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High Yield Synthesis of 12-Aminolauric Acid by "Enzymatic Transcrystallization" of ω-Laurolactam Using ω-Laurolactam Hydrolase from *Acidovorax* sp. T31

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The genes encoding ω-laurolactam hydrolases from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, and *Sphingomonas* sp. U238 were cloned and sequenced. Nucleotide and amino acid sequence analysis of the four genes indicated that the primary structures of these ω-laurolactam hydrolases are significantly similar to the 6-aminohexanoate-cyclic-dimer hydrolase (EC 3.5.2.12). These genes were expressed in *Escherichia coli*, and the ω-laurolactam hydrolysing activity of the recombinant enzymes was compared with that of 6-aminohexanoate-cyclic-dimer hydrolase from *Arthrobacter* sp. KI72. The enzyme from *Acidovorax* sp. T31 was most successfully expressed in *E. coli*. Cell-free extract of the recombinant strain was used for the synthesis of 12-aminolauric acid from ω-laurolactam by "enzymatic transcrystallization," because crystalline ω-laurolactam added into the enzyme solution was converted to crystalline 12-aminolauric acid (≧97.3% yield). Under the optimum conditions, 208 g/l of 12-aminolauric acid was produced in 17 h. The resulting pure product was identical to authentic 12-aminolauric acid.

Key words: enzymatic transcrystallization; ω-laurolactam; 12-aminolauric acid; *Acidovorax* sp.; 6-aminohexanoate-cyclic-dimer hydrolase

Enzymes are biocatalysts not only responsible for the metabolism of natural compounds in organisms, but also utilized for the industrial production of natural and non-natural compounds. New microbial enzymes have been successfully developed for the production of important chemicals in Japan and other countries. Recent studies on microbial transformation has been based mostly on screening for microbial enzymes catalyzing new reactions, or on screening of known enzymes for an unknown activity with synthetic substrates.[1] Enzymatic production of various amino acids, inosinic acid,[2,3] acrylamide,[4] nicotinic acid,[5] nicotinamide,[6] *etc.*, has been successfully industrialized. Most of those enzymes were obtained by screening from new microorganisms from soil or stock cultures. In our previous report, we proposed a new enzymatic method for the production of 12-aminolauric acid by hydrolyzing ω-laurolactam (Fig. 1), a chemical widely used in the synthesis of nylon, adhesives, and hardening agents.[7] Although there are some harsh chemical processes for the preparation of 12-aminolauric acid in industry,[8–11] no report has appeared on mild, *e.g.*, enzymatic production of this chemical. Hydrolysis of the intramolecular amide bond of ω-laurolactam has been possible only by incubation with 6 N HCL at high temperatures.[12]

Recently, we isolated several ω-laurolactam degrading microorganisms from soil samples that were capable of growing in a medium containing ω-laurolactam as a sole source of carbon and nitrogen. Five bacterial strains were selected according to their unique activities, *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224, and *Sphingomonas* sp. U238. Among these bacterial strains, ω-laurolactam hydrolase from *Rhodococcus* sp. U224 was purified and its enzymatic properties have been characterized.[12] The enzyme acts on ω-octalactam and ω-laurolactam, but other lactam compounds (2-azetidinone, 2-pyrolidone, 2-pipelidone, ε-caprolactam, and ω-heptalactam), amides, and amino acid amides are not active as substrates. The primary structure of the enzyme from *Rhodococcus* sp. U224 was revealed and shown to be significantly similar to 6-aminohexanoate-cyclic-dimer hydrolase (Acd hydrolase) from *Arthrobacter* sp. KI72 (formally *Flavobacterium* sp. KI72), the strain known as nylon oligomers degrading microorganism.[13–15] The deduced amino acid sequence of two enzymes has five substitutions. The enzyme from *Rhodococcus* sp. U224 was active toward 6-aminohexanoate-cyclic-dimer, and the relative activity was 35.6% of that toward ω-laurolactam.

