# Asymmetric Synthesis of L-α-Methylcysteine with the Amidase from *Xanthobacter flavus* NR303

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Received: January 21, 2005; Accepted: April 8, 2005

**Abstract:** An amidase acting on D, L-α-methylcysteinamide was purified from *Xanthobacter flavus* NR303. The enzyme acted L-stereoselectively on D,L-α-methylcysteinamide to yield L-α-methylcysteine. Based on the N-terminal amino acid sequence of the amidase, the gene encoding the enzyme was cloned from the genomic DNA of *X. flavus* and sequenced. Analysis of 4840 bp of the genomic DNA revealed the presence of an open reading frame (*mcaA*) which encodes the amidase. This enzyme is composed of 355 amino acid residues (molecular mass, 38555 Da). The intact cells of the *Escherichia coli* transformant could be used for the L-stereoselective hydrolysis of racemic α-methylcysteinamide. It was activated in the presence of $Mn^{2+}$, and had maximal activity at pH 7.0 and 55 °C. The *E. coli* transformant catalyzed the synthesis of L-α-methylcysteine from D, L-α-methylcysteinamide in a yield of 80% with high optical purity (>98% ee).

**Keywords:** amidase; enzymes; hydrolysis; α-methylcysteinamide; stereoselectivity; *Xanthobacter flavus*

## Introduction

Amidases (acylamide amidohydrolases, EC 3.5.1.4) catalyze the hydrolysis of the carboxyl amide bonds to liberate carboxylic acids and ammonia. Recently, various kinds of stereoselective amidases from microbial origin have been reported and received much attention because of their potential use for the industrial production of optically active compounds.[1–3] *S*-Enantiomer-selective amidases from *Brevibacterium* sp. R312,[4] *Pseudomonas chlororaphis* B23,[5,6] and *Rhodococcus rhodochrous* J-1[7–9] were found to be involved in nitrile metabolism with genetically linked nitrile hydratases.[7] *S*- and *R*-enantiomer-selective amidases, which seemed not to be related to the nitrile metabolism, were also found in *Agrobacterium tumefaciens* d3[10] and *Comamonas acidovorans* KPO-2771-4,[11] respectively. These enantiomer-selective amidases can be used for the production of optically active 2-arylpropionic acids, the non-steroid anti-inflammatory drugs, from the corresponding racemic amides. *S*-Stereoselective amino acid amidases from *Pseudomonas putida* ATCC 12633,[12] *Ochrobactrum anthropi* NCIMB 40321,[13] *Mycobacterium neoaurum* ATCC 25795[14] and *Pseudomonas azotoformans* IAM1603,[15] and the *R*-stereoselective amino acid amidases from *O. anthropi* C1–38,[16,17] *O. anthropi* SV3,[18,19] *Arthrobacter* sp. NJ-26,[20] *Brevibacillus borstelensis* BCS-1[21] and *Pseudomonas* sp. MCI3434[22] were found to be useful for the production of enantiomerically pure amino acids and their derivatives from the corresponding racemic amino acid amides. The genes coding for the above amidases have been isolated and their primary structures revealed, except for the *S*-stereoselective amino acid amidases from *P. putida* ATCC 12633, *O. anthropi* NCIMB 40321 and *M. neoaurum* ATCC 25795 and the *R*-stereoselective amino acid amidase from *Arthrobacter* sp. NJ-26.

Enantiomerically pure L-α-methylcysteine is an important chiral building block for pharmacologically active compounds such as a nitric oxide synthase inhibitor.[23] The synthesis of L-α-methylcysteine through *N*-formylthiazolidine derived from (*R*)-cysteine and pivalaldehyde has been reported.[24] This synthesis was carried out under strict conditions at −78 °C.

In this study, we screened for microorganisms that can hydrolyze D, L-α-methylcysteinamide and found the hydrolytic (amidase) activity in *Xanthobacter flavus* NR303. The amidase purified from cells of the strain hydrolyzed L-stereoselectively D,L-α-methylcysteinamide to form L-α-methylcysteine (Figure 1). The gene coding for the enzyme was isolated and expressed in *Escherichia coli* host.

