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The Screening, Characterization, and Use of ω-Laurolactam Hydrolase: A New Enzymatic Synthesis of 12-Aminolauric Acid

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Several ω-laurolactam degrading microorganisms were isolated from soil samples. These strains were capable of growing in a medium containing ω-laurolactam as sole source of carbon and nitrogen. Among them, five strains (T7, T31, U124, U224, and U238) were identified as *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224, and *Sphingomonas* sp. U238, respectively. The ω-laurolactam hydrolyzing enzyme from *Rhodococcus* sp. U224 was purified to homogeneity, and its enzymatic properties were characterized. The enzyme acts on ω-octalactam and ω-laurolactam, but other lactam compounds, amides and amino acid amides, cannot be substrates. The enzyme gene was cloned, and the deduced amino acid sequence showed high homology with 6-aminohexanoate-cyclic-dimer hydrolase (EC 3.5.2.12) from *Arthrobacter* sp. KI72 and *Pseudomonas* sp. NK87. Enzymatic synthesis of 12-aminolauric acid was performed using partially purified ω-laurolactam hydrolase from *Rhodococcus* sp. U224.

Key words: hydrolysis; ω-laurolactam; 12-aminolauric acid; 6-aminohexanoate-cyclic-dimer hydrolase; *Rhodococcus* sp.

12-Aminolauric acid is an important chemical used in the production of nylon,[1)] adhesives, and hardening agents. It can be produced by reduction of 11-cyanoundecanoic acid in the presence of a noble metal catalyst or by hydrolysis of ω-laurolactam with HCl at high temperature and pressure.[2)] Both of these chemical processes have drawbacks, in that the preparation of 11-cyanoundecanoic acid and purification after hydrolysis are tedious.[3–6)] In this study, we focused on a novel ω-laurolactam hydrolyzing enzyme from microbial sources to develop a novel enzymatic method of 12-aminolauric acid preparation from ω-laurolactam under mild conditions (Fig. 1).

Amidases (acylamide amidohydrolase, EC 3.5.1.4) are hydrolases acting on carboxyl amide bonds, liberating carboxylic acids and ammonia. Many microbial amidases have been characterized and applied in the synthesis of useful compounds. Aliphatic amidases acting on aliphatic amides with short acyl chains have been found in *Arthrobacter* sp. J-1,[7)] *Pseudomonas aeruginosa*,[8)] *Brevibacterium* sp. R312,[9)] *Helicobacter pylori*,[10)] and *Bacillus stearothermophilus* BR388.[11)] Penicillin amidases (penicillin amidehydrolase, EC 3.5.1.11) hydrolyze penicillins to give carboxylic acid and 6-aminopenicillanic acid, and the reverse reaction has been applied in the semi-synthesis of penicillins.[12)] Acyl derivatives of *N*-acyl amino acids can be hydrolyzed by aminoacylase (*N*-acylamino-acid amidohydrolase EC 3.5.1.14), which specifically cleaves the acyl residues of *N*-acylated amino acids. The chiral specificity of aminoacylases allows their utilization in the industrial production of stereoisomers from racemates.[13)] We have discovered D-aminopeptidase (EC 3.4.11.19) from *Ochrobacterium anthropi* SCRC C1-38,[14)] Alkaline D-peptidase (EC 3.4.11.-) from *Bacillus cereus* DF4-B,[15)] and D-amino acid amidese from *O. anthropi* SV3,[16)] which act on D-amino acid amides or D-amino acid containing peptides, and have applied them in the synthesis of D-amino acids from racemic amino acid amides. Several microbial enzymes catalyzing the hydrolysis of cyclic amides, lactam compounds, have also been studied, including β-lactamase,[17)] γ-lactamase,[18–21)] amino-ε-caprolactam hydrolase,[22,23)] and 6-aminohexanoate-cyclic-dimer hydrolase.[24,25)] But there has been no report on an enzyme that hydrolyzes ω-laurolactam, a large cyclic amide with 12 carbon atoms.

In this report, we describe the isolation of several ω-laurolactam degrading microorganisms from soil and the identification of five strains among them. We also report the purification, characterization, and gene cloning of ω-laurolactam hydrolase from *Rhodococcus* sp. U224. Enzymatic synthesis of 12-aminolauric acid was

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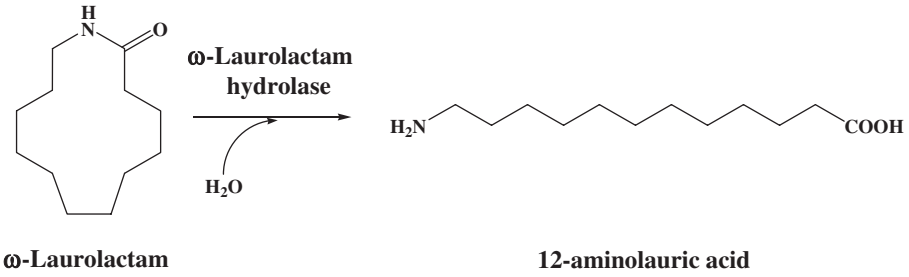

Fig. 1. Hydrolysis Reaction of ω-Laurolactam to Form 12-Aminolauric Acid.

carried out using partially purified enzyme from *Rhodococcus* sp. U224. This is the first report on the synthesis of 12-aminolauric acid from ω-laurolactam by a bacterial enzyme.

Materials and Methods

Screening for ω-laurolactam degrading microorganisms from soil. Basal agar medium contained 1% 12-aminolauric acid, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $MnCl_2 \cdot 4H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract, and 1.5% agar, and was adjusted to pH 6.4. Each of the soil samples was suspended in distilled water, streaked onto the basal agar medium, and incubated at 30 °C for 1 week. Strains forming clear zones around the colonies due to the consumption of 12-aminolauric acid were isolated and transferred to the same agar medium. The isolated colonies were then transferred to agar medium containing 1% ω-laurolactam dissolved in DMSO, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $MnCl_2 \cdot 4H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract and 1.5% agar (pH 6.4). Strains forming clear zones exhibiting ω-laurolactam degradation were isolated and transferred to the liquid medium to cultivate them at 30 °C for 12 h with shaking at 200 stroke/min. Hydrolysis of ω-laurolactam in the liquid culture was monitored by thin layer chromatography (TLC) using the developing solvent *n*-buthanol/acetic acid/water = 4/1/1 (v/v), and was visualized with ninhydrin.