Kitahara *et al.* have reported a procedure for L-malic acid production using fumarate hydratase (EC 4.2.1.2) from *Lactobacillus brevis*.[16] The enzyme, catalyzing the reversible hydration reaction of fumaric acid to L-malic acid, is one of the enzymes involved in the tricarboxylic acid cycle. To alter the equilibrium constant of the reaction catalyzed by the enzyme, the use of divalent-methallo fumarate as a substrate had a remarkable effect. The equilibrium of this reaction shifts further to the

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Abbreviation: Acd hydrolase, 6-aminohexanoate-cyclic-dimer hydrolase

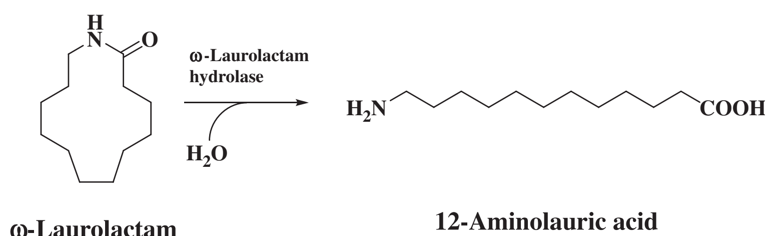

Fig. 1. Enzymatic Hydrolysis of ω-Laurolactam to 12-Aminolauric Acid.

L-malic acid side by increasing the amount of $CaCl_2$. The solubility of Ca-fumarate and that of Ca-L-malate were low, and crystals of the substrates were apparently converted into crystals of the product in the reaction mixture (*i.e.*, crystalline substrate → solution of substrate → solution of product → crystalline product). The authors have proposed a term, "enzymatic transcrystallization," for this category of reactions.

In this report, we describe the cloning and expression of these genes encoding ω-laurolactam hydrolase in *Escherichia coli*. In order to determine whether the Acd hydrolase from *Arthrobacter* sp. KI72 is able to hydrolyze ω-laurolactam, we constructed the Acd hydrolase gene from *Arthrobacter* sp. KI72 (F-*nylA*) by site-direct mutagenesis of the ω-laurolactam hydrolase gene from *Rhodococcus* sp. U224, since the enzyme from *Rhodococcus* sp. U224 and *Arthrobacter* sp. KI72 had high levels of amino acid identities. The ω-laurolactam hydrolase gene from *Acidovorax* sp. T31 was overexpressed in *E. coli* JM109, and the enzyme was used for enzymatic transcrystallization of ω-laurolactam and high yield synthesis of 12-aminolauric acid. As far as we know, this is the first report of the conversion of a non-natural compound, such as ω-laurolactam by enzymatic transcrystallization.

Materials and Methods

Materials. Escherichia coli JM109 (*e14*$^-$ (*mcr*A$^-$), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, ($r_K^- m_K^-$), *sup*E44, *rel*A1, λ^-, Δ (lac-proAB), [F′*tra*D36, *pro*AB, *lac*I^{q}ZΔM15]) was used as a host strain for DNA manipulation and expression. Plasmids pUC19 (Takara Bio, Ohtsu, Japan) and pT7-Blue (Merck Japan, Tokyo, Japan) were used as vectors for *E. coli*. The oligonucleotides were purchased from Hokkaido System Science (Tsukuba, Japan). ω-Laurolactam and 12-aminolauric acid were from Tokyo Kasei Kogyo (Tokyo, Japan). The chemicals used in this work were ordinary commercial products.

Analytical methods. The amounts of 12-aminolauric acid in the reaction mixture were assayed by analytical HPLC, equipped with a Mightysil RP-18 GP 150-4.6 column (Kanto Chemical, Tokyo, Japan), using 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 was 10–11 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.

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Biospin AVANCE II 400 (Bruker Biospin, Rheinstetten, Germany) spectrometer. An FT-IR spectrum was recorded with a PerkinElmer Spectrum 100 (PerkinElmer Japan, Yokohama) spectrometer. Melting points were measured with a Yanagimoto micro melting point apparatus (Yanagimoto, Tokyo, Japan).