  DOI: 10.1002/adsc.200505028 

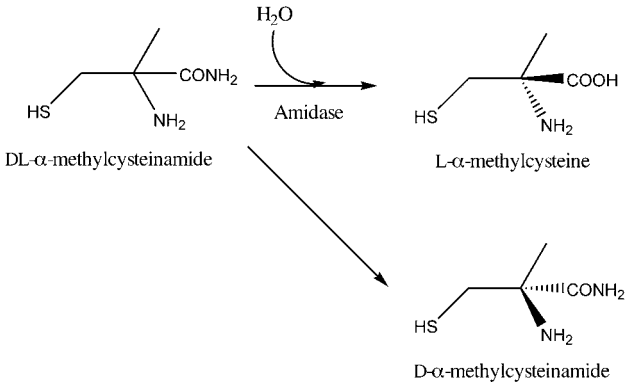


**Figure 1.** Stereoselective hydrolysis of D, L-α-methylcysteinamide by the amidase from *X. flavus* NR303.

## Results and Discussion

### Identification of the Strain NR303

An amidase, acting on D,L-α-methylcysteinamide, was detected in the strain NR303. In order to identify the strain, the nucleotide sequence of 16S rRNA gene was analyzed and compared with those of related strains. The homology scores between the NR303 and the most related strains are 100% for *Xanthobacter flavus*, 99.4% for *Xanthobacter autotrophicus* and 98.5% for *Xanthobacter agilis*. So we infer that the NR303 is *X. flavus*. The characteristics of *X. flavus* and strain NR303 are listed in Table 1.

**Table 1.** Comparison of strain NR303 with *X. flavus*

| Characteristics | Strain NR303 | *X. flavus* |
|---|---|---|
| Shape | Rod | Coccobacilli or rod |
| Pleomorphism | + | + |
| Motility | − | − |
| Gram stain | Negative | Negative |
| Urease | + | − |
| Oxidase | + | + |
| Catalase | + | + |
| Nitrate reduction | + | + |
| Indole production | − | − |
| Growth at | | |
| 37 °C | + | + |
| 45 °C | − | − |
| Sole carbon source | | |
| Gluconate, malate, citrate | + | + |
| Glucose, manose, mannitol, maltose | − | + |

### Purification and Characterization of the L-Stereoselective Amidase from *X. flavus* NR303

Various nitrogen and carbon sources were tested, and the highest level of activity was obtained after culture in an optimized medium, basal medium, containing glycerol and urea. HPLC analysis showed that the *X. flavus* NR303 cells acted on D,L-α-methylcysteinamide to produce L-α-methylcysteine. The wild-type enzyme was purified to homogeneity through a 5-step purification procedure as described in the Experimental Section (Table 2). The enzyme was purified 45-fold with a yield of 43% from the cell-free extract of *X. flavus* NR303 by measuring the enzyme activity with D,L-α-methylcysteinamide as the substrate. Enhancement of the amidase activity of the enzyme during the DEAE-Toyopearl chromatography raised the possibility of the presence of an inhibitor against the enzyme in the soluble fraction. The final preparation gave a single band on SDS/PAGE with a molecular mass of ~40 kDa. The purified enzyme catalyzed the hydrolysis of D,L-α-methylcysteinamide with strict L-stereoselectivity.

### Cloning and Characterization of the Amidase Gene, *mcaA*

To obtain information about the primary structure of the amidase, its N-terminal amino acid sequence was analyzed by Edman degradation and found to be ASRPLTPPPYSPPDPAWLEGSIMAARGEAKGRAGERYEIT. A BLAST [25] search of a protein database indicated that the N-terminal amino acid sequence of the amidase did not show significant similarity to any known proteins. The primers used for cloning part of the amidase gene, named *mcaA*, by PCR were designed based on the sequence of PPDPAWLE and GERYEIT. An 87-bp DNA was PCR-amplified with the primers and the chromosomal DNA prepared from *X. flavus* NR303, and used as a probe for Southern and colony hybridiza-

**Table 2.** Purification of amidase from *X. flavus* NR303.

| Step | Total protein [mg] | Total activity [U] | Specific activity [U/mg] | Yield [%] |
|---|---|---|---|---|
| Cell-free extract | 4270 | 20 | 0.0046 | 100 |
| Ammonium sulfate | 3090 | 16 | 0.0053 | 82 |
| DEAE-Toyopearl | 1510 | 32 | 0.021 | 161 |
| Butyl-Toyopearl | 228 | 24 | 0.11 | 122 |
| Gigapite | 40.0 | 8.5 | 0.21 | 43 |

  

tions to obtain the recombinant plasmid which contain inserts of 4.8 kb. The plasmid was named pMCA1-Eco.

