

A novel *R*-stereoselective amidase from *Pseudomonas* sp. MCI3434 acting on piperazine-2-*tert*-butylcarboxamide

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A novel amidase acting on (*R,S*)-piperazine-2-*tert*-butylcarboxamide was purified from *Pseudomonas* sp. MCI3434 and characterized. The enzyme acted *R*-stereoselectively on (*R,S*)-piperazine-2-*tert*-butylcarboxamide to yield (*R*)-piperazine-2-carboxylic acid, and was tentatively named *R*-amidase. The N-terminal amino acid sequence of the enzyme showed high sequence identity with that deduced from a gene named PA3598 encoding a hypothetical hydrolase in *Pseudomonas aeruginosa* PAO1. The gene encoding *R*-amidase was cloned from the genomic DNA of *Pseudomonas* sp. MCI3434 and sequenced. Analysis of 1332 bp of the genomic DNA revealed the presence of one open reading frame (*ramA*) which encodes the *R*-amidase. This enzyme, RamA, is composed of 274 amino acid residues (molecular mass, 30 128 Da), and the deduced amino acid sequence exhibits homology to a carbon–nitrogen hydrolase protein (PP3846) from *Pseudomonas putida* strain KT2440 (72.6% identity) and PA3598 protein from *P. aeruginosa* strain PAO1 (65.6% identity) and may be classified into a new subfamily in the carbon–nitrogen hydrolase family consisting of aliphatic amidase, β -ureidopropionase, carbamylase, nitrilase, and so on. The amount of *R*-amidase in the supernatant of the sonicated cell-free extract of an

Escherichia coli transformant overexpressing the *ramA* gene was about 30 000 times higher than that of *Pseudomonas* sp. MCI3434. The intact cells of the *E. coli* transformant could be used for the *R*-stereoselective hydrolysis of racemic piperazine-2-*tert*-butylcarboxamide. The recombinant enzyme was purified to electrophoretic homogeneity from cell-free extract of the *E. coli* transformant overexpressing the *ramA* gene. On gel-filtration chromatography, the enzyme appeared to be a monomer. It had maximal activity at 45 °C and pH 8.0, and was completely inactivated in the presence of *p*-chloromercuribenzoate, *N*-ethylmaleimide, Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Ag^+ , Cd^{2+} , Hg^{2+} or Pb^{2+} . RamA had hydrolyzing activity toward the carboxamide compounds, in which amino or imino group is connected to β - or γ -carbon, such as β -alaninamide, (*R*)-piperazine-2-carboxamide (*R*)-piperidine-3-carboxamide, D -glutaminamide and (*R*)-piperazine-2-*tert*-butylcarboxamide. The enzyme, however, did not act on the other amide substrates for the aliphatic amidase despite its sequence similarity to RamA.

Keywords: amidase; hydrolysis; piperazine-2-*tert*-butylcarboxamide; *Pseudomonas* sp.; stereoselectivity.

Amidases (acylamide amidohydrolases, EC 3.5.1.4), which are hydrolases acting on carboxyl amide bonds to liberate carboxylic acids and ammonia, have received much attention in applied microbiological field. Amidases from various microorganisms have been characterized to date. Aliphatic (wide-spectrum) amidases acting on aliphatic amides with short acyl chains were found in *Pseudomonas aeruginosa* [1], *Brevibacterium* sp. R312 [2], *Helicobacter pylori* [3] and *Bacillus stearothermophilus* BR388 [4]. Pyrazinamidase/nicotinamidase confers susceptibility to the antituberculous

drug pyrazinamide in *Mycobacterium* [5]. Many different kinds of microbial amidases with stereoselectivity have also been reported and some of them have been applied for the production of optically active compounds from the corresponding racemic amides [6–8]. *S*-enantiomer-selective amidases in *Brevibacterium* sp. R312 [9], *Pseudomonas chlororaphis* B23 [10] and *Rhodococcus rhodochromus* J1 [11] are involved in nitrile metabolism with a genetically linked nitrile hydratase. *S*- and *R*-enantiomer-selective amidase, which appeared not to be related to nitrile metabolism, were also found in *Agrobacterium tumefaciens* d3 [12] and *Comamonas acidovorans* KPO-2771-4 [13], respectively. *S*-Stereoselective amino acid amidases from *Pseudomonas putida* ATCC 12633 [14], *Ochrobactrum anthropi* NCIMB 40321 [15], and *Mycobacterium neoaurum* ATCC 25795 [16] can be used for the enzymatic production of (*S*)-amino acids from the corresponding racemic amino acid amides. *R*-Stereoselective amino acid amidases from *O. anthropi* SV3 [17], *Arthrobacter* sp. NJ-26 [18] and *Brevibacillus borstelensis* BCS-1 [19] were also used for the production of (*R*)-amino acids from racemic amino acid amides. The genes coding for the above amidases have been isolated and

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Abbreviations: NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole; ORF, open reading frame.

Enzymes: acylamide amidohydrolases (EC 3.5.1.4).

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their primary structures revealed, except for the *S*-stereoselective amino acid amidases of the three microorganisms and the *R*-stereoselective amino acid amidase from *Arthrobacter* sp. NJ-26. Although these amidases show a wide variety of substrate specificities, there is no report on the hydrolysis of amides containing a bulky substituent at the amide nitrogen, such as *tert*-butylcarboxamide. This inability to hydrolyze the bulky amides hindered the wide use of amidases for the production of complex compounds.

Enantiomerically pure piperazine-2-carboxylic acid and its *tert*-butylcarboxamide derivative are important chiral building blocks for some pharmacologically active compounds such as *N*-methyl-*D*-aspartate antagonist for glutamate receptor [20], cardioprotective nucleoside transport blocker [21], and HIV protease inhibitor [22]. (*S*)-Piperazine-2-carboxylic acid has been prepared by kinetic resolution of racemic 4-(*tert*-butoxycarbonyl)piperazine-2-carboxamide with leucine aminopeptidase [21] or racemic piperazine-2-carboxamide with *Klebsiella terrigena* DSM9174 cells [23]. There is no report on the kinetic resolution of (*R,S*)-piperazine-2-*tert*-butylcarboxamide.

In this study, we screened for microorganisms that can hydrolyze piperazine-2-*tert*-butylcarboxamide and found the hydrolytic (amidase) activity in *Pseudomonas* sp. MCI3434. The amidase purified from cells of the strain hydrolyzed *R*-stereoselectively piperazine-2-*tert*-butylcarboxamide to form (*R*)-piperazine-2-carboxylic acid (Fig. 1), and was tentatively named R-amidase. The gene coding for the R-amidase was isolated, sequenced and expressed in an *Escherichia coli* host. The recombinant protein was purified and characterized, and found to be a novel amidase with a unique substrate specificity.