Cloning of the gene encoding ω-laurolactam hydrolase from Cupriavidus *sp. T7,* Acidovorax *sp. T31,* Cupriavidus *sp. U124, and* Sphingomonas *sp. U238.* Cloning and sequencing of the gene encoding ω-laurolactam hydrolase from *Rhodococcus* sp. U224 was done previously.[12] The gene sequence showed high similarity with F-*nylA*. Therefore, cloning of four genes from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, and *Sphingomonas* sp. U238 were done in the same way. Each of the four strains was cultivated in 3 ml of TGY medium at 30 °C for 12 h. Each genomic DNA was prepared from cells harvested from the culture by the method of Saito and Miura.[17] The coding region of ω-laurolactam hydrolase was amplified by PCR using forward (5′-^{139}AGAAGGAGCGCGACAGTGAGCAAGGTGGAC^{168}-3′) and reverse (5′-^{1650}CATCTCCCGCAAGCATCAGGCCGCTGGGATC^{1620}-3′) primers. The two primers were designed on the basis of the flanking region of F-*nylA*. 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 each extracted DNA, 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 Gel-M™ gel extraction kit from Viogene (Sunnyvale, CA, USA), and then ligated into pT7-Blue using T4 ligase (New England BioLabs Japan, Tokyo, Japan). Recombinant plasmids, pTT7, pTT31, pTU124, and pTU238, contained the respective genes for the enzyme from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, and *Sphingomonas* sp. U238. Nucleotide sequence analysis of the inserted fragment was performed using an ABI PRISM 310 Genetic analyzer (Applied Biosystems Japan, Tokyo). The nucleotide sequences from strains T7, T31, U124, and U238 reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database under the accession nos. AB444712, AB444713, AB444714, and AB444715, respectively. The deduced amino acid sequences of the enzymes were analyzed by BLAST searches of the databases at the National Center for Biotechnology Information of Japan.

Construction of expression vectors of ω-laurolactam hydrolase. Modified DNA fragments coding for ω-laurolactam hydrolase from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224, and *Sphingomonas* sp. U238 were obtained by PCR using forward (5′-ACAGCGAAGCTTTAAGGAGGAAtagACAATGAGCAAGGTGGACCTTTGG-3′) and reverse (5′-GGAGGACGTCTAGATCAGGCCGCTGGGATC-3′) primers. The sense primer contained a *Hin*dIII recognition site (underlined sequence), a ribosome binding site (double-underlined sequence), and a TAG stop codon (lower case letters) in-frame with the *lacZ* gene in pUC19, and the start codon was modified GTG to ATG (dashed line). The antisense primer contained an *Xba*I site (underlined sequence). PCR was performed by the method described above. The amplified PCR product was separated by agarose-gel electrophoresis, purified with a Gel-M™ gel extraction kit (Viogene), and digested with *Hin*dIII (Takara Bio) and *Xba*I (Takara Bio). The DNA was then ligated into pUC19 using T4 ligase, yielding pT7, pT31, pU124, pU224, and pU238 vectors, and then used to transform *E. coli* JM109.

*Construction of F-*nylA *by site-directed mutagenesis.* To construct the Acd hydrolase expression plasmid, five mutations (V118T, T125N,

G171A, F312Y, and R420Q) were introduced into pU224 with a QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, Tokyo, Japan) using pU224 as a template. The reaction mixture for the mutagenesis contained 2.5 µl of QuikChange® Multi reaction buffer, 1 µl of dNTP mix, 9.5 µl of distilled H_2O, 50 ng of ds-DNA template, pU224, four sense and four antisense primers each at 50 ng, and 1 µl of QuikChange® Multi enzyme blend. The four primers were as follows: primer 1 5′-AAAACTAATACACCGGAGATGGGCAAT-CAG-3′ (for V118T and T125N), primer 2 5′-CGCACGGCAATGAC-GCGGCAGGTTCCGTGC-3′ (for G171A), primer 3 5′-CTTCCTC-AAGGACTACTCGACGATTTGCGA-3′ (for F312Y), and primer 4 5′-TCTCGGGCAGTCTGCAGATGCTGGCCTTCA-3′ (for R420Q). These designed primers contained mutation points (double-underlined). Thirty cycles were performed, each consisting of a denaturing step at 95 °C for 1 min, an annealing step at 55 °C for 1 min, and an elongation step at 65 °C for 8 min. The amplified product was incubated with 1 µl of *Dpn* I (10 units/µl) at 37 °C for 1 hr, and then used for the transformation of *E. coli* JM109. The plasmid was extracted and sequenced to make sure occurrences of each mutation, V118T, T125N, G171A, F312Y, and R420Q, and the plasmid was named pF-*nylA*.

Expression of recombinant enzymes. Recombinant *E. coli* JM109 strains harboring each of the expression vectors, pT7, pT31, pU124, pU224, pU238, and pF-*nylA*, were cultured at 37 °C for 8 h with shaking (200 rpm) in a test tube containing 5 ml of LB broth containing 80 µg/ml of ampicillin. After 8 h of cultivation, IPTG (final concentration, 0.5 mM) was added to the culture broth and the cultivation temperature was shifted to 30 °C. The *E. coli* transformants were totally cultivated for 20 h. Cells of 1 ml culture were harvested by centrifugation (15,000 × *g*, 5 min, 4 °C) and suspended in 1 ml of 100 mM potassium phosphate buffer (pH 7.0). Cell suspensions were then disrupted by Multi Beads Shocker (Yasui Instruments, Osaka, Japan). For removal of intact cells and cell debris, the lysate was centrifuged at 15,000 × *g* for 10 min at 4 °C to yield the cell-free extract. The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 10 mM of ω-laurolactam dissolved in toluene, and an appropriate amount of the enzyme.