The nucleotide sequence of the inserted fragment was determined, and an open reading frame (ORF) was present in this region. The structural *mcaA* gene consists of 1065 bp and codes for a protein of 355 amino acids with a predicted molecular mass of 38555 Da, which is consistent with the value estimated from the relative mobility of the purified amidase on SDS/PAGE. A potential ribosome-binding site (AGGAGA) was located just seven nucleotides upstream from the start codon ATG. Alignment by the protein databases using the BLAST program showed that the deduced primary structure of McaA is similar to those of putative acetamidase/formamidase as summarized in Table 3.

### Production of the McaA in *E. coli* and Optical Resolution of Racemic α-Methylcysteinamide by the Recombinant *E. coli* Cells

The direction of *mcaA* transcription was opposite to that of the *lac* promoter in pMCA1-Eco. *E. coli* JM109 transformed by the recombinant plasmid exhibited no amidase activity toward D, L-α-methylcysteinamide. These findings suggest that RNA polymerase in *E. coli* cannot recognize the promoter for *mcaA* or that there is a possible regulatory gene in the inserted fragment of pMCA1-Eco. To express the *mcaA* gene in *E. coli*, we improved the sequence upstream from the ATG start codon by PCR, with chromosomal DNA from *X. flavus* NR303 as a template. A TAG stop codon was added in-frame with the *lacZ* gene in pUC19 to avoid the formation of a possible fusion protein, and a ribosome-binding site (AGGAGG) was also added 9 bp upstream from the ATG start codon to enhance the translation. The resultant plasmid, pMCA1, in which the *mcaA* gene was under the control of the *lac* promoter of the pUC19 vector, was introduced into *E. coli* JM109 cells. When *E. coli* JM109 harboring pMCA1 was cultured in Luria-Bertani medium[26] supplemented with ampicillin and isopropyl β-D-thiogalactopyranoside for 12 h at 37 °C, a protein band (40 kDa) corresponding to McaA purified from *X. flavus* NR303 was produced (Figure 2).

L-α-Methylcysteine was produced by the action of McaA expressed in *E. coli* cells, L-stereoselectively and stoichiometrically from racemic α-methylcysteinamide resulting in the formation of a half concentration of L-α-methylcysteine. From 1% D, L-α-methylcysteinamide (weight/volume), 0.5% L-α-methylcysteine was synthesized at 30 °C and pH 7.0 in 24 h using 1% (weight dry cells/weight D, L-α-methylcysteinamide) *E. coli* cells with 5 ppm $Mn^{2+}$ ($MnCl_2 \cdot 4\,H_2O$). The effect of concentration of $Mn^{2+}$ on the activity of *E. coli* JM109 harboring pMCA1 is shown in Table 4.

The *E. coli* cells produced L-α-methylcysteine with high optical purity (>98% ee). Traces of D-α-methylcysteine were observed.

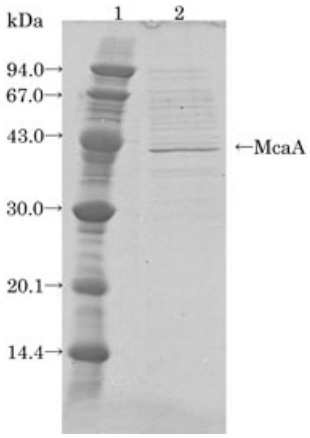


**Figure 2.** SDS/PAGE of the supernatant of the sonicated cell-free extract of the *E. coli* transformant. Lane 1, molecular mass standards [phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa)]; Lane 2, the cell-free extract of the *E. coli* transformant.

**Table 3.** The homology of McaA with other strains.

| Strain | Homology | Accession number |
|---|---|---|
| *Rhodopseudomonas palustris* | 76% identical over 327 amino acids | NP_947758 |
| *Desulfitobacterium hafniense* | 48% identical over 306 amino acids | ZP_00098942 |
| *Bacillus cereus* | 35% identical over 284 amino acids | NP_978922 |
| *Rhodobacter sphaeroides* | 35% identical over 297 amino acids | ZP_00207763 |
| *Ferroplasma acidarmanus* | 33% identical over 311 amino acids | ZP_00306227 |
| *Bacillus anthracis* | 34% identical over 289 amino acids | YP_028678 |
| *Picrophilus torridus* | 31% identical over 286 amino acids | YP_022950 |
| *Pyrococcus abyssi* | 32% identical over 294 amino acids | NP_126599 |
| *Chloroflexus aurantiacus* | 34% identical over 303 amino acids | ZP_00356595 |
| *Thermoanaerobacter tengcongensis* | 32% identical over 287 amino acids | NP_623495 |
| *Sulfolobus solfataricus* | 32% identical over 312 amino acids | NP_344131 |
| *Synechococcus elongates* | 33% identical over 281 amino acids | ZP_00164608 |

