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Purification and Gene Cloning of an Enantioselective Thioesterification Enzyme from *Brevibacterium ketoglutamicum* KU1073, a Deracemization Bacterium of 2-(4-Chlorophenoxy)propanoic Acid

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We succeeded in the purification and gene cloning of a new enzyme, α-methyl carboxylic acid deracemizing enzyme 1 (MCAD1) from *Brevibacterium ketoglutamicum* KU1073, which catalyzes the (*S*)-enantioselective thioesterification reaction of 2-(4-chlorophenoxy)propanoic acid (CPPA). The cloned gene of MCAD1 contained an ORF of 1,623 bp, encoding a polypeptide of 540 amino acids. In combination with cofactors ATP, coenzyme A (CoASH), and Mg^{2+}, MCAD1 demonstrated perfect enantioselectivity toward CPPA. The optimal pH and temperature for reaction were found to be 7.25 and 30 °C. Under these conditions, the K_m and k_{cat} values for (*S*)-CPPA were 0.92 ± 0.17 mM and $0.28 \pm 0.026\ s^{-1}$ respectively. The results for substrate specificity revealed that MCAD1 had highest activity toward fatty acid tails with a medium chain-length (C_8). This result indicates that MCAD1 should be classified into a family of medium-chain acyl-CoA synthetase. This novel activity has never been reported for this family.

Key words: *Brevibacterium ketoglutamicum*; enantioselective thioesterification; acyl-CoA synthetase; deracemization

The preparation of optically pure enantiomers is very important, for the physiological activity of a compound often depends on the absolute configuration of chiral centers in the molecule. In the case of 2-aryloxypropanoic acids, only the (*R*)-enantiomers show herbicide activity.[1,2] Additionally, whereas (*R*)-2-(4-chlorophenoxy)propanoic acid (CPPA) lowers the level of serum cholesterol and prevents platelet aggregation, the (*S*)-antipode leads to muscle irritability and spasms by inhibiting chloride channels.[3] Hence a number of approaches to prepare optically active 2-aryloxypropanoic acid have been made.[4]

The usual biocatalytic method to obtain enantiomerically enriched compounds from racemates is kinetic resolution, but the maximum yield of the desired enantiomer is limited theoretically to 50%. To overcome this disadvantage, the deracemization process has been deeply investigated.[5,6] Deracemization inverts the chirality of one enantiomer in a racemic mixture to the other antipode, resulting in one optically active compound. In this case, the starting material and the product are the same compound except for their chirality. Successful application of this process yields a pure, optically active compound that is approximately equal to the originally synthesized racemate. Thus, deracemization is a novel strategy for the preparation of optically active compounds.

There have been only a few reports on biocatalytic systems, including the use of rat liver and fungi, *Cordyceps militaris* and *Verticillium lecanii*, that cause the deracemization reaction.[7–10] These are capable of inverting the chirality of the (*R*)-enantiomers of 2-arylpropanoic acids to the corresponding (*S*)-antipode. Through thorough investigations of rat-related enzymes it became clear that three enzymes are involved in this biotransformation system (Fig. 1).[11–14] In these three mechanistic reactions, only the long-chain acyl-CoA synthetase-catalyzed reaction proceeds in an enantioselective manner. Thus, the enantiomeric ratio of the acid shifts to the (*S*)-form with the progress of the reaction.

Recently, we reported a novel biocatalytic deracemization of CPPA and its derivatives using the whole-cell system of *Nocardia diaphanozonaria* JCM3208.[15] Substrate specificity investigation and the detection of metabolic intermediates suggested that this deracemization process competitively inhibits the β-oxidation pathway. This indicates that an acyl-CoA thioester is involved in this process.[16] Although we tried enzyme purification on the assumption that an enantioselective thioesterification enzyme is also involved in this deracemization process, the trial was not successful due to the instability of the enzymes in the cell-free extract.

In this report, we describe the isolation of several new microorganisms that exhibited deracemization activity of CPPA. In addition, we succeeded in the purification and gene cloning of an enantioselective thioesterification enzyme, α-methyl carboxylic acid deracemizing enzyme 1 (MCAD1), from *Brevibacterium ketoglutamicum* KU1073.

Materials and Methods

Chemicals were purchased from standard suppliers and were used without further purification unless otherwise noted. (*R*)- and (*S*)-CPPA were prepared from a racemic mixture by a diastereomeric salt

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Abbreviations: CoASH, coenzyme A; CPPA, 2-(4-chlorophenoxy)propanoic acid; DTT, dithiothreitol; MCAD1, α-methyl carboxylic acid deracemizing enzyme 1; PMSF, phenylmethylsulfonyl fluoride; PPB, potassium phosphate buffer

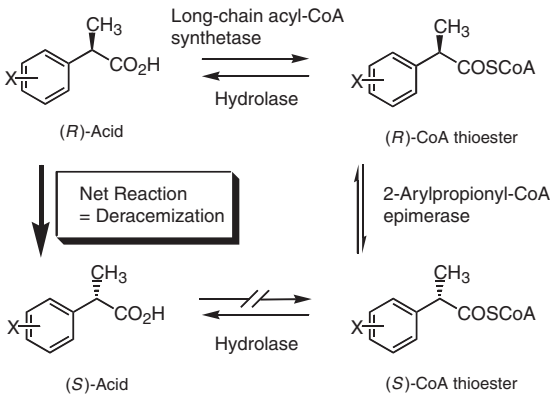

Fig. 1. Proposed Reaction Mechanism for the Deracemization Reaction of 2-Arylpropanoic Acid in Rat Liver.

formation method using optically active 1-(1-naphthyl)ethylamine as a counter amine. Authentic samples of 2-aryloxypropanoyl-CoA were chemically synthesized by a previously described method.[17] 2-Methyl-3-phenylpropanoic acid (MPPA) and 2-phenylthiopropanoic acid were synthesized by a published procedure.[15] A Bradford assay was done to determine protein concentrations using bovine serum albumin as standard.[18]