Materials and methods

Bacterial strains, plasmids and culture conditions

Pseudomonas sp. MCI3434 (TPU7190 of Toyama Prefectural University) was selected as a microorganism capable of degrading (*R,S*)-piperazine-2-*tert*-butylcarboxamide and used as the source of enzyme and chromosomal DNA. *E. coli* JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*)/F' [*traD36*, *proAB*⁺, *lacI*^f, *lacZ* Δ M15]) 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. *Pseudomonas* sp. MCI3434 was grown in medium I containing 10 g Bonito extract (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 10 g disodium DL-malate *n*-hydrate, 3 g K₂HPO₄ and 1 g KH₂PO₄ in 1 L distilled water, pH 7.0. Recombinant *E. coli* JM109 was cultured in Luria-Bertani medium [24] containing ampicillin (80 μ g mL⁻¹). To induce expression of the gene

under the control of the *lac* promoter, isopropyl thio- β -D-galactoside was added to a final concentration of 0.5 mM.

Purification of the R-amidase from *Pseudomonas* sp. MCI3434

Pseudomonas sp. MCI3434 was subcultured at 30 °C for 16 h in a test tube containing 5 mL of medium I. The subculture (5 mL) was then inoculated into a 2 L Erlenmeyer flask containing 500 mL of medium I. After an 8 h incubation at 25 °C with reciprocal shaking, the cells were harvested by centrifugation at 10 000 *g* for 20 min at 4 °C and washed with 0.9% (w/v) NaCl. All the purification procedures were performed at a temperature lower than 4 °C. The buffer used was potassium phosphate (pH 7.0) containing 0.1 mM dithiothreitol and 5 mM 2-mercaptoethanol. Washed cells (125 g, wet weight) from 25 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 15 000 *g* for 20 min at 4 °C, and the resulting supernatant was used as the cell-free extract. The cell-free extract was dialyzed for 24 h against three changes of 10 mM buffer. The dialyzed enzyme solution was then applied to a column (6 \times 15 cm) of DEAE-Toyopearl 650M (Tosoh Corp.) previously equilibrated with 10 mM buffer. After the column had been washed with 2 L of 10 mM buffer, the enzyme was eluted with a 10 mM buffer containing 0.1 M NaCl. 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% ammonium sulfate saturation and applied to a column (3 \times 12 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, 500 mL each) in 10 mM buffer. The active fractions were combined and dialyzed against 10 L of 10 mM buffer for 12 h. The dialyzed enzyme was applied to a column (2 \times 15 cm) of DEAE-Toyopearl 650M previously equilibrated with 10 mM buffer. After the column had been washed with 150 mL of 10 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.2 M, 150 mL each) in 10 mM buffer. The active fractions were combined and dialyzed against 10 L of 10 mM buffer for 12 h. The dialyzed enzyme was applied to a column (3 \times 15 cm) of Gigapite (Seikagaku Kogyo, Tokyo, Japan) previously equilibrated with 10 mM buffer. The column was washed with 10 mM buffer, fractions of 10 mL were collected, and the active fractions were combined and concentrated. The enzyme solution was applied to a Superdex 200 HR 10/30 column (Amersham Biosciences K.K., Tokyo, Japan) equilibrated with 10 mM buffer containing 150 mM NaCl and eluted with the same buffer. The active fractions were collected and dialyzed against 10 L of 10 mM buffer for 12 h. For the determination of the N-terminal amino acid sequence of the enzyme, the purified enzyme was covalently bound to Sequelone-arylamine and -diisothiocyanate membranes and then analyzed with a Prosequencer 6625 automatic protein sequencer (Millipore, MA, USA).

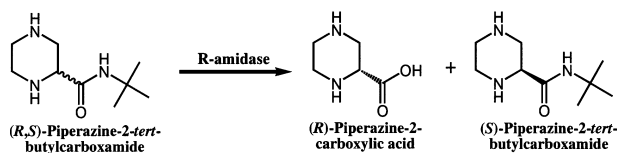


Fig. 1. Stereoselective hydrolysis of racemic piperazine-2-*tert*-butylcarboxamide by the R-amidase (RamA) from *Pseudomonas* sp. MCI3434.

Cloning of the *Pseudomonas* sp. MCI3434 R-amidase gene, *ramA*

For routine work with recombinant DNA, established protocols were used [24]. Restriction endonucleases were purchased from Takara Shuzo and alkaline phosphatase from shrimp was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chromosomal DNA was prepared from *Pseudomonas* sp. MCI3434 by the method of Misawa *et al.* [25]. An oligonucleotide sense primer, 5'-TACCGCAAGACCCACCT(C/G)-3', and an antisense primer, 5'-TCCGGGAAGCTCGA(C/T)GTC-3', were synthesized on the basis of the amino acid sequence of the conserved region of proteins whose N-terminal sequence is homologous to that of the R-amidase from *Pseudomonas* sp. MCI3434. The ExpandTM high fidelity PCR system from Roche Diagnostics was used for the PCR. The reaction mixture for the PCR contained 50 μ L of Expand HF buffer with 1.5 mM MgCl₂, each dNTP at a concentration of 0.2 mM, the sense and antisense primers each at 1 μ M, 2.5 U of Expand HF PCR system enzyme mix and 0.5 μ g of chromosomal DNA from *Pseudomonas* sp. MCI3434 as a template. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 30 s (initial cycle 2 min 30 s), an annealing step at 55 °C for 30 s and an elongation step at 72 °C for 2 min. The PCR product (125 bp) was radiolabeled with [α -³²P]dCTP using a Rediprime II DNA labeling system (Amersham Biosciences) and used as a probe for the R-amidase-encoding gene, *ramA*, of *Pseudomonas* sp. MCI3434. Chromosomal DNA of *Pseudomonas* sp. MCI3434 was completely digested with *FbaI* or *PstI*. Southern hybridization showed a 5.5 kb band from *FbaI* digestion and a 2.1 kb band from *PstI* digestion that hybridized with the probe. DNA fragments of 5.0–6.0 kb from the *FbaI* digestion and 2.0–2.2 kb from the *PstI* digestion were recovered from 0.7% (w/v) agarose gel by use of a QIAquickTM gel extraction kit from QIAGEN (Tokyo, Japan) and ligated into *Bam*HI or *Pst*I-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.* [26], and screened for the existence of the *ramA* gene by colony hybridization with the probe. Positive *E. coli* transformants carried an 8.5 kb plasmid designated pRTB1-Fba, or 5.1 kb plasmid designated pRTB1-Pst. Southern hybridization toward the two plasmids digested with various restriction endonucleases and preliminary nucleotide sequencing suggested that a 1.3 kb *NaeI-FbaI* fragment in pRTB1-Fba contained the entire *ramA* gene.

DNA sequence analysis

Nested unidirectional deletions were generated from a plasmid containing the 1.3 kb *NaeI-FbaI* fragment with the Kilo-Sequence deletion kit (Takara Shuzo). An automatic plasmid isolation system (Kurabo, Osaka, Japan) was used to prepare the double-stranded DNAs for sequencing. Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method [27] with M13 forward and reverse oligonucleotides as primers. Sequencing reactions were carried out with a Thermo SequenaseTM

cycle sequencing kit and dNTP mixture with 7-deaza-dGTP from Amersham Biosciences, 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 [28].