  

**Table 4.** Effect of concentration of $Mn^{2+}$ on the activity of *E. coli* transformant.[a]

| Concentration of $Mn^{2+}$ [ppm] | Activity [U/mg-cell] |
|---|---|
| 0 | 0.15 |
| 5 | 0.20 |
| 10 | 0.23 |
| 50 | 0.45 |
| 100 | 1.2 |
| 500 | 1.1 |

[a] The cell reaction with 0–500 ppm $Mn^{2+}$ ($MnCl_2 \cdot 4\,H_2O$) was carried out using 1% (weight dry cells/weight D,L-α-methylcysteinamide) *E. coli* cells prepared from the 16 h culture at 37 °C. The reaction mixture was incubated at 30 °C and the reaction was stopped at 60 min.

### Characterization of McaA

When the concentration of $Mn^{2+}$ was 100 ppm, the level of *E. coli* JM109 harboring pMCA1 activity toward D,L-α-methylcysteinamide was 1.185 U/mg-cell. The addition of $Mn^{2+}$ is effective to activate the activity of *E. coli* JM109 harboring pMCA1. No metal ion was detected by a Polarized Zeeman Atomic Absorption Spectrophotometer 180-80 (Hitachi Ltd., Tokyo, Japan) in McaA (data not shown), so $Mn^{2+}$ is an activator for McaA.

To study the substrate specificity, the cells of *E. coli* JM109 harboring pMCA1 was used to hydrolyze various amides, and the activity was assayed. L-Valine, L-α-phenylglycine, L-*tert*-leucine, L-phenylalanine and L-α-aminobutyric acid were produced in a similar manner by the enzymatic kinetic resolution of amino acid amides by using the *E. coli* transformant. However, when the cells of *E. coli* JM109/pMCA1 were incubated with propionamide and butyramide, propionic acid and butyric acid were not formed.

The effects of pH and temperature on the activity of *E. coli* JM109 harboring pMCA1 are shown in Figure 3 and Figure 4. The *E. coli* cells produced L-α-methylcysteine with high optical purity (>98% ee). The cells showed maximal activity at pH 7.0 and 55 °C.

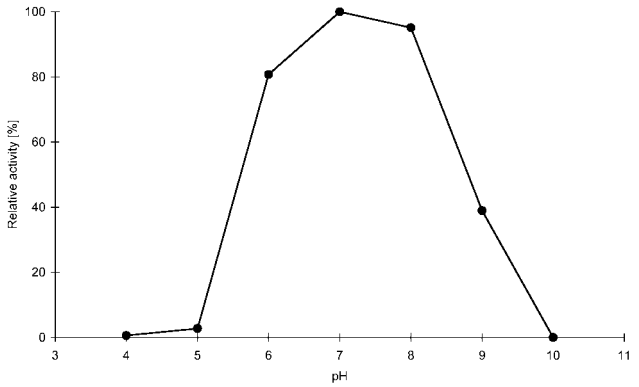


**Figure 3.** Effect of pH on the activity of *E. coli* transformant. The cell reaction with 100 ppm $Mn^{2+}$ ($MnCl_2 \cdot 4\,H_2O$) was carried out in the pH range 4–10 using 1% (weight dry cells/weight D, L-α-methylcysteinamide) *E. coli* cells prepared from the 16 h culture at 37 °C. The reaction mixture was incubated at 30 °C and the reaction was stopped at 60 min.

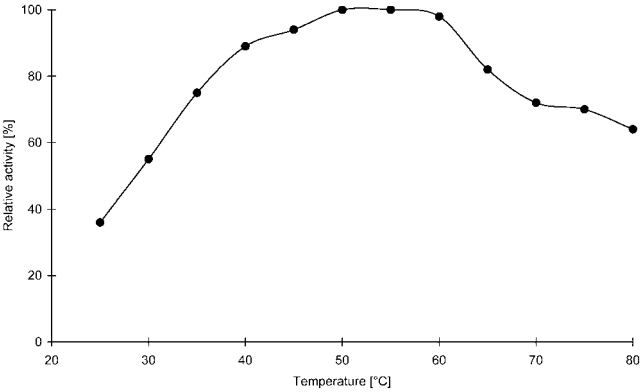


**Figure 4.** Effect of temperature on the activity of *E. coli* transformant. The cell reaction with 10% (weight/volume) D,L-α-methylcysteinamide and 100 ppm $Mn^{2+}$ ($MnCl_2 \cdot 4\,H_2O$) was carried out in the temperature range 25–80 °C using 1% (weight dry cells/weight D,L-α-methylcysteinamide) *E. coli* cells prepared from the 16 h culture at 37 °C. The reaction mixture was incubated at 25–80 °C and the reaction was stopped at 90 min.