Screening of microorganisms that deracemize CPPA. Microbial strains were obtained from a glycerol stock solution of cells at Keio University. The microorganism was cultured with shaking in 10 ml of *N. diaphanozonaria* medium for 48 h at 30 °C.[16] The cells were harvested by centrifugation (4,500 × *g*, 10 min, 4 °C) and then washed with 0.1 M potassium phosphate buffer (PPB) (pH 7.0). They were resuspended in 5 ml of 0.1 M PPB containing (±)-CPPA (5 mg, 0.1% w/v) in a test tube. The tube was shaken at 30 °C for 72 h. The reaction mixture was then filtered through celite. The celite was washed with ethyl acetate (EtOAc), and the washing was combined with the filtrate. After acidification with 2 M HCl, the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residual acid was converted to the corresponding methyl ester with diazomethane, and this was followed by a conventional work-up. The residue was purified by pTLC (hexane/EtOAc = 9/1) to yield the methyl ester of the starting acid as a colorless oil. The enantiomeric excess (ee) of the ester was determined by high-performance liquid chromatography (HPLC) with a Daicel Chiralcel OJ column (Daicel, Japan). The conditions and elution profiles for HPLC analyses were described previously.[16]

General procedure for the deracemization reaction of CPPA by cell-free extract from isolated bacteria. The microorganism was cultured with shaking in 10 ml of *N. diaphanozonaria* medium for 48 h at 30 °C. These pre-cultured cells were then added to 90 ml of fresh medium and cultivated at 30 °C for 48 h (second cultivation). In the case of *B. ketoglutamicum*, the second incubation lasted for 24 h. The cells were then harvested and suspended in 9.1 ml of 100 mM MOPS-NaOH buffer (4 °C, pH 7.25) containing 1 mM DTT. They were disrupted by French press. The cell debris was removed by centrifugation (14,500 × *g*, 10 min, 4 °C). Three hundred µl of ethylene glycol (final concentration, 3% v/v), 360 µl of cofactor solution (final concentration, 10 mM of ATP, 2 mM of CoASH, and 10 mM of $MgCl_2$), and 240 µl of substrate solution (final concentration, 1 mM of (±)-CPPA, pH 7.0) were added (total volume, 10 ml) to this cell-free extract, and the mixture was incubated for 24 h at 30 °C. The reaction was quenched by the addition of 1 ml of 2 M HCl, and was then extracted by diethyl ether (Et_2O). The ee and yield of the product were confirmed after conversion to a methyl ester by treatment with diazomethane.

Purification of enantioselective thioesterification enzyme from B. ketoglutamicum. All purification steps were carried out at 4 °C and were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Enzyme active fractions were determined by the index of ee improvement of unreacted carboxylic acid using a previously reported principle and (±)-CPPA as the starting substrate.[19] The unreacted carboxylic acid was prepared by the method described in the section on MCAD1 activity assay by HPLC method. The ee was confirmed by chiral phase HPLC using a Daicel Chiralcel OJ column. Separation was achieved by employing an isocratic mobile phase consisting of hexane/2-propanol/trifloroacetic acid (TFA) (95/5/0.1) at a flow rate of 0.5 ml/min at 254 nm. Under these conditions, the elution profile for CPPA was 25.9 and 31.9 min for the (*R*)- and (*S*)-forms respectively.

The cell pellet was harvested from 2 liters of culture broth (100 ml × 20), suspended in 100 ml Buffer A (100 mM MOPS-NaOH buffer, pH 7.25 containing 1 mM DTT), and subjected to disruption by French press. The cell debris was removed by centrifugation (14,500 × *g*, 10 min, 4 °C). To remove nucleic acid, 112 µl of 2% protamine sulfate was added to the cell-free extract (112 ml). After stirring for 15 min, the precipitate was removed by centrifugation (14,500 × *g*, 20 min, 4 °C). Solid ammonium sulfate was carefully added to the supernatant with gentle stirring until the solution reached 40% saturation. After equilibration for 30 min, the mixture was centrifuged, and the supernatant was separated. To this supernatant, ammonium sulfate was again added to 70% saturation. The solution was allowed to equilibrate for 30 min, and the resulting precipitate was collected by centrifugation (14,500 × *g*, 20 min, 4 °C). The precipitate was dissolved in Buffer A and dialyzed for 4 h. The dialyzed fraction was loaded onto a pre-equilibrated Toyopearl HW-75F column (250 ml) and eluted with Buffer A. The fractions were collected in 7 ml portions. To the collected active fractions, ammonium sulfate was added to 20% saturation. The ammonium sulfate solution was applied to a Toyopearl Butyl-650M column (100 ml), pre-equilibrated with Buffer A containing a 20% saturation of ammonium sulfate. After washing of the column, the enzyme was eluted using a linear gradient from 20 to 0% saturation of ammonium sulfate. The fractions were collected in 12 ml portions. The active fractions were combined, concentrated to 22 ml with an amicon filter, and applied to a Toyopearl DEAE-650M column (50 ml) pre-equilibrated with Buffer A. After washing of the column, the enzyme was eluted using a linear gradient from 0 to 300 mM NaCl. The fractions were collected in 12 ml portions. The active fractions were combined, concentrated to 20 ml with an amicon filter, and loaded onto a Macro-Prep Ceramic Hydroxyapatite Type I column (10 ml), pre-equilibrated with a mixture of Buffer A and 100 mM PPB (pH 7.0) (volume ratio, 3:1). After washing of the column, the enzyme was eluted using a linear gradient (volume ratio, 3:1 to 1:3) of a mixture of Buffer A and 100 mM PPB (pH 7.0). The fractions were collected in 12 ml portions. The combined active fractions were dialyzed for 4 h against Buffer A containing 200 mM NaCl. The volume of the solution was concentrated to 22 ml with an amicon filter. The dialyzed enzyme solution was loaded onto a DEAE-Sepharose CL-6B column (50 ml), pre-equilibrated with Buffer A containing 200 mM NaCl. After washing of the column, the enzyme was eluted using a linear gradient from 200 to 300 mM NaCl. The fractions were collected in 12 ml portions. The active fractions were combined, and the solution was concentrated to 8 ml with an amicon filter to obtain the purified enzyme.

The N-terminal amino acid sequence of the purified enzyme was performed by Edman degradation using a protein sequencer (Applied Biosystems, Model 491, USA).

Cloning and sequencing of the MCAD1 gene. Total genomic DNA was isolated from *B. ketoglutamicum* by the method reported by Saito and Miura.[20] To identify a partial, internal sequence of the MCAD1 gene, PCR was performed, using genomic DNA as a template with forward (ATGTTGAGCACCATGCAGGACGCGC) and reverse (GCTTCTGTTCGAACTTGCCGACGCTG) primers. The forward primer was designed according to the revealed N-terminal amino acid sequence. The reverse primer was designed based on the internal sequence of a putative acyl-CoA synthetase from *Nocardia farcinica* IFM10152 (GenBank accession no. BAD55557). The N-terminal amino acid sequence of this protein showed high homology (84.6%) to that of MCAD1, according to a FASTA search.