Expression of the *ramA* gene in *E. coli*

A modified DNA fragment coding for the R-amidase was obtained by PCR. The reaction mixture for the PCR contained in 50 μ L of 10 mM Tris/HCl, pH 8.85, 25 mM KCl, 2 mM MgSO₄, 5 mM (NH₄)₂SO₄, each dNTP at a concentration of 0.2 mM, a sense and an antisense primer each at 1 μ M, 2.5 U of *Pwo* DNA polymerase (Roche Diagnostics) and 0.1 μ g of plasmid pRTB1-Fba as a template DNA. The PCR cycle was the same as that described above. The sense primer contained an *Hind*III-recognition site (underlined sequence), a ribosome-binding site (double underlined sequence), and a TAG stop codon (lowercase letters) in-frame with the *lacZ* gene in pUC19, and spanned positions 244–271 in the sequence from GenBank with accession number AB154368. The antisense primer contained an *Xba*I site (underlined sequence) and corresponded to the sequence from 1060 to 1088. The two primers were as follows: sense primer, 5'-GGCTCAAA GCTTTAAGGAGGAAtagGAGATGAAAATTGAATT GGTGCAACTGG-3'; antisense primer, 5'-CATAGTG TTTCTAGACTTCATTGGCTGGC-3'. The amplified PCR product was digested with *Hind*III and *Xba*I, separated by agarose gel electrophoresis and purified from the gel. The amplified DNA was inserted downstream of the *lac* promoter in pUC19, yielding pRTB1EX, and which was then used to transform *E. coli* JM109 cells.

Purification of R-amidase, RamA from *E. coli* transformant

E. coli JM109 harboring pRTB1EX was subcultured at 37 °C for 12 h in a test tube containing 5 mL Luria-Bertani medium supplemented with 80 μ g·mL⁻¹ ampicillin. The subculture (5 mL) was then inoculated into a 2 L Erlenmeyer flask containing 500 mL Luria-Bertani medium supplemented with 80 μ g·mL⁻¹ ampicillin and 0.5 mM isopropyl thio- β -D-galactoside. After a 16 h incubation at 37 °C with rotary shaking, the cells were harvested by centrifugation at 8000 *g* for 10 min at 4 °C and washed with 0.9% (w/v) NaCl. All the purification procedures were performed at a temperature lower than 5 °C. The buffer used throughout this purification was Tris/HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol, 5 mM 2-mercaptoethanol and 0.1 mM ethylenediaminetetraacetic acid. Washed cells from a 2.5 L culture were suspended in 100 mM buffer and disrupted by sonication for 10 min. For the removal of intact cells and cell debris, the sonicate was centrifuged at 15 000 *g* for 20 min at 4 °C. After centrifugation, the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 0–40% saturation was collected by centrifugation and dissolved in 20 mM buffer. The resulting enzyme solution was dialyzed against 10 L of the same buffer for 24 h. The dialyzed solution was applied to a column (2.5 \times 7 cm) of DEAE-Toyopearl 650M previously equilibrated with 20 mM buffer.

After the column had been washed thoroughly with 20 mM buffer, followed by the same buffer containing 50 mM NaCl, the enzyme was eluted with 100 mL of 20 mM buffer containing 100 mM NaCl. The active fractions were combined and dialyzed against 10 L of 20 mM buffer for 12 h. The enzyme solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences KK) previously equilibrated with 20 mM buffer. After the column had been washed with 30 mL of 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in 20 mM buffer using an ÄKTA-FPLC system (Amersham Biosciences KK). The active fractions were combined and dialyzed against 10 L of 20 mM buffer for 12 h and used for characterization.

Enzyme assay

During the purification of the amidase from *Pseudomonas* sp. MCI3434, the enzyme assay was carried out with (*R,S*)-piperazine-2-*tert*-butylcarboxamide as a substrate. The reaction mixture (0.1 mL) contained 10 μmol potassium phosphate buffer (pH 7.0), 5.4 μmol (*R,S*)-piperazine-2-*tert*-butylcarboxamide and an appropriate amount of the enzyme. After the reaction was performed at 30 °C for 5–10 h, the piperazine-2-carboxylic acid formed was derivatized with 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) by the addition of 100 μL of 0.1% NBD-Cl in methanol, 100 μL of 0.1 M NaHCO₃, and 500 μL of H₂O to the reaction mixture. After incubation at 55 °C for 1 h, the amount of derivatized piperazine-2-carboxylic acid was determined with an HPLC apparatus equipped with an ODS-80Ts column (0.46 × 150 cm; Tosoh Corp., Tokyo, Japan) at a flow rate of 0.6 mL·min⁻¹, using as a solvent system methanol/5 mM H₃PO₄ (2 : 3, v/v). The eluate was detected spectrofluorometrically with an excitation wavelength of 503 nm and an emission wavelength of 541 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol piperazine-2-carboxylic acid·min⁻¹ from (*R,S*)-piperazine-2-*tert*-butylcarboxamide under the above conditions. Protein was determined by the method of Bradford [29] 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 100 μmol potassium phosphate buffer (pH 7.0), 10 μmol (*R,S*)-piperazine-2-*tert*-butylcarboxamide and an appropriate amount of the cell or enzyme. The reaction was performed at 30 °C and stopped by the addition of 1 mL of ethanol. The amount of each enantiomer of the piperazine-2-carboxylic acid formed in the reaction mixture was determined with an HPLC apparatus equipped with a Sumichiral OA-5000 column (0.46 × 15 cm; Sumika Chemical Analysis Service, Osaka, Japan) at a flow rate of 1.0 mL·min⁻¹, using as a solvent system 2 mM CuSO₄. The absorbance of the eluate was monitored at 254 nm.

(*R,S*)-Piperazine-2-carboxamide was used as a substrate during the purification and characterization of recombinant RamA from *E. coli* transformant. The reaction mixture (1 mL) contained 100 μmol Tris/HCl buffer (pH 8.0), 20 μmol (*R,S*)-piperazine-2-carboxamide and an

appropriate amount of the enzyme. The reaction was performed at 30 °C for 5–15 min and stopped by the addition of 1 mL ethanol. The amount of piperazine-2-carboxylic acid formed in the reaction mixture was determined with the HPLC apparatus equipped with a Sumichiral OA-5000 column as described above. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol piperazine-2-carboxylic acid·min⁻¹ from (*R,S*)-piperazine-2-carboxamide under the above conditions.

Enzyme activity toward other amide and nitrile compounds was determined by measuring the formation of ammonia. The amount of ammonia produced was colorimetrically determined by the phenol/hypochlorite method [30] using Conway microdiffusion apparatus [31]. Enzyme activity toward dipeptides was determined by measuring the production of amino acids by thin-layer chromatography with a solvent system (1-butanol/acetic acid/water; 4 : 1 : 1). The amounts of β-alanine, D-glutamic acid amide and D-glutamine were quantitatively assayed by HPLC as described for the (*R,S*)-piperazine-2-carboxylic acid. The amounts of (*R,S*)-piperidine-3-carboxylic acid and piperidine-4-carboxylic acid were assayed after derivatization with NBD-Cl by the same method described for (*R,S*)-piperazine-2-carboxylic acid.