### Synthesis of L-α-Methylcysteine Hydrochloride

L-α-Methylcysteine hydrochloride was synthesized from D, L-α-methylcysteinamide by the method described in the Experimental Section. The total yield from L-α-methylcysteinamide was 80% and stereoselectivity was >98% ee.

As McaA has become abundantly available using the DNA technique, *E. coli* cells producing McaA may be used in the optical resolution of D,L-α-methylcysteinamide to yield L-α-methylcysteine and D-α-methylcysteinamide. McaA is the enzyme useful for the enzymatic optical resolution of D,L-α-methylcysteinamide carried out under mild conditions. This synthetic method may be applicable to large-scale preparation of L-α-methylcysteine and L-amino acids.

## Conclusion

In this study, we purified an amidase from *X. flavus* NR303 acting L-stereoselectively on D,L-α-methylcysteinamide, cloned its structural gene, *mcaA*, and investigated the activity of *E. coli* JM109 harboring pMCA1.

The *E. coli* JM109 harboring pMCA1 acted on D,L-α-methylcysteinamide with strict L-stereoselectivity to form L-α-methylcysteine. The addition of $Mn^{2+}$ is effec-

  

tive to activate the activity of *E. coli* JM109 harboring pMCA1. It had maximal activity at pH 7.0 and 55 °C. The *E. coli* transformant catalyzed the synthesis of L-α-methylcysteine from D,L-α-methylcysteinamide in a yield of 80%.

The amidase is the first enzyme useful for the enzymatic optical resolution of D,L-α-methylcysteinamide. We assume that the procedures proposed here can be applicable to the synthesis of a wide variety of enantiomerically pure alpha-amino acids.

## Experimental Section

### Synthesis of D,L-α-Methylcysteinamide Hydrochloride

2,2,4-Trimethylthiazolidine-4-carboxamide hydrochloride was synthesized as described previously.[27] 2,2,4-Trimethylthiazolidine-4-carboxamide hydrochloride (90 g, 0.43 mol) was dissolved in distilled water (1 L) at room temperature and the solution was stirred at 105 °C for 3 h. The mixture was evaporated and dried under vacuum to leave a white solid; yield: 70 g (0.41 mol, 95%). $^1$H NMR (90 MHz, $D_2O$): δ = 3.19 (d, 1 H), 2.95 (d, 1 H), 1.64 (s, 3H); $^{13}$C NMR (22.6 MHz, $D_2O$): δ = 173.78 (s), 62.31 (s), 31.73 (t), 22.21 (q); MS (70 eV): *m/e* (relative intensity) = 135 (5), 90 (100), 87 (35), 73 (26), 57 (14), 42 (26), 36 (17).

### Bacterial Strains, Plasmids and Culture Conditions

*X. flavus* NR303 was selected as a microorganism capable of degrading D,L-α-methylcysteinamide and used as the source of enzyme and chromosomal DNA. *E. coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*)/F′ [*traD*36, *proAB*$^+$, *lacI*$^q$, *lacZ*ΔM15]) was used as a host for the recombinant plasmids. Plasmids pBluescriptII SK(−) (Toyobo, Osaka, Japan) and pUC19 (Takara Shuzo, Kyoto, Japan) were used as cloning vectors. *X. flavus* NR303 was grown in basal medium containing 3 g $(NH_4)_2SO_4$, 1.4 g $KH_2PO_4$, 3 g $Na_2HPO_4$, 0.3 g $NaHCO_3$, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 1 mL vitamin mixture solution (0.02 g/L biotin, 4 g/L calcium pantothenate, 4 g/L pyridoxine hydrochloride, 4 g/L thiamin hydrochloride, 2 g/L *p*-aminobenzoic acid, 0.02 g/L folic acid, 20 g/L inositol, 4 g/L nicotinic acid, 2 g/L riboflavin), 1 g urea, 3.5 mL trace element solution [6 g/L iron(III) citrate *n*-hydrate, 2 g/L $ZnSO_4 \cdot 7\,H_2O$, 1 g/L $MnCl_2 \cdot 4\,H_2O$, 0.1 g/L $CuSO_4 \cdot 5\,H_2O$, 0.1 g/L potassium iodide, 0.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, 0.1 g/L $CoCl_2 \cdot 6\,H_2O$, 0.2 g/L $H_3BO_3$, 5 g/L NaCl, 4 g/L $CaCl_2 \cdot 2\,H_2O$], 10 g glycerol in 1 L distilled water, pH 7.0. Recombinant *E. coli* JM109 was cultured in Luria-Bertani medium containing ampicillin (50 g/mL). To induce the gene under the control of the *lac* promoter, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.005 mM.