The amplified 1.5 kbp PCR product was purified, inserted into pGEM-T easy vector (Promega, USA), and used for the transformation of *E. coli* XL10-Gold ultracompetent cells (Stratagene, USA). The positive clones were isolated and sequenced using an ABI Prism 3100 DNA sequencer (Applied Biosystems, Japan).

For hybridization, 80 ng of genomic DNA was digested using *Acc* I, *Kpn* I, *Sac* I, *Hin*d III, *Xba* I, *Pst* I, *Eco*R I, *Sal* I, *Bam*H I, *Xho* I, *Hin*c II, and *Sph* I. This mixture was then transferred to a Hybond-N+ membrane using a G capillary blotter (TAITEC, Japan). Hybridization was done at 60 °C for 13 h using the AlkPhos Direct labeling and detection System with CDP-Star (GE Healthcare, UK) using the 1.5 kbp PCR product described above as probe. The 0.9 and 1.0 kbp fragments, digested with *Hin*c II and *Sph* I respectively, showed strong signal intensities. These fragments were isolated and inserted into a pUC19 *Hin*c II or *Sph* I/BAP vector and used in the transformation of *E. coli* XL10-Gold ultracompetent cells. The positive clones were isolated and sequenced to confirm the overall ORF sequences.

Nucleotide sequence data analysis and alignment were conducted using a BioEdit program. Sequence similarity to MCAD1 was analyzed using the NCBI FASTA program.

Plasmid construction and expression of MCAD1. The MCAD1 gene was amplified from genomic DNA by PCR with forward (GGAATTC*CATATG*TTGAGCACCATGCAGGACGCGC) and reverse (TGC*TCTAGA*AGTACGTCGACATCGCTTTTCACACGG) primers. These primers were designed to contain artificially introduced *Nde* I and *Xba* I sites (italic form) in the 5′ and 3′ ends of the MCAD1 gene respectively. Underlined ATG represents the initial codon. The amplified 1.6 kbp fragment was subcloned to pGEM T-easy vector. The subcloned vector was first digested with *Nde* I and *Xba* I, and then purified and inserted into a pColdI expression vector (Takara, Japan). The resulting plasmid, pColdI-MCAD1, was used to express the recombinant MCAD1 protein. *E. coli* BL21(DE3) cells were transformed with pColdI-MCAD1, cultivated, and treated to express MCAD1 following the manufacturer's instructions.

After recombinant gene expression, the cells were collected, disrupted by sonication (20 kHz, 30 s × 10 times) in 5 ml of 50 mM PPB (pH 7.0) containing 300 mM NaCl, and centrifuged (14,500 × *g* for 10 min, 4 °C). The enzyme was purified from the supernatant using a TALON Metal Affinity Resin (2 ml) (Clontech, USA) following the manufacturer's instructions, and identified by SDS–PAGE. The active fractions were combined and dialyzed overnight against Buffer A.

Molecular weight determination of recombinant MCAD1. The molecular weight of the native enzyme was measured by gel-filtration chromatography. The enzyme was subjected to ÄKTA FPLC (Pharmacia Biotech, USA) on a Superdex 75 10/300 GL at a flow rate of 0.5 ml/min, using 50 mM PPB (pH 7.2) containing 150 mM NaCl at room temperature. Eluent absorbance was monitored at a wavelength of 280 nm. The relative mobility of the enzyme was compared to those of the proteins from the Gel Filtration Standard kit (Bio-Rad, USA). The molecular weight of the subunit of the enzyme was estimated by SDS–PAGE on the basis of relative mobility as compared with those of standard proteins from Perfect Protein Markers (Novagen, USA) and Pre-stained SDS–PAGE standards (Bio-Rad, USA).

MCAD1 activity assay by the HPLC method. The rate of thioester formation was determined from the yields of unreacted carboxylic acid and thioester product. Because the yield of the thioester in the aqueous layer could not be determined as they were, the thioester bond was hydrolyzed and the yield of the resulting free acid was measured. The reaction mixture contained 100 mM MOPS-NaOH (pH 7.25), 1 mM (±)-CPPA, 10 mM ATP, 2 mM CoASH, 10 mM $MgCl_2$, 3% w/v ethylene glycol, and an appropriate amount of MCAD1 protein. The total volume was brought to 500 µl. The mixture, containing all components except the MCAD1 protein, was preincubated at 30 °C for 5 min. The reaction was initiated by the addition of the enzyme and incubated at 30 °C for 1 h. The reaction was quenched by adding 100 µl of 2 M HCl and 700 µl of Et_2O. After shaking, the reaction mixture was centrifuged at 17,610 × *g* for 10 min, and two layers were separated.

To measure the yield of unreacted carboxylic acid, the separated organic layer was concentrated *in vacuo*. The yield was determined by HPLC using a COSMOSIL 5C18-ARII (150 mm × 4.6 mm) column at room temperature and (±)-MPPA as internal standard. Separation was achieved by employing an isocratic mobile phase consisting of H_2O/acetonitrile/TFA (2/1/0.05%) at a flow rate of 0.5 ml/min, detected at 254 nm. Under these conditions, the retention times of MPPA and CPPA were 33.8 and 40.9 min respectively.

To measure the yield of thioester product, to the separated aqueous layer was added 120 µl of 2 M NaOH and this was incubated at room temperature for 30 min. The reaction mixture was then acidified by adding 120 µl of 2 M HCl and extracted the hydrolyzed product by 700 µl of Et_2O. The separated organic layer was concentrated *in vacuo*. The yield, which was taken for the yield of thioester product, was analyzed by HPLC as described above.

MCAD1 activity assay by the spectrophotometric method. The activity of MCAD1 was also spectrophotometrically observed. This assay measures the initial rate of AMP formation by coupling the reaction of MCAD1 with adenylate kinase, pyruvate kinase, and lactate dehydrogenase, following NADH oxidation at 340 nm with a spectrophotometer (U-2800A, Hitachi, Japan).[21–23] The standard reaction mixture for this assay contained 100 mM MOPS-NaOH (pH 7.25), 1 mM DTT, 10 mM ATP, 10 mM $MgCl_2$, 1 mM (*S*)-CPPA, 2 mM CoASH, 1 mM phosphoenolpyruvic acid, 0.2 mM NADH, 40 µg/mL adenylate kinase, 20 µg/mL pyruvate kinase, 20 µg/mL lactate dehydrogenase, and 120 µg of MCAD1. The total volume was brought to 500 µl. The mixture, containing all components except the MCAD1 protein, was preincubated at room temperature (27 ± 2 °C) for 3 min. The reaction was then initiated by addition of the enzyme.