Enzyme assay. Each colony of microorganisms having ω-laurolactam hydrolyzing activity was cultivated in 3 ml of TGY medium (0.5% polypepton, 0.5% yeast extract, 0.1% KH_2PO_4, and 0.1% glucose, pH 7.0) at 30 °C for 12 h (200 stroke/min). Cells were then harvested by centrifugation (15,000 × *g*, 10 min, 4 °C) and suspended in 50 µl of 0.1 M potassium phosphate buffer (pH 7.0). The reaction mixture (1 ml) contained 50 µl of the cell suspension, 850 µl of 0.1 M potassium phosphate buffer (pH 7.0), and 100 µl of 100 mM ω-laurolactam dissolved in DMSO. The reaction was carried out with shaking (200 rpm) at 30 °C for 48 h.

During the purification of ω-laurolactam hydrolase from *Rhodococcus* sp. U224, the reaction mixture (1 ml) contained 700 µl of 0.1 M potassium phosphate buffer (pH 7.0) and 200 µl of 50 mM ω-laurolactam dissolved in toluene, and 100 µl, an appropriate amount, of the enzyme. The reaction mixture was shaken (200 rpm) at 30 °C for 30 min, the reaction was stopped by adding 100 µl of 2 N $HClO_4$ and then an aliquot of the mixture (100 µl) was taken and diluted by mixing it with 800 µl H_2O. The amount of 12-aminolauric acid formed in a sample (10 µl) was measured with an HPLC apparatus equipped with a Cosmosil $5C_{18}$MS-II column (φ 0.46 × 15 cm) (Nacalai Tesque, Kyoto, Japan) with an isocratic elution of 20 mM H_3PO_4/40% MeOH at a flow rate of 1.0 ml/min. UV detection was done at 210 nm. The column temperature was 40 °C. The retention time of 12-aminolauric acid was about 9.5 min. One unit of enzyme activity was defined as the amount of the enzyme that produced 1 µmol of 12-aminolauric acid from ω-laurolactam per min under the above conditions. Specific activity was expressed as unit/mg of protein. To determine the hydrolysis activity of the enzyme toward ω-octalactam, the amount of 8-aminooctanoic acid formed was measured with an HPLC apparatus equipped with a Mightysil RP-18 GP 150-4.6 column (Kanto Chemical, Tokyo) with an isocratic elution of 10 mM H_3PO_4/10% MeOH at a flow rate of 1.0 ml/min. UV detection was done at 210 nm. The column temperature was 30 °C. Hydrolysis of the other lactams was monitored by TLC. Hydrolysis of amide compounds and amino acid amides was evaluated by determination of ammonia.[26)]

Time course of ω-laurolactam hydrolysis of isolated microorganisms. Hydrolysis of ω-laurolactam by each isolate was measured every 2 h up to 12 h, 24 h, and 48 h from the beginning of the reaction. The 12-aminolauric acid formed was analyzed by the method described above.

Purification of ω-laurolactam hydrolase from Rhodococcus *sp. U224. Rhodococcus* sp. U224 was used as the source of the enzyme. The strain was grown on a TGY agar plate at 30 °C for 2 d. A loopful of the strain was inoculated into 5 ml of TGY medium and incubated at 30 °C for 24 h with reciprocal shaking (200 stroke/min). Preculture (5 ml) was added to 500 ml of the same medium in a 2 liters Sakaguchi flask. Cultivation was carried out at 30 °C for 72 h with shaking (96 stroke/

min), and cells were harvested by centrifugation (15,000 × *g*, 10 min, 4 °C). Unless otherwise stated, all purification procedures were performed at below 4 °C. Potassium phosphate buffer (20 mM, pH 7.0) was used throughout the purification. The washed cells (139.9 g wet weight obtained from 30 liters of culture) were suspended in 600 ml of 20 mM buffer. The cells were disrupted by sonication for 30 min (19 kHz, Insonator model 201M, Kubota, Tokyo). For removal of intact cells and cell debris, the lysate was centrifuged at 15,000 × *g* for 15 min at 4 °C to yield cell-free extract. To remove nucleic acid, protamine sulfate (final concentration, 0.05%) was added to the cell-free extract. After stirring for 30 min on ice, the mixture was centrifuged at 15,000 × *g* for 10 min at 4 °C. Solid ammonium sulfate was added carefully to the supernatant with gentle stirring until 30% saturation was reached. After equilibration for 30 min, the supernatant after centrifugation was removed, and ammonium sulfate was added again to 60% saturation. The solution was allowed to equilibrate for 30 min, and the resulting precipitate was collected after centrifugation (15,000 × *g* for 15 min at 4 °C). The precipitate was dissolved in 20 mM buffer and dialyzed overnight against the same buffer. The dialyzed solution was applied to a DEAE-Toyopeal 650 M (Tosoh, Tokyo) column (2.5 × 15 cm), which had been equilibrated with 20 mM buffer. After the column was washed thoroughly with 20 mM buffer, the enzyme was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM buffer. The dialyzed active fractions were then brought to 30% ammonium sulfate saturation and were added to a Butyl-Toyopearl 650 M (Tosoh) column (1.5 × 10 cm) equilibrated with 20 mM buffer saturated with 30% ammonium sulfate. After the column was washed with the same buffer, the active fractions were eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 20 mM buffer. The dialyzed active fractions were combined, concentrated with a Centricon (Amicon, Beverly, MA) and applied to a column of Mono Q HR 5/5 (GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM buffer. The enzyme was eluted with a gradient of 0–0.5 M NaCl in 20 mM buffer with Äkta FPLC™ (GE Healthcare) at 0.8 ml/min. The dialyzed active fractions were combined, concentrated with the Centricon, and applied to a column of Superdex 200 HR 10/30 (GE Healthcare) equilibrated with 20 mM buffer containing 150 mM NaCl. The column was eluted by FPLC at 0.5 ml/min, and the active fractions were collected.

Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. For column chromatography, the protein concentration was determined by measuring the absorbance at 280 nm.

SDS-polyacrylamide gel electrophoresis (SDS–PAGE), molecular weight and N-terminal amino acid sequence analysis. SDS–PAGE was carried out by the method of Laemmli[27] using an electrophoresis unit supplied by Atto (Tokyo). The protein solution was mixed with an equal volume of sample buffer containing 10% 2-mercaptoethanol and 4% SDS, and heated at 100 °C for 10 min to denature the proteins. The treated protein solution was loaded on a gel consisting of 11.9% of acrylamide, 0.35 M of Tris–HCl (pH 8.8), 0.01% SDS, 0.12% *N*,*N*,*N*,*N*-tetramethylethylenediamine, and 0.07% ammonium peroxodisulfate, and electrophoresed. The protein bands were stained with Coomassie Brilliant Blue R-250.

The molecular weight of the native enzyme was measured by gel-permeation chromatography. The enzyme was subjected to HPLC on a Superdex 200 HR 10/30 at a flow rate of 0.5 ml/min, using 20 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl at room temperature. The absorbance at 280 nm of the effluent was recorded. The relative mobility of the enzyme was compared with those of standard proteins: glutamate dehydrogenase (290,000), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000) and cytochrome *c* (12,400). The molecular weight of the subunit of the enzyme was estimated by SDS–PAGE, calculating on the basis of relative mobility as compared with those of the standard proteins: phosphorylase (97,400), bovine serum albumin (66,267), aldolase (42,200), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000).

Amino acid sequence analysis of the amino terminus of the purified enzyme was performed at Apro Life Science Institute (Tokushima, Japan) with a Procise 494 HT protein sequencing system.

Cloning of the gene encoding ω-laurolactam hydrolase from Rhodococcus *sp. U224.* N-terminal amino acid sequence (30 residues) analysis of the ω-laurolactam hydrolase purified from *Rhodococcus* sp. U224 suggested the possible identity of the enzyme with 6-aminohexanoate-cyclic-dimer hydrolase from *Arthrobacter* sp. (formerly *Flavobacterium* sp.) KI72 and *Pseudomonas* sp. NK87.[28] *Rhodococcus* sp. U224 was cultivated in 3 ml TGY medium at 30 °C for 12 h. Genomic DNA was prepared from cells harvested from the culture by the method of Saito and Miura.[29] The coding region of ω-laurolactam hydrolase was amplified by PCR using forward (5′-^{139}AGAAGGAGCGCGACA<u>GTG</u>AGCAAGGTGGAC^{168}-3′) and reverse (5′-^{1650}CATCTCCCGCAAGCA<u>TCA</u>GGCCGCTGGGATC^{1620}-3′) primers. The two primers were designed on the basis of the flanking region of *nylA* from *Arthrobacter* sp. KI72.[28] The superscript numbers were based on the sequence in accession no. M26953 in the DDBJ/EMBL/GenBank nucleotide sequence databases. The forward primer contained a GTG start codon (underlined sequence), and the reverse primer contained a TGA stop codon (underlined sequence). The reaction mixture of PCR contained 0.25 µl of TaKaRa Ex *Taq* (5 units/µl), 5 µl of 10 × Ex *Taq* buffer, 4 µl of dNTP mixture (2.5 mM

each), 2 µl of forward primer (100 pmol/µl), 2 µl of reverse primer (100 pmol/µl), 1 µl of the extracted genomic DNA (100–250 µg/µl), and 35 µl of sterilized water. Thirty cycles were performed, each consisting of a denaturation step at 95 °C for 30 s (initial cycle 3 min), an annealing step at 55 °C for 15 s, and an extention step at 72 °C for 1 min (final cycle 5 min). The amplified PCR product was separated by agarose-gel electrophoresis, purified with a Gel-M™ gel extraction kit from Viogene (Sunnyvale, CA) then ligated into pT7-Blue using T4 ligase (New England Biolabs Japan, Tokyo). Nucleotide sequence analysis of the inserted fragment was performed using ABI PRISM 310 Genetic analyzer (Applied Biosystems Japan, Tokyo). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession no. AB426241. The deduced amino acid sequence of the enzyme was analyzed by BLAST searches of the databases at the National Center for Biotechnology Information.

Results

Isolation and identification of ω-laurolactam degrading microorganisms

ω-Laurolactam degrading microorganisms were screened, and strains forming clear zone on basal agar medium containing 12-aminolauric acid were isolated from soil samples from Toyama, Ishikawa, and Yamaguchi Prefectures, Japan, and active sludge from Toyama Prefecture. Among these strains, six strains isolated from Toyama Prefecture and 67 strains from Yamaguchi Prefecture formed clear zones on basal medium containing 0.5% ω-laurolactam. None of the strains isolated from the other soil samples formed clear zones on ω-laurolactam medium. The ω-laurolactam hydrolyzing activities of these strains were investigated using ω-laurolactam as a substrate. Four strains from Toyama Prefecture and 29 strains from Yamaguchi Prefecture gave spots with the same R_f as 12-aminolauric acid by TLC. Of the 33 strains, five, T7, T31, U124, U224, and U238 were selected for further characterization.