Analytical measurements

To estimate the molecular mass of the enzyme, the sample (3 μg) was subjected to HPLC on a TSK G-3000 SW column (0.75 × 60 cm; Tosoh Corp.) at a flow rate of 0.6 mL·min⁻¹ with 0.1 M sodium phosphate (pH 7.0) containing 0.1 M Na₂SO₄ at room temperature. The absorbance of the eluate was monitored at 280 nm. The molecular mass of the enzyme was then calculated based on relative mobility (retention time) using the standard proteins glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa) (Oriental Yeast Co., Tokyo, Japan). SDS/PAGE analysis was performed by the method of Laemmli [32]. Proteins were stained with Brilliant blue G and destained in ethanol/acetic acid/water (3 : 1 : 6, v/v/v).

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 AB154368.

Results

Purification and characterization of the *R*-stereoselective amidase from *Pseudomonas* sp. MCI3434

An amidase, acting on piperazine-2-*tert*-butylcarboxamide was detected in *Pseudomonas* sp. MCI3434. Various nitrogen and carbon sources were tested, and the highest level of activity was obtained after culture in an optimized medium, Medium I, containing Bonito extract and DL-malate. No amide compounds enhanced the amidase activity in the cells, suggesting a constitutive expression of the amidase. HPLC analysis with a Sumichiral OA-5000 column showed that the *Pseudomonas* sp. MCI3434 cells acted on racemic

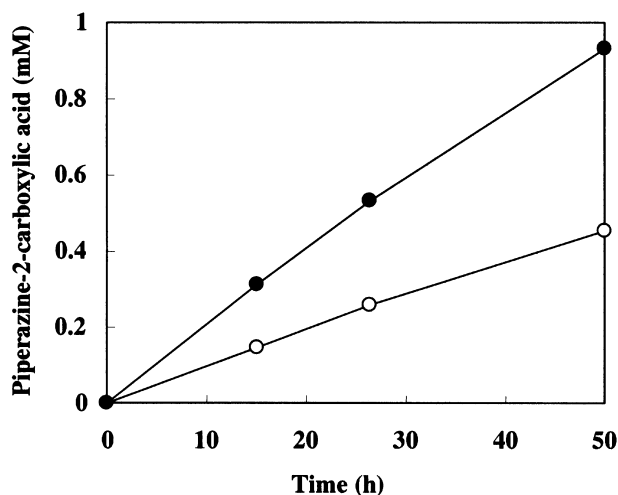


Fig. 2. Hydrolysis of racemic piperazine-2-*tert*-butylcarboxamide by cells of *Pseudomonas* sp. MCI3434. *Pseudomonas* sp. MCI3434 was cultured in 200 mL of medium I for 12 h at 30 °C. The cells were then harvested, washed with 0.9% NaCl, and suspended in 3 mL of 0.1 M potassium phosphate (pH 7.0). The reaction mixture contained 10 mM of piperazine-2-*tert*-butylcarboxamide, 150 μ L of the cell suspension and 0.1 M of potassium phosphate (pH 7.0) in a total volume of 200 μ L, and was incubated at 30 °C. The reaction was stopped at a specific time and the concentration of each enantiomer of piperazine-2-carboxylic acid formed was determined using HPLC with a Sumichiral OA-5000 column as described in Materials and methods. ●, (*R*)-piperazine-2-carboxylic acid; ○, (*S*)-piperazine-2-carboxylic acid.

piperazine-2-*tert*-butylcarboxamide to produce (*R*)- and (*S*)-piperazine-2-carboxylic acid, with a preference for the *R*-form (Fig. 2).

To investigate the stereoselectivity of the hydrolytic activity toward the substrate, the amidase was purified from a cell-free extract of *Pseudomonas* sp. MCI3434 as described in Materials and methods with a recovery of 0.07% (Table 1). The final preparation gave a single band on SDS/PAGE with a molecular mass of \approx 29.5 kDa. The molecular mass of the native enzyme was about 36 kDa according to gel-filtration chromatography, indicating that the native enzyme was a monomer. The purified enzyme catalyzed the hydrolysis of piperazine-2-*tert*-butylcarboxamide with strict *R*-stereoselectivity (Fig. 3). This result suggests that the strain can express another amidase acting

Table 1. Purification of R-amidase from *Pseudomonas* sp. MCI3434. *R,S*-Piperazine-2-*tert*-butylcarboxamide was used as a substrate for total and specific activity.

Step	Total (mg)	Total protein (mU)	Specific activity ($\text{mU}\cdot\text{mg}^{-1}$)	Yield activity (%)
Cell-free extract	6700	400	0.06	100
DEAE-Toyopearl (first)	1520	230	0.151	57.5
Butyl-Toyopearl	107	32	0.299	8.0
DEAE-Toyopearl (second)	25	31	1.24	7.8
Gigapite	2.5	6.4	2.56	1.6
Superdex 200 HR10/30	0.012	0.29	24.2	0.07

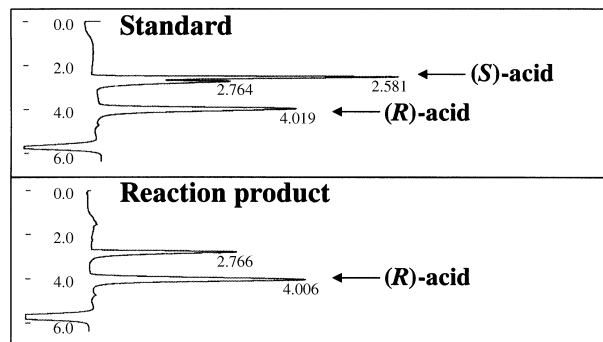


Fig. 3. Stereochemical analysis of piperazine-2-carboxylic acid produced by the purified R-amidase. The reaction mixture contained 10 mM of piperazine-2-*tert*-butylcarboxamide, 2 μ g of the purified R-amidase, and 0.1 M of potassium phosphate (pH 7.0) in a total volume of 200 μ L, and was incubated at 30 °C for 10 h. The stereochemistry of the piperazine-2-carboxylic acid formed was determined using HPLC with Sumichiral OA-5000 column as described in Materials and methods. The substrate amides were not detected in these HPLC conditions.

on the racemic substrate with *S*-stereoselectivity or without stereoselectivity. The *R*-stereoselective enzyme was tentatively named R-amidase.

Cloning and characterization of the R-amidase gene, *ramA*

To obtain information about the primary structure of the R-amidase, its N-terminal amino acid sequence was analyzed by Edman degradation and found to be Met-Ala-Ile-Glu-Leu-Val-Gln-Leu-Ala-Gly-Arg-Asp-Gly-Asp. A BLAST search of a protein database indicated that the N-terminal amino acid sequence of R-amidase showed high sequence identity (11 of 14 amino acid residues identical) with that of a hypothetical hydrolase encoded by the PA3598 gene found in the complete genome sequence of *Pseudomonas aeruginosa* strain PAO1 [33]. The deduced amino acid sequence of PA3598 has similarity with the putative hydrolase Q9L104 from *Streptomyces coelicolor* A3(2) [34] and a hypothetical 31.2 kDa protein, YPQQ in the *pqqf* 5' region of *Pseudomonas fluorescens* CHA0 [35]. Two highly conserved regions among the PA3598, Q9L104, and YPQQ sequences, Tyr106-Arg-Lys-Thr-His-Leu and Asp142-(Ile or Val)-Glu-Phe-Pro-Glu (numbering of the first residues are based on the PA3598 sequence), were considered to be also present in the R-amidase sequence. The primers used for cloning part of the R-amidase gene, named *ramA*, by PCR were designed based on the conserved regions. A 125-bp DNA was PCR-amplified with the primers and the chromosomal DNA prepared from *Pseudomonas* sp. MCI3434 and used as a probe for Southern and colony hybridizations to obtain the recombinant plasmids pRTB1-Fba and pRTB1-Pst which contain inserts of 5.3 and 2.1 kb, respectively. Southern hybridization of the two plasmids digested with various restriction endonucleases and preliminary nucleotide sequencing showed that a 1.3-kb *NaeI-FbaI* fragment in pRTB1-Fba contained the entire *ramA* gene. Two inserts from pRTB1-