Kinetic analysis of recombinant MCAD1. The kinetic parameters were calculated by a spectrophotometric method. The k_{cat} and K_m values were evaluated by Michaelis–Menten analysis using GraphPad prism, version 5.01 (GraphPad, USA). The k_{cat} values were expressed as turnover numbers per subunit (Mr of the subunit, 61,700). Kinetic studies were performed with various concentrations of the substrate (0.1–1.25 mM). Each assay was repeated five times.

Purification and identification of 2-(4-chlorophenoxy)propanoyl-CoA. 2-(4-Chlorophenoxy)propanoyl-CoA was purified using solid phase extraction cartridges (Chromabond C 18 ec, Macherey-Nagel, Germany),[24] and were confirmed by HPLC and TOF-MS analysis.

HPLC analysis was performed using a Senshu Pak ODS-1251 (250 mm × 4.6 mm) column at room temperature, and was detected at 232 nm. Separation was achieved by employing an isocratic mobile phase consisting of a 50 mM solution of KH_2PO_4 (pH 5.5)/methanol (1/1) at a flow rate of 0.6 ml/min. Under these conditions, the retention time of (*S*)-2-(4-chlorophenoxy)propanoyl-CoA was 22.6 min.

Mass spectra were obtained using a LCT-Premier (Waters, USA). Data were acquired in negative ESI mode, and leucine enkephalin was selected as a reference compound for high-accuracy, exact mass measurement.

Results and Discussion

Screening of microorganisms capable of deracemizing CPPA and the enzyme stability of cell-free extracts

Microorganisms deracemizing CPPA were screened and obtained from stock cultures at Keio University. Of approximately 300 cultured species, only *Mycobacterium smegmatis* KU1047, *B. ketoglutamicum*, and *Pseudomonas aeruginosa* KU1097 were found to show deracemization activity. The yields and ee values of the recovered substrate were compared and displayed (Fig. 2, Table 1). The enantioselectivity of the microorganisms obtained were found to be similar; the racemate of CPPA was deracemized to the (*R*)-enantiomer.

Deracemization activity was also observed in the cell-free extract of *B. ketoglutamicum* when the necessary cofactors for the acyl-CoA synthetase-catalyzed formation of acyl-CoA thioester (ATP, CoASH, and Mg^{2+}) were added to the reaction mixture. Notably, 50% of maximum activity was still observed after 7 d, but no activity was detected when any one of the above cofactors was absent. In other microorganisms, however, deracemization activity were not detected even immediately after cell disruption.

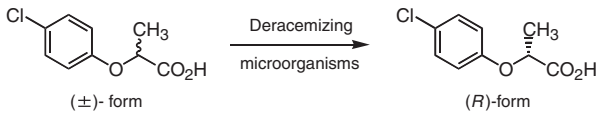

Fig. 2. Deracemization Reaction of CPPA by Selected Microorganisms: *N. diaphanozonaria* JCM3208, *M. smegmatis* KU1047, *B. ketoglutamicum* KU1073, and *P. aeruginosa* KU1097.

Table 1. Deracemization Reaction of CPPA by Screened Microorganisms

	Whole cells[a]		Cell-free extract[a]	
	yield (%)	ee (%)	yield (%)	ee (%)
N. diaphanozonaria JCM3208	95	97 (*R*)	95	0 (rac.)
M. smegmatis KU1047	98	47 (*R*)	95	0 (rac.)
B. ketoglutamicum KU1073	90	72 (*R*)	98	24 (*R*)
P. aeruginosa KU1097	85	36 (*R*)	97	0 (rac.)

[a]Reaction conditions are described under "Materials and Methods."

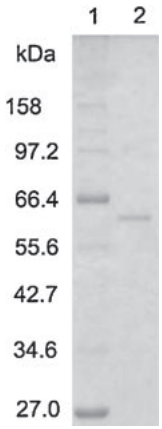

Fig. 3. SDS–PAGE Analysis of an Enantioselective Thioesterification Enzyme, MCAD1, from *B. ketoglutamicum* KU1073.

Proteins were separated on a 10% polyacrylamide gel in the presence of 0.01% SDS. Lane 1, molecular weight standards; lane 2, purified MCAD1.

Table 2. Purification of MCAD1 from *B. ketoglutamicum* KU1073

Purification step	Protein (mg)	Total activity[a] (nmol/min)	Yield (%)	Specific activity[a] (nmol/min/mg)	Purification (fold)
Cell-free extract	1660	240	100	0.145	1.0
Ammonium sulfate precipitation	839	317	132	0.338	2.3
HW-75F	605	250	104	0.414	2.9
Butyl Toyopearl	24	25.3	11	1.06	7.3
DEAE Toyopearl	2.9	17.3	7.2	6.06	41.8
Hydroxyapatite	0.58	9.4	3.9	16.1	111
DEAE Sepharose	0.11	1.77	0.7	15.8	109

[a]The activity of thioester formation was determined by the method of HPLC assay described under "Materials and Methods."

Purification of enantioselective thioesterification enzyme (MCAD1) from B. ketoglutamicum

In the case of rat liver studies, long-chain acyl-CoA synthetase catalyzed enantioselective thioester formation is the first step in the deracemization process.[14] We have proposed that a similar enzyme should work in *B. ketoglutamicum*, because the deracemization activity in the cell-free extract was detected only in the presence of cofactors for the formation of acyl-CoA synthetase (ATP, CoASH, and Mg^{2+}). Hence the enantioselective thioesterification enzyme for CPPA was selected as the target protein for purification. This protein was tentatively named α-methyl carboxylic acid deracemizing enzyme 1 (MCAD1), to the effect that it is an enzyme relating to the deracemization reaction of α-methyl carboxylic acid.

For enzyme purification, a simple procedure to assay the index of ee improvement of unreacted acid was used. This reported method was first employed to determine whether firefly luciferase has a capacity for enantioselective thioester formation toward 2-arylpropanoic acid.[19] The racemate is used as a starting material, and if the enzyme has the ability to distinguish between the absolute configuration of *R* and *S*, the ee of the unreacted carboxylic acid increases. Based on this principle, collected fractions exhibiting activity can be selected easily during enzyme purification.