The taxonomical characteristics of strain T7 were as follows: Rod cells (0.5–0.6 × 1.0 µm), non-spore-forming, gram negative. Motility, positive. The most variable region of the 16S rDNA sequence revealed 99.7% identity to *Cupriavidus taiwanensis*[30] (the highest value). These characteristics indicate that strain T7 belongs to the genus *Cupriavidus* sp., because it was rod-shaped, gram-negative, motility-positive, catalase-positive, oxidase-positive, and nitrate reduction-positive.

The taxonomical characteristics of strain T31 were as follows: Rod cells (0.6–0.7 × 1.0–1.5 µm), non-spore-forming, gram negative. Motility, negative. The most variable region of the 16S rDNA sequence revealed 99.7% identity to *Acidovorax temperans*[31] (the highest value). These characteristics indicate that strain T31 belongs to the genus *Acidovorax* sp., because it was a rod-shaped, gram-negative, non-fermentation bacterium, catalase-positive, and oxidase-positive.

The taxonomical characteristics of strain U124 were as follows: Rod cells (0.5–0.6 × 0.7–0.8 µm), non-spore-forming, gram negative. Motility, positive. The most variable region of the 16S rDNA sequence revealed 98.5% identity to *Cupriavidus necator*[32] (the highest value). These characteristics indicate that strain U124 belongs to the genus *Cupriavidus* sp., because it was rod-shaped, gram-negative, motility-positive, catalase-positive, and oxidase-positive.

The taxonomical characteristics of strain U224 were as follows: Irregular rod cells (0.8–1.0 × 3.0–5.0 µm), non-spore-forming, gram positive. Motility, positive. The most variable region of the 16S rDNA sequence revealed 99.5% identity to *Rhodococcus wratislaviensis*[33] (the highest value). These characteristics indicate that strain U224 belongs to the genus *Rhodococcus* sp., because it was irregularly rod-shaped, gram-positive, non-spore forming, motility-negative, catalase-positive, and oxidase-negative.

The taxonomical characteristics of strain U238 were as follows: Rod cells (0.8 × 1.0–2.0 µm), non-spore-forming, gram negative. Motility, negative. Growth on nutrient agar, smooth, entire, convex, round, yellow. Anaerobic growth, negative. Gelatin liquefaction, negative. The most variable region of the 16S rDNA sequence revealed 100.0% identity to *Sphingomonas subarctica*[34] (the highest value). These characteristics indicate that strain U238 belongs to the genus *Sphingomonas* sp., because it was yellow, non-motile, gram-negative, catalase-positive, and oxidase-positive.

Time course of ω-laurolactam hydrolysis by the various isolated microorganisms

Figure 2 shows the time course of the production of 12-aminolauric acid from 10 mM ω-laurolactam by the five strains. *Cupriavidus* sp. T7 gave a maximum yield of 12-aminolauric acid (1.8 mM) after 4 h of incubation, but the product decreased gradually, possibly due to further metabolism. *Acidovorax* sp. T31 gave a maximum yield of 12-aminolauric acid (7.1 mM) after 12 h of incubation, but the product decreased gradually due to further metabolism. *Cupriavidus* sp. U124 gave a maximum yield of 12-aminolauric acid (3.2 mM) after 8 h of incubation, but the product also gradually decreased. *Rhodococcus* sp. U224 gave a maximum yield of 12-aminolauric acid (10 mM) after 24 h of incubation, and consumption of the product was not observed even after 48 h. In the case of *Sphingomonas* sp. U238, 12-aminolauric acid was not detected during 48 h by the HPLC method. Of these five strains, *Rhodococcus* sp. U224 was selected for enzyme purification, since that strain showed the least activity of 12-aminolauric acid degradation, which would interfer with accurate measurement of ω-laurolactam hydrolase activity during enzyme purification.

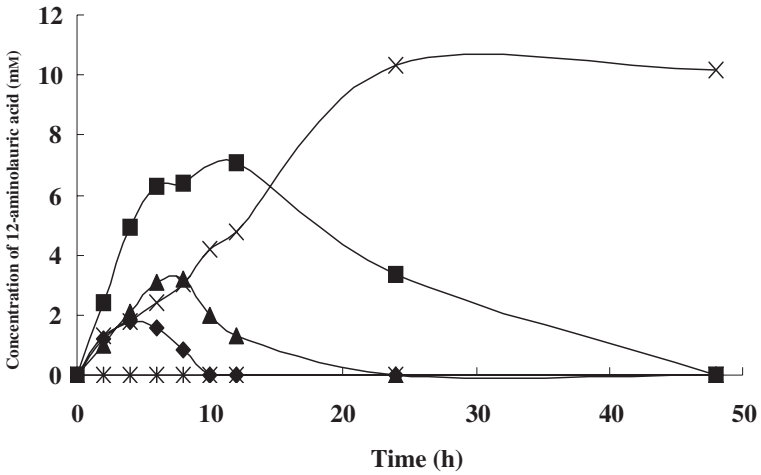

Fig. 2. Time Course of ω-Laurolactam Hydrolysis by Isolates from Soil.

A reaction mixture (1 ml) containing 100 mM of potassium phosphate buffer (pH 7.0), 10 mM of ω-laurolactam dissolved in DMSO, and cells from 1 ml of culture were incubated with shaking at 30 °C. Symbols: ◆ *Cupriavidus* sp. T7; ■ *Acidovorax* sp. T31; ▲ *Cupriavidus* sp. U124; × *Rhodococcus* sp. U224; ∗ *Sphingomonas* sp. U238.

