	98.1	ND	NT	NT

^a NT, not tested; ND, not detected.

synthesized. The primers were used to screen the cDNA library of *P. citrinum* constructed in λ ZapII. PCR yielded a single product of approximately 0.74 kb in length. It was confirmed that the 0.74-kb fragment was a portion of the whole *ker* gene, because three internal amino acid sequences (S2, S4, and S5) were found in the deduced amino acid sequence. The upstream and downstream regions of the *ker* gene were subcloned on the basis of the 0.74-kb fragment. The upstream region (0.35-kb PCR fragment) contained one internal amino acid sequence (S2) and an initiation codon (ATG), and the downstream region (0.65-kb PCR fragment) contained two internal amino acid sequences (S1 and S3) and a poly(A) region. To clone the whole *ker* gene, primer P2 (5'-ATGTCTAACGGAACCTTC-3'), including an initiation codon derived from the 0.35-kb PCR fragment, and primer P3 (5'-TCACGCAGACAGGTTCTTGGC-3'), containing a termination codon derived from the 0.65-kb PCR fragment, were synthesized. PCR performed with primers P2 and P3 gave a single product of approximately 1.0 kb in length. The nucleotide sequence revealed one open reading frame (975 bp; 325 amino acids), and the deduced amino acid sequence was identical to the partial amino acid sequences of KER determined by the peptide sequencing. The deduced molecular mass of KER was 36.6 kDa, which was similar to that estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A sequence identity search showed that KER had strong similarities with the proteins belonging to the aldoketoreductase (AKR) superfamily (4) (Fig. 1). Although the primary structure of KER showed 82% identity to *Aspergillus nidulans* glycerol dehydrogenase (2), KER did not catalyze the dehydrogenation of glycerol. KER was classified as a new member of the yeast AKRs (AKR3E1) according to the updated AKR nomenclature system.

An expression vector, pTrcKER, was constructed by inserting the whole *ker* gene into the *Nco*I/*Bam*HI site of pTrc99A

(Pharmacia Biotech, Uppsala, Sweden). *Escherichia coli* HB101 cells (pTrcKER) were cultured at 30°C in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl [pH 7.0]) containing 0.05 mg of ampicillin/ml. For induction of the gene under the control of the *trc* promoter, 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the Luria-Bertani medium. Washed cells obtained from 100 ml of culture broth were incubated in 30 ml of reaction mixture containing 1.5 mmol of potassium phosphate buffer (pH 6.5), 6.72 mmol of BAM, 0.021 mmol of NADP⁺, 210 U of glucose dehydrogenase (Amano Pharmaceutical, Nagoya, Japan), 16.3 mmol of glucose, and 15 ml of *n*-butyl acetate. The mixture was vigorously stirred at 30°C. During the reaction, the pH of the mixture was adjusted to 6.5 by adding 2 M Na₂CO₃ solution automatically. After an 11-h reaction, (*S*)-BHBM (79 g/liter) was accumulated in the organic solvent layer. The molar conversion yield and EE of (*S*)-BHBM were 88 and 96%, respectively. On the other hand, when we tested the recombinant *E. coli* cells in the water-*n*-butyl acetate two-phase system in the absence of BAM with shaking, approximately 70% of total KER activity liberated from the cells was found in the aqueous solution, and 90% of the total KER activity was found in the solution after a 4-h incubation, suggesting that *n*-butyl acetate damages the cell membrane to cause the release of proteins from the cells. Thus, the recombinant *E. coli* cells (pTrcKER) were confirmed to be a suitable biocatalyst to produce (*S*)-BHBM.

Nucleotide sequence accession number. The sequence reported in this paper has been submitted to the EMBL database and is available under accession number AX472815.

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TABLE 2. Summary of the purification of KER of *P. citrinum*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	43	98	2.3	100	1
(NH ₄) ₂ SO ₄ ^a	41	80	2.0	81	0.9
Hi-Load Phenyl	0.38	17	44	17	19
Hi-Load Q Sepharose	0.01	2	232	2.0	102
Superdex 200	0.0006	0.1	217	0.1	95

^a Supernatant obtained by the addition of (NH₄)₂SO₄ to a final concentration of 1.5 M was used.

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