Purification of Acd hydrolase from E. coli *JM109/pF-*nylA. The subculture of *E. coli* JM109/pF-*nylA* was inoculated into 500 ml of LB medium containing ampicillin in a 2-liter Sakaguchi flask and the culture broth was shaken (96 stroke/min) at 37 °C, and after 12 h of cultivation, IPTG (final concentration, 0.5 mM) was added to the culture broth and the cultivation temperature was shifted 30 °C to induce the enzyme (total cultivation time, 24 h). Cells from 5 liters of culture were suspended in the buffer described above and the suspensions were disrupted by sonication for 15 min (19 kHz, Insonator model 201M; Kubota, Tokyo). The cell-free extract was produced by centrifugation (15,000 × *g*, 15 min, 4 °C). It was fractionated with ammonium sulfate (30–60% saturation). The active pellet was dialyzed, applied to a DEAE-Toyopearl (Tosoh, Tokyo) column, and eluted with 100 mM of potassium phosphate buffer (pH 7.0) containing 0.5 M NaCl. The active fraction was dialyzed, and applied to MonoQ 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, 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. The dialyzed active fractions were combined and concentrated with the Centricon (Amicon, Beverly, MA).

Enzymatic transcrystallization of ω-laurolactam to 12-aminolauric acid using overexpressed ω-laurolactam hydrolase from Acidovorax *sp. T31.* The cultivation conditions for *E. coli* JM109/pT31 were carried out by the same method as for *E. coli* JM109/pF-*nylA*. Cells from 5 liters of culture were suspended in the buffer described above, and the suspensions were disrupted by sonication for 15 min. The cell-free extract was produced by centrifugation (15,000 × *g*, 15 min, 4 °C).

Enzymatic transcrystallization of ω-laurolactam using ω-laurolactam hydrolase from *Acidovorax* sp. T31 was carried out with 0.5 M and 1.0 M of the substrate concentrations. The reaction mixture contained 100 ml of the cell-free extract obtained from 1 liter of culture broth and 9.85 g (0.05 mol) or 19.7 g (0.1 mol) of ω-laurolactam. It was stirred at 35 °C for 18 h in the flask. After the reaction, the product was filtrated and washed with deionized water. The crystals formed were dried at 80 °C and then in a vacuum desiccator night over.

Results

Comparison of the amino acid sequences of cloned ω-laurolactam hydrolase with Acd hydrolase from Arthrobacter *sp. KI72*

The deduced amino acid sequence of ω-laurolactam hydrolase from *Rhodococcus* sp. U224 and Acd hydrolase from *Arthrobacter* sp. KI72 has only five substitutions.[12] We designed primers for PCR based on the sequence information, and successfully cloned genes encoding ω-laurolactam hydrolase from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, and *Sphingomonas* sp. U238. A 1,482-bp DNA fragment starting at GTG and terminating at the TGA codon was found in all of the nucleotide sequences encoding ω-laurolactam hydrolyzing enzymes from the four strains, although the N-terminal and C-terminal gene sequences of these genes were unclear. These open reading frames encoded 493 amino acid residues. A homology search using the BLAST program showed that in primary structure, the ω-laurolactam hydrolases from the four strains were almost identical with the F-*nylA* protein. Multiple alignments of the deduced sequences of these six ω-laurolactam hydrolases are shown in Fig. 2. These enzymes were highly homologous with each other.