Table 1. Purification of ω-Laurolactam Hydrolase from *Rhodococcus* sp. U224

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	1,200	87.2	0.07	100	1
Removal of nucleic acid	563	62.4	0.11	72	2
Ammonium sulfate fractionation (30–60%)	200	24.2	0.12	28	2
DEAE-Toyopearl	38.1	23.3	0.61	27	8
Butyl-Toyopearl	12.8	16.7	1.31	19	18
Mono Q HR 5/5	1.8	8.6	4.67	10	64
Superdex 200 10/30	0.1	0.7	6.54	1	90

Purification of ω-laurolactam hydrolase from Rhodococcus *sp. U224*

As summarized in Table 1, ω-laurolactam hydrolase from *Rhodococcus* sp. U224 was purified 90-fold to homogeneity, with an overall yield of 1.0% from the cell-free extract of the strain, involving ammonium sulfate fractionation, and ion-exchange, hydrophobic, and gel-filtration column chromatography. The final preparation gave a single band on SDS–PAGE, with a molecular weight of about 47,000 (Fig. 3). The molecular weight of the enzyme as determined by high performance gel-permention chromatography on Superdex 200 HR 10/30 was 79,500, indicating that the native enzyme was active as a dimer. The purified enzyme catalyzed the hydrolysis of ω-laurolactam to 12-aminolauric acid at 6.54 units/mg under the standard conditions.

Substrate specificity of the enzyme

To determine substrate specificity, the purified enzyme was used to hydrolyze lactams, various amide compounds, and amino acid amides. Besides ω-laurolactam, this enzyme hydrolyzes ω-octalactam and 6-aminohexanoate-cyclic-dimer (6.74% and 35.6% as comparing

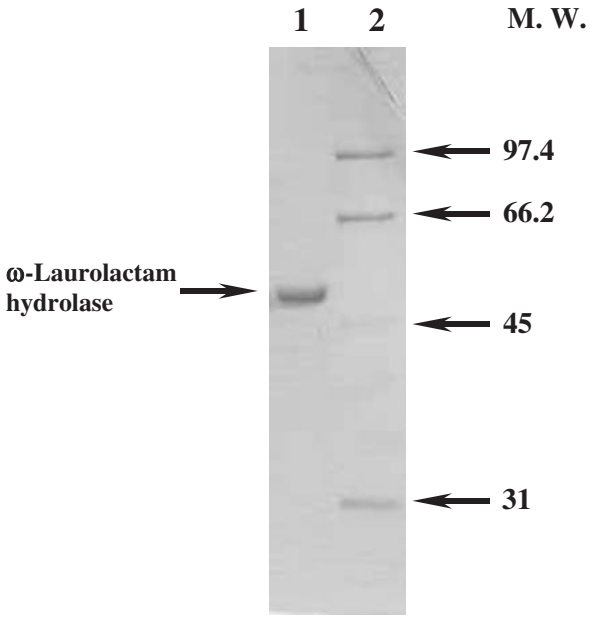

Fig. 3. SDS–PAGE of ω-Laurolactam Hydrolase from *Rhodococcus* sp. U224.

Proteins were separated on a 11.9% polyacrylamide gel in the presence of 0.01% SDS. Lanes: 1, purified ω-laurolactam hydolase; 2, molecular weight standards.

with that toward ω-laurolactam respectively). However, activity of the enzyme were not detected toward 5 lactam compounds: 2-azetidinone, 2-pyrolidone, 2-piperidone, ε-caprolactam, and ω-heptalactam. None of the compounds described as follows was judged to be active as a substrate at a detection limit below 1% between standard and sample in absorbance: acetamide, 2-cyanoacetamide, 2-iodoacetamide, benzylacetamide, phenylacetamide, propionamide, *n*-butyramide, isobutyramide, *n*-valeramide, isovaleramide, *n*-capronamide, methacrylamide, 2-thiophenecarboxamide, pyrazinecarboxamide, cyclohexanecarboxamide, maleic acid monoamide, malonamide, succinamide, nipecotamide, 2-picolinamide, crotonamide, nicotinamide, benzamide, 2-aminobenzamide, 3-aminobenzamide, 4-aminobenzamide, 4-nitrobenzamide, *N*-methylbenzamide, *p*-toluamide, *p*-chlorobenzamide, benzylbenzamide, DL-alaninamide, DL-valinamide, DL-leucinamide, DL-isoleucinamide, DL-prolinamide, DL-phenylalaninamide, DL-tryptophanamide, DL-methioninamide, glycinamide, DL-serinamide, DL-threoninamide, DL-cysteinamide, DL-glutaminamide, DL-asparaginamide, DL-tyrosinamide, DL-lysinamide, DL-argininamide, DL-histidinamide, DL-asparaginic acid amide, and DL-glutamic acid amide.

Effects of temperature and pH on enzyme activity

The effects of temperature and pH on enzyme activity were examined. Activity was measured at various temperatures in potassium phosphate buffer (pH 7.0) and at various pHs in several buffers (Fig. 4). The enzyme exhibited maximum activity at 45 °C at around pH 7.2 in potassium phosphate buffer.

Stability of the enzyme

The remaining activity of the enzyme was measured after incubation of it at various temperatures. After it had been preincubated for 30 min at various temperatures in 100 mM of potassium phosphate buffer (pH 7.0), an aliquot of each enzyme solution was taken and enzyme activity was assayed under standard conditions. The enzyme was stable up to 35 °C. After preincubation at 40 °C and at 45 °C, the remaining enzyme activity corresponded to 80% and 20% of the original activity toward the substrate respectively, and it was inactivated almost completely above 50 °C (Fig. 5A). After the enzyme was preincubated for 30 min at various pHs at 30 °C, an aliquot the each enzyme solution was taken, and the enzyme activity was assayed under the standard conditions. As shown in Fig. 5B, the enzyme was stable at pH 7.1–8.2.