As summarized in Table 2, MCAD1 from the cell-free extract of *B. ketoglutamicum* was purified 109-fold to homogeneity with an overall yield of 0.7%. Purification included ammonium sulfate precipitation, gel-filtration, hydrophobic, ion-exchange, and hydroxyapatite column chromatography. The optimal pH and temperature for the reaction were found to be 7.25 and 30 °C. The specific activity of the purified enzyme was analyzed by HPLC, and was found to be 15.8 nmol/min/mg. A solution containing 1 mM (±)-CPPA, cofactors (10 mM ATP, 2 mM CoASH, 10 mM $MgCl_2$), and 3% w/v ethylene glycol in Buffer A at 30 °C (standard condition) was used. Under these conditions, the enzyme catalyzed (*S*)-enantioselective thioester formation. The purified enzyme required cofactors (ATP, CoASH, Mg^{2+}) since no activity was detected in the absence of any one of the cofactors.

The final product afforded a single band by SDS–PAGE, and the molecular weight was determined to be approximately 60,000 Da (Fig. 3). The first 13 N-terminal amino acid residues of the purified enzyme were sequenced to be MLSTMQDAPLSIA, and these were then used in the cloning of the gene.

Cloning and sequencing of MCAD1

The complete ORF of MCAD1 contained a DNA sequence of 1,623 bp, encoding 540 amino acids. The ORF initiated and terminated with an ATG and a TGA codon respectively. The nucleotide sequence of the MCAD1 gene was submitted to the DDBJ database (accession no. AB546128). After comparison, a putative AMP-binding domain signature (PROSITE accession no. PS00455) was found in the sequences at positions 182 to 193 (Fig. 4).

The entire amino acid sequence of MCAD1 was used to search for protein sequence homology in the FASTA

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MCAD1     MLSTMQDAPLSIAQLLRHGASVHGASEVVTWTESGPRRVSYADVGKRCAQLAHALR-GLGVEGDERVATFMWNNSEHLEAYLAIPSMGAVLHTLNIRLFPEQLIHVANHAEDRVIIVDPS
EEN87350  MLSTMQDAPLSIAQLLRHGASVHGASEVVTWTESGPRRVSYADVGKRCAQLAHALR-GLGVEGDERVATFMWNNSEHLEAYLAIPSMGAVLHTLNIRLFPEQLIHVANHAEDRVIIVDPS
BAH36329  MLSTMQDAPLSIAQLLRHGASVHGASEVVTWTESGPRRVSYADVGKRCAQLAHALR-GLGVEGDERVATFMWNNSEHLEAYLTIPSMGAVLHTLNIRLFPEQLIHVANHAEDRVIIVDPS
ACY19667  MLSTMQDLPLSVAQILRHGSTFHGTAEVVTWVGGGSRRRSYAEVGRRSAQLAHALR-GLGIDGDQRVGTFMWNNAEHMEAYLAVPAMGAVLHTLNIRLFPEQLVFVANHGEDQIIIVDPT
ACU98289  ----MQDDQLSLAYLLQYGARLHANAEVATWTGSGTRKSTFAQVGKRSAQLAHALR-SLGVTGDQRVGTFMWNNTEHLECYMAIPAMGAVLHTLNIRLFPEQLVYVANHAEDHVVIVDGS
BAD55557  MLSTMQDEPLSLATLLKYAATFQGESTVSTWTGDGVRTMTYRELGAEAARLANALS-GLGIGVGDRVGTFMWNNNEHMVAYIGVPAMGAVLHALNIRLFPEQLVYVANHAEDKVVIVDGS
EEP72933  ----MMDAPLQVARILEHGATVHGTAEVVTWTGGEPRRMSYAELGRTAAQLAHALRDECGVTGDERVATFMWNNNEHLVAYFAVPSMGAVLHTLNLRLFPDQVAYIANHAEDRVVLVDST
              * *  *  *  *           * **     *       *   * ** **    *     ** ****** **   *   * ****** ** **** *    *** **    **

MCAD1     LIPLLSPQLPSLTTVKHIVVTGS-STAGLEAPEG--VQVHTYEGLIADQPTEFDWPEVDELSAAAMCYTSGTTGDPKGVVYSHRSIYLHSMQVCMSDGPNLAQGDRALAVVPQFHAMSWG
EEN87350  LIPLLSPQLPSLTTVKHIVVTGS-STAGLEAPEG--VQVHTYEGLIADQPTEFDWPEVDELSAAAMCYTSGTTGDPKGVVYSHRSIYLHSMQVCMSDGPNLAQGDRALAVVPQFHAMSWG
BAH36329  LIPLLSPQLPSLTTVKHIIVTGS-STAGLEAPEG--VQVHTYEGLIADQPTEFDWPEVDELSAAAMCYTSGTTGDPKGVVYSHRSIYLHSMQVCMSDGPNLAQGDRALAVVPQFHAMSWG
ACY19667  LLPLLNPQLPQLRTVKHVIVNGD-STDGVEAPDG--VTVHAYETLLADQPTTFDWPEVDERSAAAMCYTSGTTGDPKGVAYSHRSIYLHSMQICSTEGPALRQGDRALAVVPQFHAMSWG
ACU98289  LIPLFAKQLPEMKTVKHVVVANG-DPASLEAPSG--VTVHSYEELLSGRPETFDWPEIDERSAAAMCYTSGTTGDPKGVVYSHRSIWLHSMQICMSNTMRLSDEDRALVIVPMFHAMSWG
BAD55557  LVPMFAQLLPNMKTVRHVIVANG-DAATLQAPKG--VQVHSYSELLAGQSDSYDFPVIDERSAAAMCYTSGTTGDPKGVVYSHRSNWLHAMQVVSPSGMGFSGADSVLAIVPLFHANAWG
EEP72933  LIPLLARVIGELTTVRHVVVVGGGDPAPLTAAAGGRIAVHHWDALLAGRPEVFDWPEVDERDAAALCYTSGTTGNPRGVAYSHRSIYLHSLQVCMPEGFGLGPTDRELAIVPMFHAMSWG
          * *          ** *  *          *  *    **    *        * *  **  *** ******** * ** *****  **  *            *  *  ** ***  **