Expression of recombinant ω-laurolactam hydrolase and Acd hydrolase in E. coli

For expression of the genes from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224, and *Sphingomonas* sp. U238, the upstream regions of the start codons were modified (GTG to ATG). The six *E. coli* JM109 transformants (*E. coli* JM109 harboring pT7, pT31, pU124, pU224, pU238, or pF-*nylA*) were used for the expression of the respective recombinant enzymes. These cell-free extracts of the *E. coli* JM109 transformants were prepared as described in "Materials and Methods," and were used to assay ω-laurolactam hydrolase activity. All of them expressed ω-laurolactam hydrolase successfully, and their activity levels were different. The cell-free extracts of recombinant enzymes from *Cupriavidus* sp. T7 and *Cupriavidus* sp. U124 showed similar activity (total activity, 0.66 and 0.66 units/ml culture; specific activity, 1.22 and 1.29 units/mg protein, respectively). *E. coli* JM109/pU224 and pU238 gave lower hydrolyzing activity (total activity; 0.14 and 0.12 units/ml culture, specific activity; 0.36 and 0.29 units/mg protein, respectively) than the others, although these activities were 2 times higher than the wild strains.[12] *E. coli* JM109/pT31 showed the highest activity, of 1.14 units/ml of culture and 2.77 units/mg protein. The cultivation conditions for *E. coli* JM109/pT31 for induction of ω-laurolactam hydrolase were optimized. The highest enzyme activity (8.26 units/mg) was obtained when the recombinant strain was grown at 37 °C after 12 h of the cultivation, IPTG (final concentration, 0.5 mM) was added to the culture and the temperature was lowered to 30 °C (total

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F-nylA   1:MSKVDLWQDATAQAELVRSGEISRTELLEATIAHVQAVNPEINAVIIPLFEKARRESELASGPFAGVPYL 70
U224     1:MSKVDLWQDATAQAELVRSGEISRTELLEATIAHVQAVNPEINAVIIPLFEKARRESELASGPFAGVPYL 70
T7       1:MSKVDLWQDATAQAELVRSGEISRTELLEATIAHVQAVNPEINAVIIPLFEKARRESELASGPFAGVPYL 70
T31      1:MSKVDLWQDATAQAELVRSGEISRTELLEATIAHVQAVNPEINAVIIPLFEKARRESELASGPFAGVPYL 70
U124     1:MSKVDLWQDATAQAELVRSGEISRTELLEATIAHVQAVNPEINAVIIPLFEKARRESELASGPFAGVPYL 70
U238     1:MSKVDLWQDATAQAELVRSGEISRTELLEATIAHVQAVNPEINAVIIPLFEKARRESELASGPFAGVPYL 70