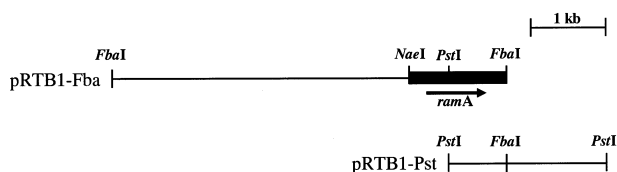


Fig. 4. Schematic view of the inserted fragments of pRTB1-Fba and pRTB1-Pst. For clarity, only restriction sites discussed in the text are shown. The location of *ramA* and its direction of transcription are indicated by an arrow. The *NaeI-FbaI* fragment sequenced in this study is indicated by a black box.

FbaI and pRTB1-Pst were also found to share a common *PstI-FbaI* region (Fig. 4).

The nucleotide sequence of the *NaeI-FbaI* fragment was determined to be 1332-bp long, and an open reading frame (ORF) was present in this region. The N-terminal sequence deduced from the ORF is consistent with the sequence determined by peptide sequencing of the purified R-amidase, except for the second lysine residue. The structural *ramA* gene consists of 822 bp and codes for a protein of 274 amino acids with a predicted molecular mass of 30 128 Da, which is consistent with the value estimated from the relative

mobility of the purified R-amidase on SDS/PAGE. A potential ribosome-binding site (AGGA) was located just seven nucleotides upstream from the start codon ATG. In the upstream region of the *ramA* translational start codon, sequences related to the -35 (TTTATT) and -10 (CATACT) consensus promoter regions were identified.

An alignment with the SwissProt and NBRF-PIR databases using the BLAST program showed that in primary structure, R-amidase is similar to the putative carbon-nitrogen hydrolase family proteins PP3846 from *Pseudomonas putida* strain KT2440 [72.6% identical over 270 amino acids [36]; TrEMBL accession number Q88G79], PA3598 from *P. aeruginosa* strain PAO1 [65.6% identical over 270 amino acids [33]; PIR accession number H83195], PP0382 from *P. putida* strain KT2440 [40.8% identical over 260 amino acids [36]; TrEMBL accession number Q88QV2], R02496 from *Sinorhizobium meliloti* strain 1021 [36.9% identical over 255 amino acids [37]; TrEMBL accession number Q92MW3], SAV6892 from *Streptomyces avermitilis* strain MA-4680 [36.2% identical over 260 amino acids [38]; TrEMBL accession number Q827N2], and a 31.2 kDa protein in the *pqqF* 5' region of *Pseudomonas fluorescens* strain CHA0 [36.8% identical over 266 amino acids [35]; SwissProt accession number YPQQ_PSEFL]. Figure 5

Fig. 5. Comparison of the amino acid sequences of the R-amidase (RamA) and homologous proteins. Identical and conserved amino acids among the sequences are marked in black and in gray, respectively. Dashed lines indicate the gaps introduced for better alignment. Invariant catalytic triad residues, glutamic acid, lysine and cysteine in the carbon-nitrogen hydrolase family are marked by asterisks. RamA, R-amidase from *Pseudomonas* sp. MCI3434; PP3846, carbon-nitrogen hydrolase PP3846 from *Pseudomonas putida* strain KT2440; PA3598, conserved hypothetical protein PA3598 from *Pseudomonas aeruginosa* strain PAO1; PP0382, carbon-nitrogen hydrolase PP0382 from *Pseudomonas putida* strain KT2440; YPQQ, carbon-nitrogen hydrolase in the *pqqF* 5' region of *Pseudomonas fluorescens* strain CHA0; SAV6892, putative hydrolase SAV6892 from *Streptomyces avermitilis* strain MA-4680; R02496, hypothetical protein R02496 from *Sinorhizobium meliloti* strain 1021.

RamA	-----MKIELVQLAGRDGDTAYNLSRTLNAIATCAAGDIDLLVF	38
PP3846	-----MKIELVQLSGRDGDTAYNLERLQAIATCAVDIDLLIF	38
PA3598	-----MKVELVQIAGRDGDTAHLERLALAAIADCAADIDELVVF	38
PP0382	-----MRIALYQGAPKPLDVPGNLQRLRHQAQLADRGQQLLVC	39
YPQQ	MSYEPATAATVAGLSVSGVKTRRVALYQCPRPLDVAGNLQRLHQVAMEATDIDLLVL	58
SAV6892	-----MRTALLQSSGRPGSIAENLKVLDAAAGRAAAGAGLVVA	39
R02496	-----MKFAALQMKSIGGDVAANLARIERAAIGASGEGASLVA	40
RamA	PETYSISCFVGG-AQLAQVAEPLHGTTLQTLQAVRQRDVAVVLQFAEVHQ-GRFYMSVVL	96
PP3846	PETQLMGFASA-QQAEIAEVPNGPSVQAQRAVHERNVSVAIGLAENDS-GTYMISLL	96
PA3598	PETHITCFPSE-DNIAALAEPLDQPTVSAQRVARERNVSAIGIAEADA-GRYMITLL	96
PP0382	PEMFLSGYNI GLAQVERLAEAADGPAAMTYVEIAQAHRIIVYGYPERGDDGAIYNSVQL	99
YPQQ	PEMFLSGYNI GLEAVGALAEAQDQPSAQRIAAIAQAAGTAI LYGYPERSDVGGIYNAVQL	118
SAV6892	PEMFLTGVAIG-DDVPRLAEPADQDSADVAETASRHGVAIAYGYPERAG-EQVFNQAQL	97
R02496	PELAIITGYNAG-EAIRRLAEPADGRIVRELGRISLKTGIIAIVAFEAQGA-DAVYNSAVH	98
RamA	VTPEG-IALQYRKTHLWPS-ERSDTPSG-DRFTTVLWRGVRVGLLICYDIELPETSRALA	153
PP3846	VTPQG-IALSRYRKTHLWPS-ERGLFQQG-DRYVATLWNGIRVGLICYDIEFPESARALG	153
PA3598	IAPDG-IALKYRKTHLWAS-DRGITTPG-DRYATLWNGIRVGLLVCFDIEFPESARALG	153
PP0382	IDAHRSLSNYRKTHLFGELDRSMFSPGADHFPVVELEGWVGLLICYDIEFPENARRLA	159
YPQQ	IDAQQRLCNRYRKTHLFGDLDRSMFSGEDDFPLVELDGWVGLLICYDIEFPENARRLA	178
SAV6892	SADGTRLANRYRKTHLFGCFERDHFETPGDQPVVQTELMGLRVGLMICYDIEFPENARRA	157
R02496	VDGDAVPVV-YRKSHLVGDYERSLFTPAEPSTRLFKHRGVTGHLICYDIEFPENARRLA	157
RamA	QLGAEVIVTNGNMDPIYG--PVHRTAIMARAQENQLFAVMVNRVGGDGLVFAAGGSMAV	211
PP3846	QLGAEELIVTNGNMDPIYG--PVHRTAIMARAQENQAFVMVNRVGGDGLVFAAGSAAV	211
PA3598	QLGAEELIVTNGNMDPIYG--PTHRTAIMARAQENQAFVMVNRVGGDGLVFAAGSAAV	211
PP0382	LDGAEELIVPTANMTPID--FTCQVTVRARAQENQCLVYANYCCGAEDEIYCCQSSII	216
YPQQ	LACAEELIVPTANMIPYD--FVADYTVRARAQENQCLVYANYCCGAEDEIYCCQSSIA	235
SAV6892	LAGTDLIVVPTAQMHPEQ--FVAQSVIPVRAFENQCLVYANYVYGGQEGEIEVGLSLTIA	214
R02496	LAGDAVIVLPTALPAGISGTFITDHIQTRAFENQCLVYANYVYHCGS--DMSEAGLSLIA	216
RamA	DPFCRVLFEAGR-DEVRHVVELDLDLKAARRDYDYLKDRRLMLSGEQTEHPDGRRELLI	270
PP3846	DPFCRTLFEAGR-AEQRVVELDLDLLRTARHEYDYLKDRRLFLTGDQSEHDDGRRELLI	270
PA3598	DPYQQLLCEAGR-EECRQIVELDLGRLQDARRDYRYLEERLLVLPGERREHPDGLRELLI	270
PP0382	GPDGSLAMAGR-DEQQLLAELHEHVYVQGRRAFPYLTDLRQELHLRKG-----	264
YPQQ	APDCSRIALAGL-DEALIIGTLDRQLMGESALNRYLSDRPELYDDLSCR-----	285
SAV6892	GPDGVARARAGH-GEELVFADAPALAASREANPYLKDRIPDLYGSLV-----	262
R02496	SPDQALAKAGSSDITLIIAEIDPQAFASRAENTYLDLIDH-----	259
RamA	GASQ	274
PP3846	P---	271
PA3598	P---	271