MCAD1     LPYAAFMIGASLIMPDRFLQPAPLAAIIASEQPTFAAAVPTIWQGLHLYLEQNPQDISSMREVLIGGSAVPPALMHAFEADHNVSVLHAWGMTETSPLGSVARPPAGVTGDAMWKYRYSQ
EEN87350  LPYAAFMIGASLIMPDRFLQPAPLAAIIASEKPTFAAAVPTIWQGLHLYLEQNPQDISSMREVLIGGSAVPPALMHAFEADHNVSVLHAWGMTETSPLGSVARPPAGVTGDAMWKYRYSQ
BAH36329  LPYAAFMIGASLIMPDRFLQPAPLAAIIASEKPTFAAAVPTIWQGLHLYLEQNPQDISSMREVLIGGSAVPPALMHAFEADHNVSVLHAWGMTETSPLGSVARPPAGVTGDAMWKYRYSQ
ACY19667  LPYAAFMIGASLIMPDRFLQPEPLAELISAEQPTFAAAVPTIWQGLHQYLEAHPQDISCMHDVLIGGSAVPPSLMHAFQDDHDIQVLQGWGMTETSPLGSVSRAPADTEGEERWRYRYTQ
ACU98289  MPYAGFMVGASMVLPDRFLQPGPIAEILAAEKPTFAGAVPTIWQGLLQHLDANPQDISHLREVVVGGSAAPPAMMHAFEERYNVPVLHAWGMTETSPMGSVARPPAGVTGQQAWEYRYTQ
BAD55557  IPYAALMSGANVVMPDRFLQPGPLLEILANLKPTFAAAVPTIWGGVLAALAAQPQDISHLRTVVVGGAAVPPSMMHAFQERHGVRVLHAWGMTETSPLGSVAHAPAGVTGEEEWAYRYTQ
EEP72933  LPYAAFLSGASLIMPDRFLQAAPIAAMIAAERPTLAGAVPSIWNDLLAYLDDNDVDTSSLREVIVGGSACPPALMHAFDERHHIDVIHAWGMTEMSPLGSVSRPPAGATGEDAWRYRYTQ
           ***    **    ******  *         ** * *** **      *     * *    *  ** * **  ****       *   ***** ** ***   **   *   * *** *

MCAD1     GRFPASVEARLIDDDGKVVAHDGVSVGELEVRGPWITGSYYG----VDAEDKFNDGWLKTGDVGSITADGFLSLSDRSKDIIKSGGEWISSVDLENAVMGHPDVTEAAVIGVPDEKWDER
EEN87350  GRFPASVEARLIDDDGKVVAHDGVSVGELEVRGPWITGSYYG----VDAEDKFNDGWLKTGDVGSITADGFLSLSDRSKDIIKSGGEWISSVDLENAVMGHPDVTEAAVIGVPDEKWDER
BAH36329  GRFPASVEARLIDDDGKVVAHDGVSVGELEVRGPWITGSYYG----VDAEDKFNDGWLKTGDVGSITADGFLSLSDRSKDIIKSGGEWISSVDLENAVMGHPDVTEAAVIGVPDEKWDER
ACY19667  GRFPASVQARLVDDLGDVVPNDGTSIGELEVSGPWITGAYYG----VDAPEKFHDGWLRTGDVGSITPDGYLTLTDRTKDIIKSGGEWISSVDLENAVMGHPDVIEAAVIGIPDAKWDER
ACU98289  GRFPANVKARLVDDDGNEVPWDGKSVGELEVKGPWIAGSYYG----DADPEKFHDGWLRTGDVGKITPDGYLTLTDRAKDVIKSGGEWISSVDLENAVMAHPAVAEAAVIGIPDEKWDER
BAD55557  GRFPASVQARLVDDAGNVVPNDGESLGELEVKGPWITGSYYSPDGSPVDPDKFDNGWLRTGDVGKISPDGYLTLVDRSKDVIKSGGEWISSVDLENAVMGHPAVAEAAVIGVPDEKWDER
EEP72933  GRVPAGVAARVVGPLGEPLPADGTSVGELEVRGPWVTARYVG--DDAPDEEKFRDGWLRTGDVGTLSPDGYLTLTDRAKDVIKSGGEWISSVELENALMAHPAVLEACVVGVPDERWDER
          ** ** * **     *     ** * ***** ***    *           **  *** *****    ** * * ** ** *********** **** * ** * ** * * **  ****