F-nylA  71:LKDLTVVSQGDINTSSIKGMKESGYRADHDAYFVQRMRAAGFVLLGKTNTPEMGNQVTTEPEAWGATRNP 140
U224    71:LKDLTVVSQGDINTSSIKGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTPEMGTQVTTEPEAWGATRNP 140
T7      71:LKDLTVVSQGDINTSSIKGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTSEMGTQVTTEPEAWGATRNP 140
T31     71:LKDLTVVSQGDINTSSIKGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTSEMGTQVTTEPEAWGATRNP 140
U124    71:LKDLTVVSQGDINTSSIKGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTSEMGTQVTTEPEAWGATRNP 140
U238    71:LKDLTVVSQGDINTSSIKGMKESGYRADHDAYFVQRMRAAGFVLLGKVNTSEMGTQVTTEPEAWGATRNP 140
                                                          *  *   *
F-nylA 141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDAAGSVRIPASVCGVVGLKPTRGRISPGPLVTDSDNVAGAA 210
U224   141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDGAGSVRIPASVCGVVGLKPTRGRISPGPLVTDSDNVAGAA 210
T7     141:WNLGRSVGGSSGGSAAAVAAALSPVAHGNDGAGSVRIPASVCGVVGLKPTRGRISPGPLVTDSDNVAGAA 210
T31    141:WNLGRSVGGSSGGSAAAVAAALSPVAHGNDGAGSVRIPASVCGVVGLKPTRGRISPGPLVTDSDNVAGAA 210
U124   141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDAAGSVRIPASVCGVVGLKPTRGRISPGPLVTDSDNVAGAA 210
U238   141:WNLGRSVGGSSGGSGAAVAAALSPVAHGNDGAGSVRIPASVCGVAGLKPTRGRISPGPLVTDSDNVAGAA 210
                         *               *            *
F-nylA 211:HEGLFARSVRDIAALLDVVSGHRPGDTFCAPTASRPYAQGISENPGSLRVGVLTHNPVGDFALDPECAAA 280
U224   211:HEGLFARSVRDIAALLDVVSGHRPGDTFCAPTASRPYAQGISENPGSLRVGVLTHNPVGDFALDPECAAA 280
T7     211:HEGLFARSVRDIAALLDVVSGHRPGDTFCAPTASRPYAQGISENPGSLRVGVLTHNPVGDFALDPECAAA 280
T31    211:HEGLFARSVRDIAALLDVVSGHRPGDTFCAPTASRPYAQGISENPGSLRVGVLTHNPVGDFALDPECAAA 280
U124   211:HEGLFARSVRDIAALLDVVSGHRPGDTFCAPTASRPYAQGISENPGSLRVGVLNHNPVGDFALDPECAAA 280
U238   211:HEGLFARSVRDIAALLDVVSGHRPGDTFCAPTASRPYAQGISENPGSLRVGVLTHNPVGDFALDPECAAA 280
                                                                *
F-nylA 281:ARGAAAALAALGHDVNDAYPEALGDRSFLKDYSTICDVAIAREIERNGELIGRPLTEDDVEWTSWEMVKR 350
U224   281:ARGAAAALAALGHDVNDAYPEALGDRSFLKDFSTICDVAIAREIERNGELIGRPLTEDDVEWTSWEMVKR 350
T7     281:ARGAAAALAALGHDVNDAYPEALGDRSFLKAFSTICDVAIAREIERNGELIGRPLTEDDVEWTSWEMVKR 350
T31    281:ARGAAAALAALGHDVNDAYPEALGDRSFLKAFSTICDVAIAREIERNGELIGRPLTEDDVEWTSWEMVKR 350
U124   281:ARGAAAALAALGHDVNDAYPEALGDRSFLKAFSTICDVAIAREIERNGELIGRPLTEDDVEWTSWEMVKR 350
U238   281:ARGAAAALAALGHDVNDAYPEALGDRSFLKDFSTIGDVAIAREIERNGELIGRPLTEDDVEWTSWEMVKR 350
                                         **   *
F-nylA 351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLLILPTVTRQTPEIGELMLAKGTDLEGRQSAFISGSLQ 420
U224   351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLLILPTVTRQTPEIGELMLAKGTDLEGRQSAFISGSLR 420
T7     351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLLILPTVTRQTPEIGELMLAKGTDLEGRQSAFISGSLR 420
T31    351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLLILPTVTRQTPEIGELMLAKGTDLEGRQSAFISGSLR 420
U124   351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLLILPTVTRQTPEIGELMLAKGTDLEGRQSAFISGSLR 420
U238   351:ADQVTGRAFAACVDELRYYAGKVERWWEAGWDLLILPTVTRQTPEIGELMLAKGTDLEGRQSAFISGSLR 420
                                                                                *
F-nylA 421:MLAFTVPFNVSGQPAISLPIGMSSDGMPIGVQIVAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRKI 490
U224   421:MLAFTVPFNVSGQPAISLPIGMSSDGMPIGVQIVAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRKI 490
T7     421:MLAFTVPFNVSGQPAISLPIGMSSDGMPIGVQIVAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRKI 490
T31    421:MLAFTVPFNVSGQPAISLPIGMSSDGMPIGVQIVAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRKI 490
U124   421:MLAFTVPFNVSGQPAISLPIGMSSDGMPIGVQIVAAYGREDLLLQVAAQLEGAMPWVARRPQLLNPSRKI 490
U238   421:MLAFTVPFNVSGQPAISLPIGMSSDGMPIGVQIVAAYGREDLLLQVAAQLEGALPWVARRPQLLNPSRKI 490
                                                                *
F-nylA 491:PAA                                                                    493
U224   491:PAA                                                                    493
T7     491:PAA                                                                    493
T31    491:PAA                                                                    493
U124   491:PAA                                                                    493
U238   491:PAA                                                                    493
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Fig. 2. Comparison of the Amino Acid Sequence of the ω-Laurolactam Hydrolase from the Five Strains with 6-Aminohexanoate-Cyclic-Dimer Hydrolase from *Arthrobacter* sp. KI72 (F-*nylA*).

The 493-amino-acid sequence is shown with F-*nylA*, *Arthrobacter* sp. KI72;[12] U224, *Rhodococcus* sp. U224;[9] T31, *Acidovorax* sp. T31 (this study); T7, *Cupriavidus* sp. T7 (this study); U124, *Cupriavidus* sp. U124 (this study); *Sphingomonas* sp. U238 (this study). The N-terminal sequence detected previously is double-underlined. Asterisks show different amino acid residues among the enzymes from the six strains. The amino acid residues mutated for construction of Acd hydrolase in U224 sequence are shaded in black.

cultivation time, 24 h). The ω-laurolactam hydrolase gene from *Rhodococcus* sp. U224 was site directed mutated to construct F-*nylA*. The recombinant enzyme from the gene can also hydrolyze ω-laurolactam with 0.26 units/ml of culture and 1.07 units/mg of protein. The purified enzyme from *E. coli* JM109/pF-*nylA* showed similar activity to ω-laurolactam hydrolase from *Rhodococcus* sp. U224 (ω-laurolactam, 5.88 units/mg; ω-octalactam, 0.21 units/mg).