shows the alignment of the primary structure of the R-amidase from *Pseudomonas* sp. MCI3434 and above sequences. All the sequences except for RamA in the figure were hypothetical proteins found in the genome sequence but yet to be characterized functionally. The most closely related characterized enzyme was *P. aeruginosa* aliphatic amidase [26.5% identical over 249 amino acids [1]; Genbank accession number M27612] which also belongs to the carbon–nitrogen hydrolase family. No significant homology was observed with the other amidases mentioned in the introduction section. The conserved motifs of the carbon–nitrogen hydrolase family [39] surrounding the probable catalytic triad, Glu40, Lys108, and Cys140 were highly conserved in the RamA sequence.

Production of the R-amidase in *E. coli* and optical resolution of racemic piperazine-2-*tert*-butylcarboxamide by the recombinant *E. coli* cells

The direction of *ramA* transcription was opposite to that of the *lac* promoter in pRTB1-Fba. *E. coli* JM109 transformed by the recombinant plasmid exhibited no amidase activity toward (*R,S*)-piperazine-2-*tert*-butylcarboxamide. These findings suggest that RNA polymerase in *E. coli* can not recognize the promoter for *ramA* or that there is a possible regulatory gene in the inserted fragment of pRTB1-Fba. To express the *ramA* gene in *E. coli*, we improved the sequence upstream from the ATG start codon by PCR, with the plasmid pRTB1-Fba as a template as described in Materials and methods. The resultant plasmid, pRTB1EX, in which the *ramA* gene was under the control of the *lac* promoter of the pUC19 vector, was introduced into *E. coli* JM109 cells. A protein band (29.5 kDa) corresponding to the R-amidase purified from *Pseudomonas* sp. MCI3434 was produced when the *lac* promoter was induced by isopropyl- β -D-thiogalactopyranoside (data not shown). When *E. coli* JM109 harboring pRTB1EX was cultured in Luria-Bertani medium supplemented with ampicillin and isopropyl- β -D-thiogalactopyranoside for 15 h at 37 °C, the level of RamA activity toward (*R,S*)-piperazine-2-*tert*-butylcarboxamide in the supernatant of the sonicated cell-free extract of the transformant was 1.81 units·mg⁻¹, which is about 30 000 times higher than that of *Pseudomonas* sp. MCI3434. The cell reaction with 0.2 M of racemic piperazine-2-*tert*-butylcarboxamide was carried out using two concentrations of *E. coli* cells (0.43 and 2.2%, weight of wet cells/volume) prepared from the 15 h culture (Fig. 6). The *E. coli* cells produced (*R*)-piperazine-2-carboxylic acid with high optical purity (> 99.5% ee) at all the reaction times tested.

Purification of RamA from *E. coli* transformant

Recombinant RamA was purified from the *E. coli* JM109 harboring pRTB1EX with a recovery of 17.9% by ammonium sulfate fractionation and DEAE-Toyopearl and MonoQ column chromatographies (Table 2). The final preparation gave a single band on SDS/PAGE with a molecular mass of \approx 29.5 kDa (Fig. 7). This value is the same as that for the R-amidase purified from *Pseudomonas* sp. MCI3434 and in good agreement with that estimated from the deduced amino acid sequence of the

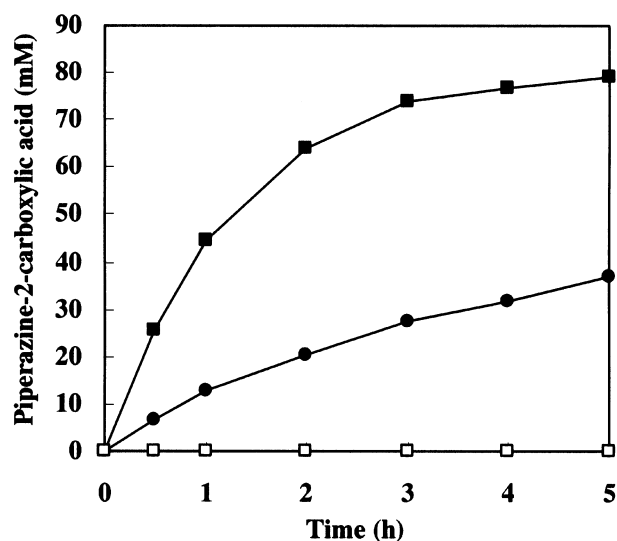


Fig. 6. Stereoselective hydrolysis of racemic piperazine-2-*tert*-butylcarboxamide by cells of *E. coli* JM109/pRTB1EX. The reaction mixture contained 0.2 M of racemic piperazine-2-*tert*-butylcarboxamide, washed *E. coli* cells prepared from the culture broth after 12 h cultivation, and 0.1 M of Tris/HCl (pH 8.0) in a total volume of 800 μ L, and was incubated at 30 °C. The reaction was stopped at a specific time and the concentration of piperazine-2-carboxylic acid formed was determined as described in Materials and methods. ●, (*R*)-acid formed with cells (0.43%, weight of wet cells/volume); ■, (*R*)-acid formed with cells (2.2%, w/v); □, (*S*)-acid formed with cells (2.2%, w/v). The cell density (0.43%, w/v) corresponds to the concentration of cells harvested from 800 μ L of culture broth used in 800 μ L of reaction mixture.