### Purification of McaA from *X. flavus* NR303

*X. flavus* NR303 was subcultured at 30 °C for 24 h in a 100-mL Erlenmeyer flask containing 20 mL of basal medium. The subculture (20 mL) was then inoculated into a 30-L jar-fermenter containing 20 L of basal medium. After a 7 days cultivation at 30 °C, the cells were harvested by centrifugation at 28000 g for 60 min at 4 °C. The buffer used was potassium phosphate (pH 7.0) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. Washed cells (240 g, wet weight) from 20 L of culture were suspended in 0.1 M buffer and disrupted by sonication for 10 min (19 kHz; Insonator model 201M; Kubota, Tokyo, Japan). The sonicate was centrifuged at 19000 g for 10 min at 4 °C, and the resulting supernatant was used as the cell-free extract. The cell-free extract was fractionated with solid ammonium sulfate. The precipitate obtained at 30–60% saturation was collected by centrifugation and dissolved in 100 mM buffer. The resulting enzyme solution was dialyzed for 24 h against three changes of 10 mM buffer. The dialyzed enzyme solution was then applied to a column (5 × 20 cm) of DEAE-Toyopearl 650M (Tosoh Corp.) previously equilibrated with 10 mM buffer. After the column had been washed with 1.2 L of 10 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.5 M, 300 mL each) in 10 mM buffer. The protein content of the eluates from the column chromatography was monitored by measuring absorbance at 280 nm. The active fractions were combined and then brought to 30% saturated with ammonium sulfate saturation and applied to a column (2.5 × 20 cm) of butyl-Toyopearl 650M (Tosoh Corp.) previously equilibrated with 10 mM buffer 30% saturated with ammonium sulfate. The column was washed with 500 mL of the same buffer, and the enzyme was eluted with a linear gradient of ammonium sulfate (30–0% saturation, 250 mL each) in 10 mM buffer. The active fractions were combined and concentrated with ultra filter, and diluted with 5 mM potassium phosphate buffer (pH 7.0). The diluted enzyme was applied to a column (2 × 18 cm) of Gigapite (Seikagaku Kogyo, Tokyo Japan) previously equilibrated with 5 mM potassium phosphate buffer (pH 7.0). The column was washed with 5 mM potassium phosphate buffer (pH 7.0), and the enzyme was eluted with a linear gradient of 5–400 mM potassium phosphate buffer (pH 7.0). The active fractions were collected for the determination of the N-terminal amino acid sequence of the enzyme. To determine the N-terminal amino acid sequence, the purified enzyme was blotted from the gel after SDS-PAGE (12.5% acrylamide) on a poly(vinylidene difluoride)membrane (PVDF, Bio-Rad, Hercules, CA) with the transblot semi-dry electrophoresis transfer cell (Nippon Eido, Tokyo, Japan), analyzed with HP G1005A Protein Sequencing Systems (Hewlett-Packard Co., Palo Alto, CA).

### Cloning of the *X. flavus* NR303 Amidase Gene, *mcaA*

For routine work with recombinant DNA, established protocols were used.[26] Restriction endonucleases were purchased from Takara Shuzo and alkaline phosphatase from shrimp was purchased from Roche Diagnostics GmbH (Mannheim, Germany). An oligonucleotide sense primer, 5′-CC(G/C)-CC(G/C)GA(C/T)CC(G/C)GC(G/C)TGGCT(G/C)G-3′, and an antisense primer, 5′-(G/C)GTGAT(C/T)TC(A/G)TA(G/C)-CG(C/T)TCGCC-3′, were synthesized on the basis of the amino acid sequence of PPDPAWLE and GERYEIT respectively, which were part of the N-terminal amino acid sequence of the purified amidase. The genomic DNA of *X. flavus* NR303 was isolated as described previously[28] and used as a template for PCR amplification with a PTC-200 thermal cycler (MJ Re-