Effects of various compounds on enzyme activity

Various compounds at a final concentration of 1 mM were added to the reaction mixture, and enzyme activity was monitored by TLC (*n*-buthanol/acetic acid/water = 4/1/1) and visualized with ninhydrin. The enzyme was inhibited almost completely by 1 mM of *p*-chloromercuribenzoic acid and Hg^{2+}. Metal ions

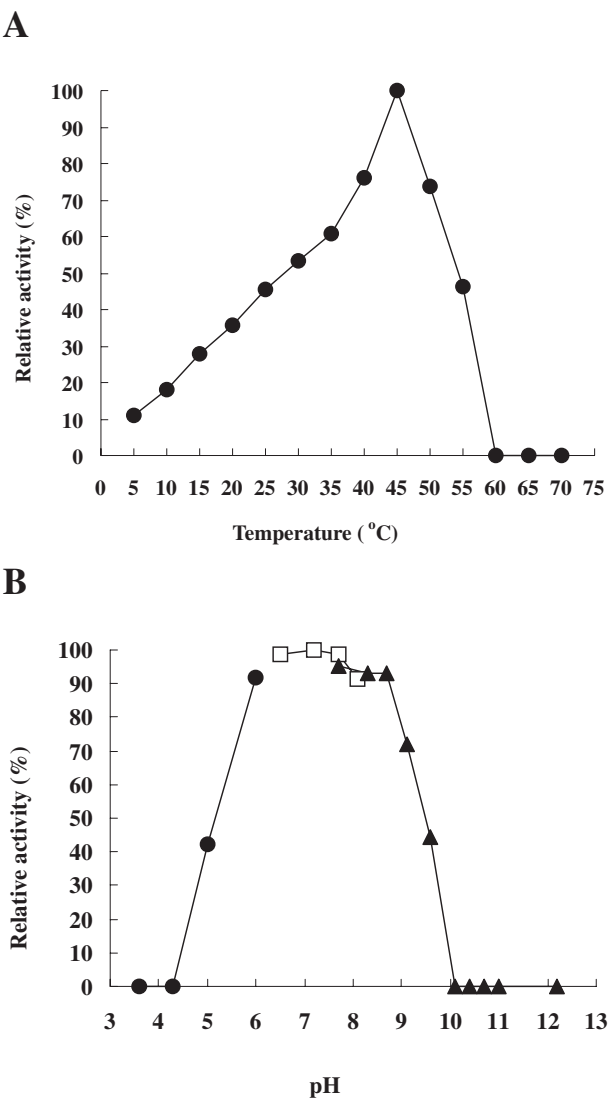

Fig. 4. Effect of Temperature (A) and pH (B) on Enzyme Activity.
In the pH-effect experiments, the following buffers (0.1 M) were used: pH 3.6–6.0, sodium acetate; pH 6.5–8.1, potassium phosphate; pH 7.7–12.2, Gly-NaCl-NaOH.

such as Ca^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+} had no effect on enzyme activity. EDTA, NaF, Tiron, ethyleneglycol tetraacetic acid, 5,5′-dithiobis-2-nitrobenzoic acid, *N*-ethylmaleimide, phenylmethane sulphonylfluoride, hydroxylamine, potassium cyanide, dithiothreitol, 2-mercaptoethanol, DL-penicillemine, D-cycloserine, potassium metabisulfite, phenylhydrazine, hydrazine, diphenylhydrazine, avidin, pepstatine, tetramethyl phenyrendiamine, and indoacetic acid had no effect on enzyme activity.

N-Terminal amino acid sequence analysis

The N-terminal amino acid sequence of the enzyme was found to be M-S-K-V-D-L-W-Q-D-A-T-A-Q-A-E-L-V-R-S-G-E-I-S-R-T-E-L-L-E-A-T (Fig. 6). A BLAST search of the protein database indicated that the N-terminal amino acid sequence of the enzyme was

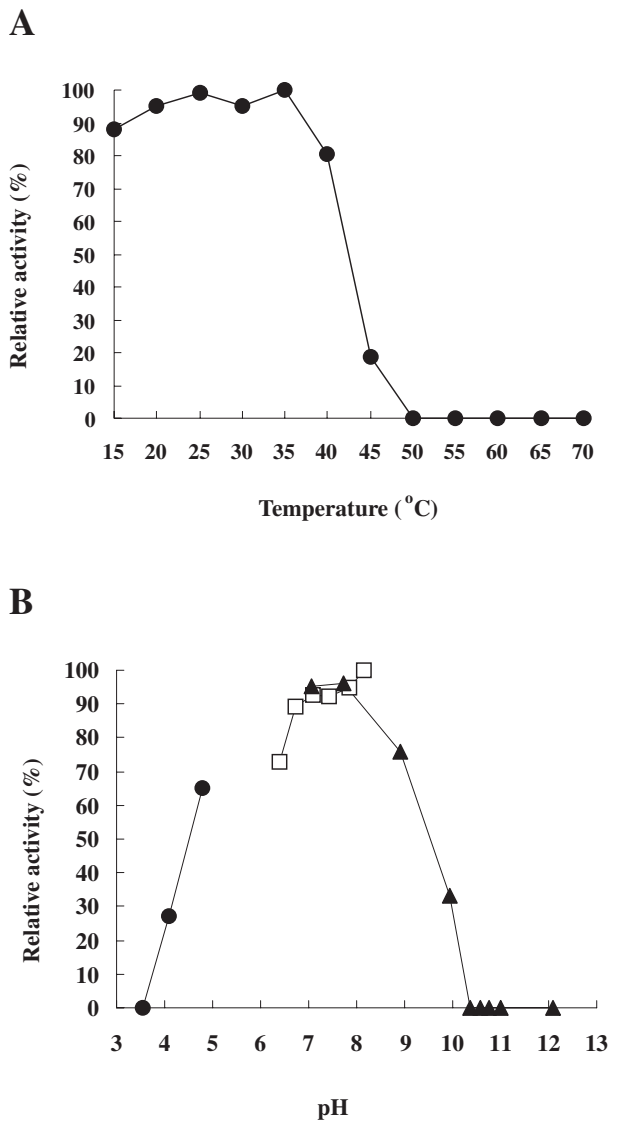

Fig. 5. Effect of Temperature (A) and pH (B) on Enzyme Stability.
In the pH-effect experiments, the following buffers (0.1 M) were used: pH 3.6–4.8 sodium acetate, pH 6.4–8.2 potassium phosphate, pH 7.0–12.1 Gly-NaCl-NaOH.

identical with that of 6-aminohexanoate-cyclic-dimer hydrolase from *Arthrobacter* sp. KI72 and from *Pseudomonas* sp. NK87.