Table 2. Purification of RamA from *E. coli* JM109 harboring pRTB1EX. Piperazine-2 carboxamide was used as a substrate for total and specific activity.

	Total protein (mg)	Total activity (U)	Specific activity (U·mg ⁻¹)	Yield (%)
Cell free extract	1890	3420	1.81	100
Ammonium sulfate	1210	3190	2.64	93.3
DEAE-Toyopearl	246	853	3.47	24.9
MonoQ HR10/10	134	614	4.59	17.9

RamA. The molecular mass of the native enzyme was again about 36 kDa according to gel-filtration chromatography, indicating that the native enzyme was a monomer. The purified enzyme catalyzed the hydrolysis of (*R,S*)-piperazine-2-carboxamide to (*R*)-piperazine-2-carboxylic acid at 4.59 U·mg⁻¹ under the standard conditions.

Effects of temperature and pH on the stability and activity of RamA

The purified enzyme could be stored without loss of activity for more than 2 months at -20 °C in the buffer containing 50% glycerol. The stability of the enzyme was examined at various temperatures. After the enzyme had been

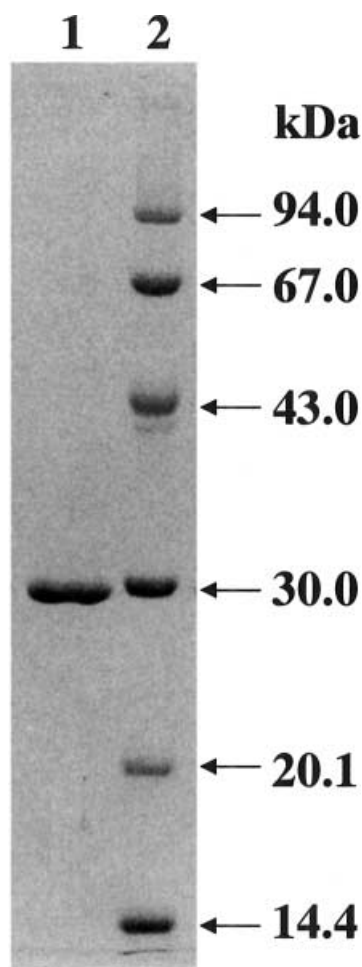


Fig. 7. SDS/PAGE of recombinant R-amidase purified from the *E. coli* transformant. Lane 1, purified enzyme (10 µg); lane 2, molecular mass standards [phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14.4 kDa)].

preincubated for 10 min, the activity was assayed with (*R,S*)-piperazine-2-carboxamide as a substrate under the standard conditions. It exhibited the following remaining activity: 55 °C, 0%; 50 °C, 2.6%; 45 °C, 87%; 40 °C, 100%; 35 °C, 100%. The stability of the enzyme was also examined at various pH values. The enzyme was incubated at 30 °C for 10 min in the following buffers (final concentration 100 mM): acetic acid/sodium acetate (pH 4.0–6.0), Mes/NaOH (pH 5.5–6.5), potassium phosphate (pH 6.5–8.5), Tris/HCl (pH 7.5–9.0), ethanolamine/HCl (pH 9.0–11.0) and glycine/NaCl/NaOH (pH 10.0–13.0). Then a sample of the enzyme solution was taken, and the remaining activity of RamA was assayed with (*R,S*)-piperazine-2-carboxamide as a substrate under the standard conditions. The enzyme was most stable in the pH range 6.0–9.0.

The enzyme reaction was carried out at various temperatures for 5 min in 0.1 M Tris/HCl (pH 8.0), and enzyme activity was found to be maximal at 45 °C. Above 45 °C, it decreased rapidly, possibly because of instability of the enzyme at the higher temperatures. The optimal pH for the activity of the enzyme was measured in the buffers

described above. The enzyme showed maximum activity at pH 9.0.

Effects of inhibitors and metal ions

The RamA solution was dialyzed against 20 mM Tris/HCl (pH 8.0). Various compounds were investigated for their effects on enzyme activity. We measured the enzyme activity under standard conditions after incubation at 30 °C for 10 min with various compounds at 1 mM. The enzyme was completely inhibited by *p*-chloromercuribenzoate, *N*-ethylmaleimide, MnSO₄, MnCl₂, CoCl₂, NiCl₂, CuSO₄, CuCl₂, ZnSO₄, ZnCl₂, AgNO₃, CdCl₂, HgCl₂ and PbCl₂ and inhibited 78% by FeCl₃ and 67% by Fe(NH₄)₂(SO₄)₂, suggesting the presence of a catalytic cysteine residue. Dithiothreitol had a little enhancing effect (138%) on the enzyme activity. Inorganic compounds such as LiBr, H₂BO₃, NaCl, MgSO₄, MgCl₂, AlCl₃, KCl, CaCl₂, CrCl₃, RbCl, Na₂MoO₄ (NH₄)₆Mo₇O₂₄, CsCl and BaCl₂ did not influence the activity. Chelating reagents, e.g. *o*-phenanthroline, 8-hydroxyquinoline, ethylenediaminetetraacetic acid and α,α' -dipyridyl had no significant effect on the enzyme. Carbonyl reagents such as hydroxylamine, phenylhydrazine, hydrazine, D,L-penicillamine and D-cycloserine were not inhibitory toward the enzyme. A serine protease inhibitor, phenylmethanesulfonyl fluoride, a serine/cysteine protease inhibitor, leupeptine and an aspartic protease inhibitor, pepstatin did not influence the activity.

Substrate specificity

To study the substrate specificity, the purified RamA was used to hydrolyze various amides, dipeptides and nitriles, and the activity was assayed (Table 3). The enzyme hydrolyzed (*R,S*)-piperazine-2-carboxamide and (*R,S*)-piperazine-2-*tert*-butylcarboxamide with strict *R*-stereoselectivity

Table 3. Substrate specificity of RamA purified from *E. coli* JM109 harboring pRTB1EX. The activity for (*R,S*)-piperazine-2-carboxamide, corresponding to 4.59 U·mg⁻¹, was taken as 100%. Amino or imino nitrogen atoms which appeared to be recognized by RamA are written in bold type.