  

search Inc., Watertown, MA). Reaction mixtures contained 150 ng of chromosomal DNA, 25 pmol of each primer, each dNTP at a concentration of 0.2 mM, and 2.5 unit of *Thermus aquaticus* DNA polymerase (EX Taq, Takara, Japan) in a volume of 50 μL. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 30 s (initial cycle 2 min), an annealing step at 61 °C for 30 s and an elongation step at 72 °C for 2 min. The PCR product (87 bp) was radiolabeled with [α-$^{32}P$]dCTP using a RediprimeII DNA labeling system (Amersham Biosciences) and used as a probe for the amidase-encoding gene of *X. flavus* NR303. Chromosomal DNA of *X. flavus* NR303 was completely digested with *Eco*RI. Southern hybridization showed a 5 kb band from *Eco*RI digestion that hybridized with the probe. DNA fragments of 4.4–6.5 kb from the *Eco*RI digestion were recovered from 0.7% (w/v) agarose gel by use of a QIAquick™ gel extraction kit from QIAGEN (Tokyo, Japan) and ligated into *Eco*RI-digested and alkaline phosphatase-treated pBluescript II SK(-) using Ligation kit version 2 from Takara Shuzo. *E. coli* JM109 was transformed with the recombinant plasmid DNA by the method of Inoue et al.,[29] and screened for the existence of the gene by colony hybridization with the probe. A positive *E. coli* transformants which carried an 7.8 kb plasmid designated pMCA1-Eco was selected for further analysis.

## DNA Sequence Analysis

The plasmid pMCA1-Eco was used as a sequencing template. Nested unidirectional deletions were generated with the Kilo-Sequence deletion kit (Takara Shuzo). Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method[30] with M13 forward and reverse oligonucleotides as primers. Sequencing reactions were carried out with a Thermo Sequenase™ cycle sequencing kit and dNTP mixture with 7-deaza-dGTP from Amersham Biosciences K.K., and the reaction mixtures were run on a DNA sequencer 4000 L (Li-cor, Lincoln, NE, USA). Both strands of DNA were sequenced. Amino acid sequences were compared with the BLAST program.

## Expression of the *mcaA* Gene in *E. coli*

A modified DNA fragment coding for McaA was obtained by PCR. The reaction mixture for the PCR contained 150 ng of chromosomal DNA from *X. flavus* as a template, 15 pmol of each primer, each dNTP at a concentration of 0.2 mM, and 1 unit of *Thermococcus kodakaraensis* DNA polymerase (KOD -Plus-, TOYOBO, Japan) in a volume of 50 μL. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 15 s (initial cycle 2 min), an annealing step at 70 °C for 30 s and an elongation step at 68 °C for 90 s. The sense primer contained a *Hin*dIII recognition site (underlined sequence), a ribosome-binding site (double underlined sequence), a TAG stop codon (lowercase letters) inframe with the *lacZ* gene in pUC19. The antisense primer contained an *Xba*I site (underlined sequence). The two primers were as follows: sense primer, 5′-CGCCAGAAGCTTTAAGGAGGAAAtagCCCATGTCCCGGCATCCCATG-3′, antisense primer, 5′-TAGGTGTCTAGACCGGAGGCTGCC-3′. The amplified PCR product was digested with *Hin*dIII and *Xba*I, separated by agarose gel electrophoresis, and then purified with QIAquick™ gel extraction kit. The amplified DNA was inserted downstream of the *lac* promoter in pUC19, yielding pMCA1, and then used to transform *E. coli* JM109 cells.

## Enzyme Assay

During the purification of McaA from *X. flavus* NR303, the enzyme assay was carried out with D,L-α-methylcysteinamide as a substrate. The reaction mixture (0.2 mL) contained 20 μmol potassium phosphate buffer (pH 7.0), 0.2% D,L-α-methylcysteinamide and an appropriate amount of the enzyme. After the reaction was performed at 30 °C for 2 h, the reaction was stopped by adding 40 μL of 0.2 M $HClO_4$ followed by vortexing. The mixture was analyzed on an HPLC apparatus equipped with a LiChrosorb 100 RP-18 column (0.4 × 25 cm; Kanto Kagaku, Tokyo, Japan) at a flow rate of 0.5 mL $min^{-1}$, using as a solvent system 50 mM $HClO_4$. The eluate was detected with refractive index detector RI-101 (Shodex, Tokyo, Japan). One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol α-methylcysteine $min^{-1}$ from D,L-α-methylcysteinamide under the above conditions. Protein was determined by the method of Bradford[31] with BSA as standard, using a kit from Bio-Rad Laboratories Ltd (Tokyo, Japan).