MCAD1     PLVAVVLRAGSETTMSELQQYLSDKVAKWQLPENWAVVQEVPKTSVGKFDKKRLRADYHEGGLEVTRL---
EEN87350  PLVAVVLRAGSETTMSELQQYLSDKVAKWQLPENWAVVQEVPKTSVGKFDKKRLRADYHEGGLEVTRL---
BAH36329  PLVAVVLRAGSETTMSELQQYLSDKVAKWQLPENWAVVQEVPKTSVGKFDKKRLRADYHEGGLEVTRL---
ACY19667  PLVAVVLREGSDTTPETLREFLSDKVAHWQLPENWTVINEVPKTSVGKFDKKRLRSQYHDGGLSVTQF---
ACU98289  PLVAVVVREGRQVTAEELREFLADKVAKWQLPENWTFVEEVPKTSVGKFDKKRLRSAHADGKLDVIHF---
BAD55557  PLVAIVLAEGATAEPTELRDFLADKFAKWQLPEHWTFISEVPKTSVGKFDKKQLRAQYANGELDVKTLA--
EEP72933  PLATVVVREGAEVTAEELRDFLAGAVARWQLPERWAFIDAVPKTSVGKFDKKVVRSRYADGGLAVRKLTTP
          **   *   *       *   *    * ***** *     ************  *     * * *
```

Fig. 4. Amino Acid Sequence Alignments of MCAD1 and Predicted Acyl-CoA Synthetases, EEN87350 (predicted medium-chain fatty acid-CoA ligase from *Rhodococcus erythropolis* SK121), BAH36329 (predicted fatty acid-CoA ligase (fadD) from *Rhodococcus erythropolis* PR4), ACY19667 (predicted AMP-dependent synthetase and ligase protein from *Gordonia bronchialis* DSM 43247), ACU98289 (predicted acyl-CoA synthetase from *Saccharomonospora viridis* P101), EEP72933 (predicted Acyl-CoA synthetase from *Micromonospora* sp. ATCC 39149), and BAD55557 (putative acyl-CoA synthetase protein from *Nocardia farcinica* IFM 10152).

The conserved residues among all the sequences are indicated by asterisks. The underlined regions represent the putative AMP-binding domain signature in MCAD1 (PROSITE accession no. PS00455).

program and the Genbank database. The results of amino acid sequence alignment are shown in Fig. 4. The MCAD1 protein sequence showed 64–99% identity to the family of acyl-CoA synthetases, which have not yet been analyzed in detail. In contrast, MCAD1 showed low homology to rat long-chain acyl-CoA synthetase (5% identity), which is involved in the deracemization process of 2-arylpropanoic acid.[14] These results imply that MCAD1 and its homology proteins constitute a new family of acyl-CoA synthetases.

Expression and enzymatic properties of recombinant MCAD1

MCAD1 was cloned in cold-inducible vector and expressed in *E. coli* with an N-terminal, fused His-tag (MNHKVHHHHHHIGGRH). As determined by SDS–PAGE, the molecular weight of the recombinant enzyme was calculated to be 61,700 Da, but the weight of the native enzyme was found to be 121,578 Da by high performance gel-filtration column chromatography. These results indicate that the native enzyme was an active dimer. In the case of the His-tag removed protein at the factor Xa site, the same result was obtained. Hence we believe that the native enzyme purified from bacterial cells and the cloned recombinant protein have the same character, and we examined using the His-tag fused recombinant proteins here.

The specific activity of purified recombinant MCAD1 toward 1 mM (±)-CPPA was assayed by HPLC, and was calculated to be 13.4 nmol/min/mg. Cofactors (ATP, CoASH, Mg^{2+}) were also required in this process. Additionally, the enzyme showed no activity toward 0.5 mM (*R*)-CPPA within the experimental error (<0.01 nmol/min/mg), while the specific activity toward 0.5 mM (*S*)-CPPA was found to be 12.9 nmol/min/mg. These findings indicate that MCAD1 shows perfect enantioselectivity in the thioesterification of CPPA, and the (*R*)-form does not affect the catalytic activity of MCAD1-catalyzed thioester formation (Fig. 5).

The thioesters were purified by solid-phase extraction,[24] and were identified by ESI-MS based on orthogonal TOF-MS. The mass spectra of (*S*)-2-(4-chlorophenoxy)propanoyl-CoA were dominated by ions that were in close agreement with the calculated mass of singly charged thioester ($[M-H]^-$ as observed 948.1208, and predicted 948.1208).[25] HPLC analysis was also carried out, and the retention time of the purified product agreed with the chemically synthesized one.

The effect of pH on enzyme activity was examined (Fig. 6A). The enzyme exhibited maximum activity at pH 7.25 in a MOPS-NaOH buffer. The optimal reaction temperature was found to be approximately 30 °C (Fig. 6B). These results are in good agreement with the wild-type enzyme purified from the bacterial cells.

Various compounds were added to the reaction system, and enzymatic activity was compared (Table 3). The enzyme was inhibited completely by solutions containing 1 mM Hg^{2+} and 10 mM Ni^{2+}, Fe^{2+}, Co^{2+}, and Cu^{2+}. *p*-Chloromercuribenzoic acid (PCMB) and Zn^{2+} also strongly affected activity. Activity was slightly decreased by the addition of *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), and certain metal ions, including Ag^+, Ca^{2+}, and Mn^{2+}. PMSF, NaN_3, and iodoacetic acid had no effect on enzyme activity.

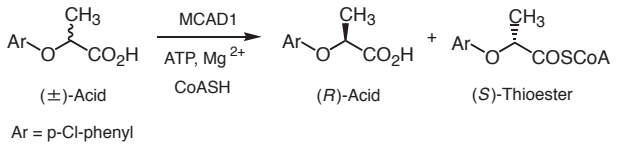

Fig. 5. MCAD1-Catalyzed Enantioselective Thioesterification of CPPA.

MCAD1 exhibited perfect enantioselectivity, and only the (*S*)-form was transformed to the thioester product in the presence of ATP, Mg^{2+}, and CoASH.

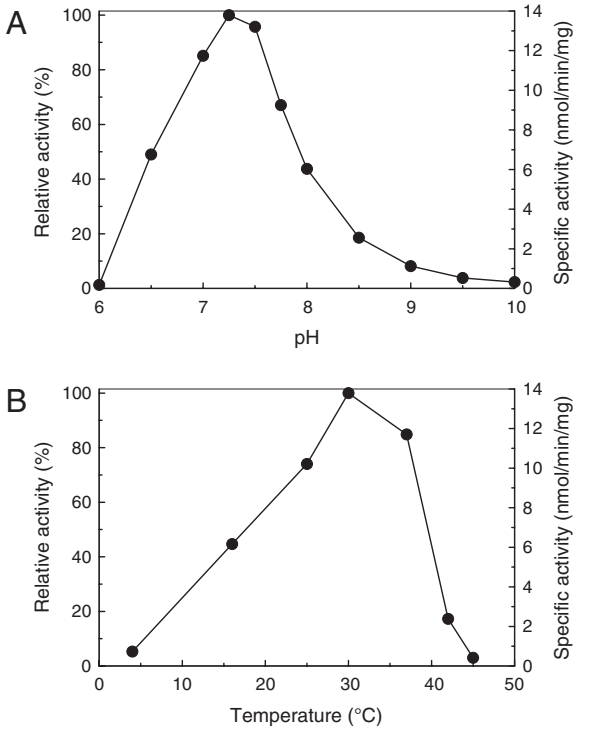

Fig. 6. Effects of pH (A) and Temperature (B) on MCAD1-Catalyzed Thioester Formation of (±)-CPPA.

Relative activity was calculated by comparing with the specific activity, using the value obtained in 100 mM MOPS-NaOH (pH 7.25) at 30 °C as standard. The specific activity of thioester formation was determined by HPLC analysis taking the final concentration of (±)-CPPA to be 1 mM. In experiments on the pH activity profile, the following buffers (100 mM) were used: pH 6.0–7.0 MOPS-NaOH; 7.0–8.0 PPB; 8.0–9.0 Tris–HCl. In the case of the temperature-effect experiments, 100 mM MOPS-NaOH (pH 7.25) was used.

Substrate specificity of recombinant MCAD1 was analyzed by a spectrophotometric method (Table 4). Likewise, MCAD1 showed perfect enantioselectivity toward CPPA. Fatty acids of various chain lengths were also subjected to this assay. As shown in Table 4, MCAD1 exhibited thioesterification activity toward fatty acids with 4 to 18 carbons. Among these compounds, the highest activity was detected in *n*-octanoic acid (C8), a medium chain-length fatty acid. These results indicate that MCAD1 is to be classified into a family of medium-chain acyl-CoA synthetases. This is the first report of enantioselective thioesterification activity of a medium-chain acyl-CoA synthetase toward 2-aryloxypropanoic acid. The enzyme can also accept 6-mercapthexanoic acid, 6-hydroxyhexanoic acid, and 11-mercaptundecanoic acid, which have a thiol or a hydroxyl group at the ω-position. In the case of