Cloning of the ω-laurolactam hydrolase gene from Rhodococcus *sp. U224*

DNA fragments encoding ω-laurolactam hydrolase from *Rhodococcus* sp. U224 were amplified using primers designed on basis of the flaking region of the 6-aminohexanoate-cyclic-dimer hydrolase gene from *Arthrobacter* sp. KI72.[26] Then the DNA fragment was inserted into pT7-Blue vector and used in nucleotide sequencing. A 1,479-bp open reading frame starting at the GTG and terminating at the TGA codon was found in the nucleotide sequences encoding ω-laurolactam hydrolase from *Rhodococcus* sp. U224. The open reading frame encodes 493 amino acid residues. The calculated molecular weight of the *Rhodococcus* sp. U224 enzyme was 52194.46, which is consistent with the value estimated from the relative mobility of the purified enzyme on SDS–PAGE (Fig. 3). A homology search using the BLAST program showed that in primary structure, the ω-laurolactam hydrolase from *Rhodococcus* sp. U224 was almost identical with *nylA* proteins from *Arthrobacter* sp. KI72 and *Pseudomonas* sp. NK87. The genes from *Rhodococcus* sp. U224 and from F-*nylA* and P-*nylA* had 10 and eight base substitutions in the coding region, which caused five and six amino acid substitutions respectively (Fig. 6).

Synthesis of 12-aminolauric acid from ω-laurolactam by the enzyme from Rhodococcus *sp. U224*

Synthesis of 12-aminolauric acid through hydrolysis of ω-laurolactam was carried out using the partially purified enzyme after a DEAE-Toyopearl column chromatography step. The reaction mixture contained 1 ml of partially purified enzyme (0.9 units) in 20 mM potassium phosphate buffer (pH 7.0) and 50 mM of ω-laurolactam in toluene (200 µl). About 10 mM of 12-aminolauric acid was synthesized in the aqueous phase in the reaction mixture after 120 min of reaction (Fig. 7).

Discussion

The intramolecular amide bond of ω-laurolactam was resistant to hydrolysis. It was cleaved only under very harsh conditions, such as incubation with 6 N HCl at 100 °C overnight, although no hydrolysis was detected under incubation at room temperature (data not shown).

Here we describe the occurrence of ω-laurolactam hydrolysis activity in several isolated microorganisms. We screened for ω-laurolactam degrading microorganisms, and isolated 33 strains from soil from Toyama and Yamaguchi Prefectures in Japan as strains capable of growing in a medium containing ω-laurolactam as sole source of carbon and nitrogen. Among the isolates, strains T7, T31, U124, U224, and U238 were identified as *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224 and *Shingomonas* sp. U238 respectively. ω-Laurolactam hydrolase from *Rhodococcus* sp. U224 was purified to homogeneity and its enzyme properties were characterized. The characteristics of the enzyme were as follows: it showed hydrolysis activity toward ω-octalactam and ω-laurolactam, but activities toward other amide compounds were not confirmed. *p*-Chloromercuribenzoic acid and Hg^{2+} strongly inhibited the activity of the enzyme, indicating the occurrence of a critical cystein residue in the active site. The gene encoding the enzyme was cloned and sequenced. Homology search analysis revealed that the amino acid deduced from the gene encoding ω-laurolactam hydrolase had high homology with the 6-aminohexanoate-cyclic-dimer hydrolase from *Arthro-*

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U224      1:MSKVDLWQDATAQAELVRSGE|SRTELLEAT|AHVQAVNPE|NAV||PLFEKARRESELASGPFAGVPYL 70
F-nylA    1:MSKVDLWQDATAQAELVRSGE|SRTELLEAT|AHVQAVNPE|NAV||PLFEKARRESELASGPFAGVPYL 70
P-nylA    1:MSKVDLWQDATAQAELVRSGE|SRTELLEAT|AHVQAVNPE|NAV||PLFEKARRESELASGPFAGVPYL 70

U224     71:LKDLTVVSQGD|NTSS|KGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTPEMGTQVTTEPEAWGATRNP 140
F-nylA   71:LKDLTVVSQGD|NTSS|KGMKESGYRADHDAYFVQRMRAAGFVLLGKTNTPEMGNQVTTEPEAWGATRNP 140
P-nylA   71:LKDLTVVSQGD|NTSS|KGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTPEMGTQVTTEPEAWGATRNP 140
                                                          *      *
U224    141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDGAGSVR|PASVCGVVGLKPTRGR|SPGPLVTDSDNVAGAA 210
F-nylA  141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDAAGSVR|PASVCGVVGLKPTRGR|SPGPLVTDSDNVAGAA 210
P-nylA  141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDAAGSVR|PASVCGVVGLKPTRGR|SPGPLVTDSDNVAGAA 210
                                              *
U224    211:HEGLFARSVRD|AALLDVVSGHRPGDTFCAPTASRPYAQG|SENPGSLRVGVLTHNPVGDFALDPECAAA 280
F-nylA  211:HEGLFARSVRD|AALLDVVSGHRPGDTFCAPTASRPYAQG|SENPGSLRVGVLTHNPVGDFALDPECAAA 280
P-nylA  211:HEGLFARSVRD|AALLDVVSGHRPGDTFCAPTASRPYAQG|SENPGSLRVGVLTHNPVGDFALDPECAAA 280