Enzymatic transcrystallization of ω-laurolactam to 12-aminolauric acid using crude recombinant enzyme from E. coli *JM109/pT31*

We discovered the occurrence of enzymatic transcrystallization in the reaction of the enzymatic hydrolysis of ω-laurolactam when ω-laurolactam hydrolase was present on the surface of the agar plate containing ω-laurolactam. The enzyme hydrolyzed crystalline ω-laurolactam in the agar plate, and crystalline 12-aminolauric acid appeared after 4 h (Fig. 3). Therefore, we investigated high yield production of 12-aminolauric acid from ω-laurolactam using ω-laurolactam hydrolase in the method of enzymatic transcrystallization.

The crude enzyme was prepared from 5 liters of culture as described in "Materials and Methods," to yield 6,000 units of total activity. The reaction mixture (100 ml) consisted of 0.05 mol (0.5 M) or 0.1 mol (1.0 M) of ω-laurolactam, 100 mM potassium phosphate buffer (pH 7.0), and the crude enzyme from 1 liter of culture broth (approximately 1,200 units). It was incubated at 35 °C with stirring. The conversion of ω-laurolactam to 12-aminolauric acid was determined by HPLC analysis. Figure 4 shows a time course of the enzymatic transcrystalization of ω-laurolactam to 12-aminolauric acid

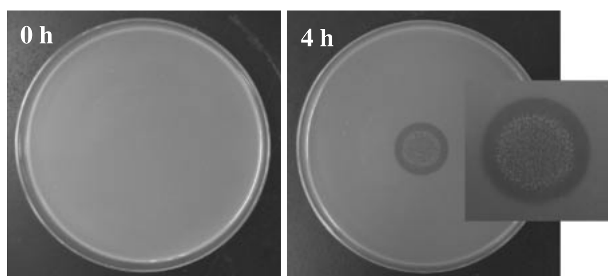

Fig. 3. Enzymatic Transcrystallization of ω-Laurolactam to 12-Aminolauric Acid.

After 4 h of incubation at room temperature, the crystalline structure of 12-aminolauric acid was made to appear by spotting ω-laurolactam hydrolase on the agar plate. The agar plate contained 20 mM of potassium phosphate buffer (pH 7.0), 0.5% of ω-laurolactam, and 1.5% agar.

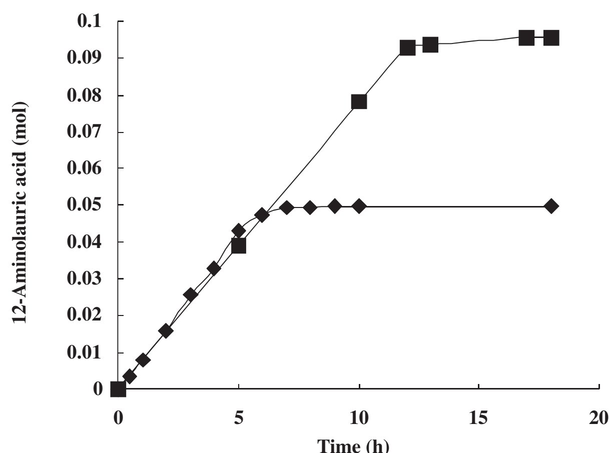

Fig. 4. Time Course of the Enzymatic Transcrystallization of ω-Laurolactam Using Recombinant ω-Laurolactam Hydrolase from *Acidovorax* sp. T31.

◆, Reaction with 0.05 mol of substrate; ■, Reaction with 0.1 mol of substrate.

using the crude enzyme. ω-Laurolactam (0.05 mol) was converted to 12-aminolauric acid (≧99.7% yield) after 9 h of incubation. When 0.1 mol of ω-laurolactam was used as a substrate, the substrate was converted to the product (≧97.3% yield) after 17 h of incubation.