	Relative activity (%)
(<i>R,S</i>)-Piperazine-2- <i>tert</i> -butylcarboxamide	9.0
(<i>R,S</i>)-Piperazine-2-carboxamide	100
(<i>R,S</i>)-Piperidine-3-carboxamide (Nipecotamide)	68.9
Piperidine-4-carboxamide (Isonipecotamide)	0.23
β -Alaninamide	108
D-Glutaminamide	27.0
L-Glutaminamide	0.35

to produce only (*R*)-piperazine-2-carboxylic acid. Besides the two substrates, the enzyme was also active towards β -alaninamide, (*R,S*)-piperidine-3-carboxamide and *D*-glutaminamide, and slightly active on *L*-glutaminamide and piperidine-4-carboxamide. When both of the glutaminamide enantiomers were used as substrates, the reaction products of hydrolysis were corresponding enantiomers of glutamic acid amides, not glutamines. Considering the structural formulae of the above substrates, RamA seemed to recognize the carboxamide substrates in which the amino or imino group is connected to β - or γ -carbon of the compounds. The enzyme could not hydrolyze the following *D*- and *L*-amino acid amides: *D*-alaninamide, *D*-valinamide, *D*-leucinamide, *D*-isoleucinamide, *D*-prolinamide, *D*-phenylalaninamide, *D*-tryptophanamide, *D*-methioninamide, *D*-serinamide, *D*-threoninamide, *D*-tyrosinamide, *D*-aspartic acid amide, *D*-glutamic acid amide, *D*-lysinamide, *D*-argininamide, *D*-histidinamide, *L*-alaninamide, *L*-valinamide, *L*-leucinamide, *L*-isoleucinamide, *L*-prolinamide, *L*-phenylalaninamide, *L*-tryptophanamide, *L*-methioninamide, *L*-serinamide, *L*-threoninamide, *L*-tyrosinamide, *L*-asparaginamide, *L*-aspartic acid amide, *L*-glutamic acid amide, *L*-lysinamide, *L*-argininamide, *L*-histidinamide, glycylglycylglycine, *L*-alanyl-L-alanine, β -alanyl-L-alanine, β -alanyl-L-histidine, glycylglycylglycylglycine, *L*-alanyl-L-alanine, *D*-alanyl-L-alanine, *D*-alanyl-L-alanine, *DL*-alanyl-*DL*-isoleucine, *DL*-alanyl-*DL*-leucine, *DL*-alanyl-*DL*-methionine, *DL*-alanyl-*DL*-phenylalanine, *DL*-alanyl-*DL*-serine, *DL*-alanyl-*DL*-valine and *L*-aspartyl-*D*-alanine. Although the RamA showed high sequence similarity with the hypothetical proteins in the carbon–nitrogen hydrolase family, the enzyme could not hydrolyze the following aliphatic amides, aromatic amides and nitriles: acetamide, propionamide, *n*-butyramide, isobutyramide, *n*-valeramide, *n*-capronamide, crotonamide, methacrylamide, cyclohexanecarboxamide, benzamide, *o*-aminobenzamide, *m*-aminobenzamide, *p*-aminobenzamide, *p*-toluamide, *p*-chlorobenzamide, *p*-nitrobenzamide, 2-picolinamide, nicotinamide, pyridine-4-carboxamide, pyrazinamide, 2-thiophenecarboxamide, phenylacetamide, indole-3-acetamide, acetonitrile, propionitrile, 3-hydroxypropionitrile, *n*-capronitrile, methacrylonitrile, crotononitrile, glutaronitrile, 2,4-dicyanobut-1-ene, β -phenylpropionitrile, cinnamonitrile, 2-cyanopiperidine, 2-cyanopiperazine, phenylacetoneitrile, 4-methoxyphenylacetoneitrile, α -methylbenzyl cyanide, 2-pyridineacetoneitrile, 3-pyridineacetoneitrile, thiophene-2-acetoneitrile, β -indoleacetoneitrile, diphenylacetoneitrile, 4-chlorobenzyl cyanide, benzonitrile, 4-chlorobenzonitrile, 4-nitrobenzonitrile, *p*-tolunitrile, anisonitrile, 2-cyanophenol, 2-cyanopyridine, 3-cyanopyridine, 4-cyanopyridine, pyrazinecarbonitrile, 3-cyanoindole, α -naphthonitrile, 2-thiophenecarbonitrile, terephthalonitrile and isophthalonitrile.

Discussion

In this paper, we purified an R-amidase from *Pseudomonas* sp. MCI3434 acting *R*-stereoselectively on (*R,S*)-piperazine-2-*tert*-butylcarboxamide, cloned its structural

gene, *ramA*, and investigated characteristics of the R-amidase using the recombinant enzyme purified from the *E. coli* transformant.

The amino acid sequence of RamA shared homology with sequences of hypothetical proteins belonging to the carbon–nitrogen hydrolase family from several bacteria and actinomycetes. Pace and Brenner called the carbon–nitrogen hydrolase family the ‘nitrilase superfamily’. Based on a comparison of amino acid sequences and biochemical properties, they classified it into 13 branches including nitrilase (branch 1), aliphatic amidase (branch 2), N-terminal amidase (branch 3), biotinase (branch 4), β -ureidopropionase (branch 5), carbamylase (branch 6), prokaryotic NAD^+ synthetase (branch 7), eucaryotic NAD^+ synthetase (branch 8), apolipoprotein *N*-acyltransferase (branch 9), Nit and Nitfhit (branch 10), NB11 (branch 11), NB12 (branch 12), and nonfused outliers (branch 13) [39]. Within most branches, there is sharp cut-off in *E*-values obtained with the BLAST program such that sequences with *E*-values greater than 1×10^{-25} can be identified as belonging to another branch. Although RamA is most closely related to the *P. aeruginosa* aliphatic amidase among the members of the 13 branches, the level of homology was not so high (26.5% identity) and the *E*-value was 2×10^{-11} between their sequences. This finding suggests that the carbon–nitrogen hydrolase family must contain a new branch, into which RamA as well as its homologous sequences, PP3846 from *P. putida* and PA3598 from *P. aeruginosa*, should be classified.

The R-amidase acted on (*R,S*)-piperazine-2-*tert*-butylcarboxamide with strict *R*-stereoselectivity to form (*R*)-piperazine-2-carboxylic acid. Moreover, R-amidase exhibited significantly unique substrate specificity with hydrolyzing activity only for seven amide compounds listed in Table 3, indicating that the enzyme prefers carboxamide compounds as substrates in which the amino or imino group is connected to β - or γ -carbon of the compounds. The enzyme did not hydrolyze the other amides, peptides, and nitriles. Although RamA shares sequence similarity with *P. aeruginosa* aliphatic amidase as mentioned above, RamA could not hydrolyze aliphatic amides with short acyl chains such as acetamide, propionamide, *n*-butyramide, isobutyramide, and *n*-valeramide which were good substrates for the aliphatic amidase.

As the R-amidase (RamA) has become abundantly available using the DNA technique, *E. coli* cells producing RamA or a purified recombinant RamA may be of use in the optical resolution of piperazine-2-*tert*-butylcarboxamide to yield (*R*)-piperazine-2-carboxylic acid and (*S*)-piperazine-2-*tert*-butylcarboxamide. Although (*S*)-piperazine-2-*tert*-butylcarboxamide, which is an important chiral building block for an HIV protease inhibitor [22], can be prepared by either diastereomeric crystallization using (*S*)-camphorsulfonic acid from its racemic form [40] or asymmetric hydrogenation of tetrahydropyrazine-*tert*-butylcarboxamide using [(*R*)-BINAP(COD)Rh]TfO catalyst [41], both chemical processes require a harsh reaction condition under high pressure using a much amount of solvents such as acetonitrile, propan-1-ol or methanol. R-Amidase is the first enzyme useful for the enzymatic optical resolution of racemic piperazine-2-*tert*-butylcarboxamide carried out under mild conditions.

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