For determination of the stereochemistry of the reaction product, a chiral-separation column was used in HPLC. The reaction mixture (1 mL) contained 0.1 mmol potassium phosphate buffer (pH 7.0), 0.2% D, L-α-methylcysteinamide and an appropriate amount of the cell or enzyme. After the reaction was performed, the α-methylcysteine formed was derivatized with formaldehyde by the addition of 0.1 mL formaldehyde (36% aqueous solution) to the reaction mixture. After incubation at 30 °C for 1 h, the amount of each enantiomer of the derivatized α-methylcysteine formed in the reaction mixture was determined with a SUMICHIRAL OA-5000 column (0.46 × 5 cm; Sumika Chemical Analysis Service, Osaka, Japan) at a flow rate of 0.5 mL $min^{-1}$, using as a solvent system 0.5/99.5 (*i*-PrOH/3 mM $CuSO_4$). The absorbance of the eluate was monitored at 320 nm.

## Nucleotide Sequence Accession Number

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB196122.

## Reaction from Amide to Acid by using Whole Cells of *E. coli* JM109/pMCA1

The cells of *E. coli* harboring pMCA1 washed once with 0.85% NaCl and used as an enzyme source. The reaction mixture (2 mL) containing 10% amide and the washed cells was adjusted to pH 7.0 with 25% ammonia solution, and was incubated. The reaction was terminated by removing the cells by centrifugation. The yields of α-methylcysteine and the stereochemistry of L-α-methylcysteine were determined by the procedure described under "Enzyme Assay".

  

### Synthesis of L-α-Methylcysteine Hydrochloride

In this procedure all solvents were deoxidized by $N_2$ bubbling and all reactions were done under a nitrogen atmosphere. D,L-α-Methylcysteinamide hydrochloride (20 g, 0.118 mmol) and $MnCl_2 \cdot 4\,H_2O$ (0.8 g, 100 ppm) were dissolved in distilled water (182 mL) and the pH was adjusted to pH 7.0 with 25% ammonia solution. After addition of *E. coli* JM109/pMCA1 (15 mg) prepared from the culture broth after 16 h cultivation in Luria-Bertani medium the reaction mixture was allowed to stand at 50 °C for 19 h. NaOH (4.7 g, 0.118 mmol) was added to the mixture. The aqueous solution was dehydrated by azeotropic distillation with *i*-butyl alcohol (302 g) at 75 °C for 28 h under 74 mmHg. Acetone (62 g, 1.1 mol) was added in the reaction mixture and stirred at 100 °C for 2.5 h at atmospheric pressure. The solution was heated at 120 °C for 2 h, and then salts were precipitated and removed by filtration. The filtrate was allowed to stand at 5 °C until the white solid was precipitated. The solid was (*R*)-2,2,4-trimethylthiazolidine-4-carboxylic acid; yield: 8.5 g (0.049 mmol, 83%). $^1$H NMR (90 MHz, $D_2O$): δ = 3.70 (d, 1 H), 3.30 (d, 1 H), 1.83 (s, 3 H), 1.81 (s, 3 H), 1.71 (s, 3H); MS (70 eV): *m/e* (relative intensity) = 175 (13), 160 (48), 142 (6), 130 (62), 114 (41), 101 (27), 83 (65), 73 (57), 58 (99), 42 (100).

(*R*)-2,2,4-Trimethylthiazolidine-4-carboxylic acid (8.5 g, 0.049 mmol) was dissolved in 4% hydrochloric acid (42.5 mL) and the solution was dehydrated by azeotropic distillation with cyclohexane (110 g) at 90 °C at atmospheric pressure. The white solid was precipitated, and then the solid was filtered and dried under vacuum. The solid was L-α-methylcysteine hydrochloride; yield: 8.0 g (0.047 mmol, 80%). $^1$H NMR (90 MHz, $D_2O$): δ = 3.17 (d, 1 H), 2.86 (d, 1 H), 1.57 (s, 3H); $^{13}$C NMR (22.6 MHz, $D_2O$): δ = 178.04 (s), 55.75 (s), 38.53 (t), 23.50 (q); MS (70 eV): *m/e* (relative intensity) = 90 (35), 88 (78), 73 (23), 56 (15), 42 (100), 36 (13).

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