Table 3. Effects of Various Reagents on the MCAD1-Catalyzed Thioesterification Reaction of (±)-CPPA

Reagent	Conc. (mM)	Relative activity[b] (%)
Control[a]	—	100
PMSF	10	97
NaN_3	10	94
Iodoacetic acid	1	92
N-Ethylmaleimide	1	69
5,5′-Dithiobis(2-nitrobenzoic acid)	1	47
PCMB	1	27
$AgNO_3$	1	53
$ZnCl_2$	1	26
$HgCl_2$	1	N.D.
$NiSO_4$	10	N.D.
$FeSO_4$	10	N.D.
$CoCl_2$	10	N.D.
$CuCl_2$	10	N.D.
$CaCl_2$	10	80
$MnCl_2$	10	75

N.D., not detected; Relative activity could not be calculated because the specific activity was under the limit of detection.
[a]Control contained enzyme solution without any reagents.
[b]The activity under the control condition (13.8 nmol/min/mg) was taken to be 100%. The specific activity of thioester formation was determined by the method of HPLC assay with the final concentration of (±)-CPPA taken to be 1 mM.

6-aminohexanoic acid (Ahx), however, thioesterification activity was decreased dramatically. Interestingly the catalytic activity was recovered when the ω-amine was protected by Boc or Cbz protecting group. This result indicates that the substitution of ω-position is important to determine the substrate accessibility of this enzyme. Additionally, hydrocinnamic acid and α-lipoic acid were also accepted by this enzyme with high efficiency. In addition, (±)-2-phenoxypropanoic acid, (±)-2-phenylthiopropanoic acid, and (±)-MPPA were also converted to thioester, which are accepted by the whole-cell deracemization system of *N. diaphanozonaria* JCM 3208.[16] In the cases of 2-phenoxypropanoic acid and 2-phenylthiopropanoic acid, the results of chiral phase HPLC analysis revealed that its enantioselectivity was the same as that of CPPA. In the case of MPPA, both enantiomers were converted to thioesters speedily with little precedence over (*R*)-enantioselectivity. These results indicate that the substrate specificity of MCAD1 might be similar to that of the thioesterification enzyme of *N. diaphanozonaria*, though its enzyme was not yet been isolated. MCAD1 also catalyzed the thioesterification reaction of 2-phenylpropanoic acid (toward *R*), though the specific activity was one-tenth of that of (*S*)-CPPA and the enantiodifferentiational activity toward 2-phenylpropanoic acid was not sufficient. These results differ from those reported for rat long-chain acyl-CoA synthetase.

Anthraquinone-2-carboxylic acid, 2-anthracenecarboxylic acid, 1-pyrenebutyric acid, ferrocenecarboxylic acid, retinoic acid, *trans*-cinnamic acid, caffeic acid, malonic acid, and phenylalanine were ineffective substrates.

Kinetic studies of (*S*)-CPPA, *n*-octanoic acid, and α-lipoic acid were also conducted. The efficiency of thioester formation was calculated from of the data obtained by spectrophotometric analysis. The K_m and k_{cat} values were determined by Michaelis-Menten fitting

Table 4. Substrate Specificity of MCAD1-Catalyzed Thioesterification Reaction

Carboxylic acid[a]	Relative activity[b] (%)
(*S*)-CPPA	7
(*R*)-CPPA	N.D.
Acetic acid (C2)	N.D.
n-Propanoic acid (C3)	0.4
n-Butanoic acid (C4)	34
n-Hexanoic acid (C6)	46
n-Octanoic acid (C8)	100
n-Decanoic acid (C10)	67
n-Dodecanoic acid (C12)	43
n-Butyldecanoic acid (C14)[c]	30
n-Hexadecanoic acid (C16)[c]	26
n-Octadecanoic acid (C18)[c]	15
6-Mercaptohexanoic acid	89
6-Hydroxyhexanoic acid	71
Ahx	0.5
11-Mercaptoundecanoic acid	59
Boc-Ahx	6
Cbz-Ahx	58
α-Lipoic acid	82
Hydrocinnamic acid	36
(±)-2-Phenoxypropanoic acid	4
(±)-2-Phenylthiopropanoic acid	4
(±)-MPPA	38
(*S*)-2-Phenylpropanoic acid	0.6
(*R*)-2-Phenylpropanoic acid	0.3
Benzoic acid	0.3
Anthraquinone-2-carboxylic acid	N.D.
2-Anthracenecarboxylic acid	N.D.
1-Pyrenebutyric acid	N.D.
Ferrocenecarboxylic acid	N.D.
Retinoic acid	N.D.
trans-Cinnamic acid	N.D.
Caffeic acid	N.D.
Malonic acid	N.D.
Phenylalanine	N.D.

N.D., not detected; Relative activity could not be calculated because the specific activity was under the limit of detection.
[a]The final concentration of carboxylic acid was 1 mM.
[b]Relative activity was calculated by comparison with specific activity, taking the value toward *n*-octanoic acid (C8) (248 nmol/min/mg) as standard. The specific activity of thioester formation was determined by spectrophotometric assay.
[c]To dissolve the substrate, activity was measured when 0.2% Triton X-100 was added to the reaction mixture.

Table 5. Kinetic Parameters of MCAD1 Catalyzed Thioesterification Reaction

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
(*S*)-CPPA	0.92 ± 0.17	0.28 ± 0.026	0.28
α-Lipoic acid	0.30 ± 0.027	2.1 ± 0.080	7.0
n-Octanoic acid (C8)	0.077 ± 0.009	6.6 ± 0.27	86.0

The specific activity of thioester formation was determined by spectrophotometric assay.

(Table 5). These results also confirmed that MCAD1 is a suitable enzyme to catalyze the thioester formation of fatty acids with a medium chain-length.

In this report, we describe the isolation of several new microorganisms that exhibited deracemization activity of CPPA. Additionally, we purified and cloned a new enzyme, MCAD1, from *B. ketoglutamicum*. This protein was found to catalyze the (*S*)-enantioselective thioesterification reaction of CPPA in a practically perfect

manner. Investigation of substrate specificity revealed that this enzyme shows high enzymatic activity toward fatty acids possessing a medium chain-length. MCAD1 shares very low homology with long-chain acyl-CoA synthetase, which has been found to catalyze the enantioselective thioester formation of 2-arylpropanoic acid in rats. Specific activity toward 2-arylpropanoic acid was also relatively low. Thus, MCAD1 is to be classified in a family of medium-chain acyl-CoA synthetases. This activity has not previously been reported.

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