U224    281:ARGAAAALAALGHDVNDAYPEALGDRSFLKDFST|CDVA|ARE|ERNGEL|GRPLTEDDVEWTSWEMVKR 350
F-nylA  281:ARGAAAALAALGHDVNDAYPEALGDRSFLKDYST|CDVA|ARE|ERNGEL|GRPLTEDDVEWTSWEMVKR 350
P-nylA  281:ARGAAAALAALGHDVNDAYPEALGDRSFLKDYLT|CDVA|ARE|ERNGEL|GRPLTEDDVEWTSWEMVKR 350
                                            **
U224    351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLL|LPTVTRQTPE|GELMLAKGTDLEGRQSAF|SGSLR 420
F-nylA  351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLL|LPTVTRQTPE|GELMLAKGTDLEGRQSAF|SGSLQ 420
P-nylA  351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLL|LPTVTRQTPE|GELMLAKGTDLEGRHTAL|SGSLR 420
                                                          ** *     *
U224    421:MLAFTVPFNVSGQPA|SLP|GMSSDGMP|GVQ|VAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRK| 490
F-nylA  421:MLAFTVPFNVSGQPA|SLP|GMSSDGMP|GVQ|VAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRK| 490
P-nylA  421:MLAFTVPFNVSGQPA|SLP|GMSSDGMP|GVQ|VAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRK| 490

U224    491:PAA                                                                   493
F-nylA  491:PAA                                                                   493
P-nylA  491:PAA                                                                   493
```

Fig. 6. Comparison of the Amino Acid Sequence of ω-Laurolactam Hydrolase from *Rhodococcus* sp. U224 (U224) with 6-Aminohexanoate-Cyclic-Dimer Hydrolase from *Arthrobacter* sp. KI72 (F-*nylA*) and *Pseudomonas* sp. NK87 (P-*nylA*).

Underlining indicates the detected N-terminal amino acid sequences. Asterisks show different amino acid residues between ω-laurolactam hydrolase from *Rhodococcus* sp. U224 and 6-aminohexanoate-cyclic-dimer hydrolase from *Arthrobacter* sp. KI72 and from *Pseudomonas* sp. NK87.

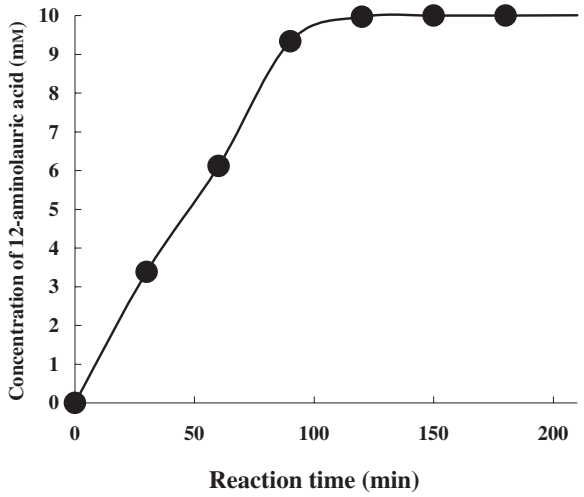

Fig. 7. Time Course of Hydrolysis of ω-Laurolactam by the Partially Purified Enzyme of *Rhodococcus* sp. U224.

A reaction mixture (1 ml) containing 20 mM potassium phosphate buffer (pH 7.0), 10 mM of ω-laurolactam dissolved toluene (200 µl), and 0.9 units of the enzyme was incubated at 30 °C. The concentration of 12-aminolauric acid (●) was determined by HPLC.

bacter sp. KI72 and *Pseudomonas* sp. NK87 (Fig. 6). The enzymatic properties of ω-laurolactam hydrolase from *Rhodococcus* sp. U224 are described above, and were similar to the characteristics of 6-aminohexanoate-cyclic-dimer hydrolase.[25)]

Nylon-6 is produced from ε-caprolactam, a cyclic amide with six carbons, by ring cleavage polymerization. During the polymerization reaction, some molecules fail to polymerize and remain as linear oligomers, while others undergo head-to-tail condensation forming cyclic oligomers. Kinoshita *et al.*[24,25)] reported a 6-aminohexanoate-cyclic-dimer hydrolase from *Arthrobacter* sp. KI72 (formerly *Achromobacter guttatus* KI72), that grows with 6-aminohexanoate-cyclic-dimer, one of the by-products from a nylon-6 factory, as the sole source of carbon and nitrogen. It has been reported that the enzyme was active only toward 6-aminohexanoic-acid-cyclic dimer forming 6-aminohexanoyl-6-aminohexanoic acid, and that it was not effective on various cyclic amides, cyclic diamides, amides, oligopeptides, or casein. The degradation of nylon-oligomers is highly dependent on specific enzymes, 6-aminohex-

anoate-cyclic-dimer hydrolase (EI),[25] 6-aminohexanoate-dimer hydrolase (EII),[35] and endo-type 6-aminohexanoate-oligomer hydrolase (EIII),[36] and the responsible genes are encoded on plasmid pOAD2 (45,519 bp) in *Arthrobacter* KI72.[37] The gene encoding ω-laurolactam hydrolase was cloned from the genomic DNA of *Rhodococcus* sp. U224. A plasmid extraction from the strain U224 was tried, but no any plasmids such as pOAD2 were detected in the strain. It is not clear whether the gene encoding ω-laurolactam hydrolase is isolated on the genomic DNA or the plasmid DNA. On the other hand, 6-aminohexanoate-cyclic-dimer hydrolase was found to be an inducible enzyme from the observation that the cell extract did not hydrolyze the cyclic dimer when the cells were harvested from nutrient broth without the cyclic dimer. However, all ω-laurolactam degrading microorganisms hydrolyzed ω-laurolactam when the cells were harvested from nutrient broth with or without ω-laurolactam. This suggests that ω-laurolactam hydrolase is a constitutive enzyme.

Hydrolysis of ω-laurolactam by microorganisms has never before been reported. This study suggests that the microorganisms screened can perhaps be used effectively in the production of 12-aminolauric acid from ω-laurolactam, since it is difficult to prepare it by chemical reaction. A possible mechanism by which an enzyme is active towards synthetic substances such as ω-laurolactam is that a non-natural compound is hydrolyzed as an analogue of a physiological substrate, but this is not clear.

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