Isolation and identification of 12-aminolauric acid

To isolate the 12-aminolauric acid produced, the reaction mixture was treated as described in "Materials and Methods." The yield of 12-aminolauric acid was measured by weight and calculated by the ideal weight of the product. Crystalline ω-laurolactam (9.9 g; 0.05 mol) was converted to 10.6 g of the crystalline product (0.049 mol), and the isolated yield was 98.3%. When 19.7 g of the substrate (0.1 mol) was used, it was converted to 20.8 g of the product (0.097 mol) and the isolated yield was 96.6%.

The product was identified as 12-aminolauric acid by comparing the infrared, ^{1}H and ^{13}C NMR spectra of the product with those of authentic 12-aminolauric acid. The analytical data were as follows: m.p. 185–186 °C, ^{1}H NMR [trifluoro acetic acid (TFA)] δ_{ppm} 3.27 (2H, t, $J = 7.6$ Hz), 2.55 (2H, t, $J = 7.6$ Hz), 1.84 (2H, tt, $J = 7.4$, 7.6 Hz), 1.78 (2H, tt, $J = 7.6$, 7.5 Hz), 1.38–1.47 (14H, m), ^{13}C NMR (TFA) δ_{ppm} 183.18, 40.88, 33.02, 28.32, 28.26, 28.20, 28.05, 27.96, 27.86, 26.37, 25.04, 23.77. All analytical data indicated that there were no other by-product materials, such as the substrate (Fig. 5).

Discussion

In this report, we describe the cloning and expression in *E. coli* of ω-laurolactam hydrolase from *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224, and *Sphingomonas* sp. U238 and high-yield production of 12-aminolauric acid from ω-laurolactam using recombinant ω-laurolactam hydrolase from *Acidovorax* sp. T31 by enzymatic transcrystallization. In addition, the Acd hydrolase gene from *Arthrobacter* sp. KI72, which is significantly similar to the ω-laurolactam hydrolase gene from *Rhodococcus* sp. U224, was constructed by site-directed mutagenesis of the latter gene.

We are interested in whether Acd hydrolase, which is similar to our ω-laurolactam hydrolase, has also ω-laurolactam hydrolyzing activity. There were three ways to obtain Acd hydrolase enzyme: (i) purification of the enzyme from *Arthrobacter* sp. KI72, (ii) Cloning of the Acd hydrolase gene from *Arthrobacter* sp. KI72, and (iii) construction of the Acd hydrolase gene from the ω-laurolactam hydrolase gene. We selected the third strategy, by introducing five point mutations into the ω-laurolactam hydrolase gene from *Rhodococcus* sp. U224. In Table 1, some enzymatic properties of ω-laurolactam hydrolase from *Rhodococcus* sp. U224 are summarized and compared with Acd hydrolase from *Arthrobacter* sp. KI72.[12,14,18] The ω-laurolactam hydrolase from *Rhodococcus* sp. U224 resembled that of *Arthrobacter* sp. KI72 with respect to specific activity toward ω-laurolactam, ω-octalactam, and 6-aminohexanoate-cyclic-dimer, molecular weight, subunit structure, and inhibitors. We found for the first time that the constructed Acd hydrolase from *Arthrobacter* sp. KI72 is active toward ω-laurolactam and ω-octalactam, indicating that these two enzymes shares high similarity in their properties. There has been no report that Acd hydrolase is active toward these lactam compounds.

Arthrobacter sp. KI72 has been studied as a model of how microorganisms evolved specific enzymes that degrade nylon oligomers.[18–24] Since Acd hydrolase has been found to be one of the nylon oligomers degrading enzymes encoded on plasmid pOAD2, it has been considered that the enzymes degrading nylon oligomers are active specifically toward 6-aminohexanoate-cyclic-dimer.[14] There is a hypothesis for the birth of nylon oligomer degradation mechanisms that wild-type cells can be maintained in the starved condition for a long period, since the nylon oligomer has no detectible toxicity toward microorganisms. After the cells accumulated the required genetic alteration to make a cryptic region active, cells grew in the nylon oligomer medium.[18,25] On the other hand, we screened for ω-laurolactam degrading microorganisms in soil samples from Toyama Prefecture and from an industrial site in Yamaguchi Prefecture in Japan.[12] Among them, *Cupriavidus* sp. T7 and *Acidovorax* sp. T31, having very similar genes to F-*nylA* in their chromosomes, were isolated from the soil samples at Toyama Prefecture University without any appreciable exposure to indus-

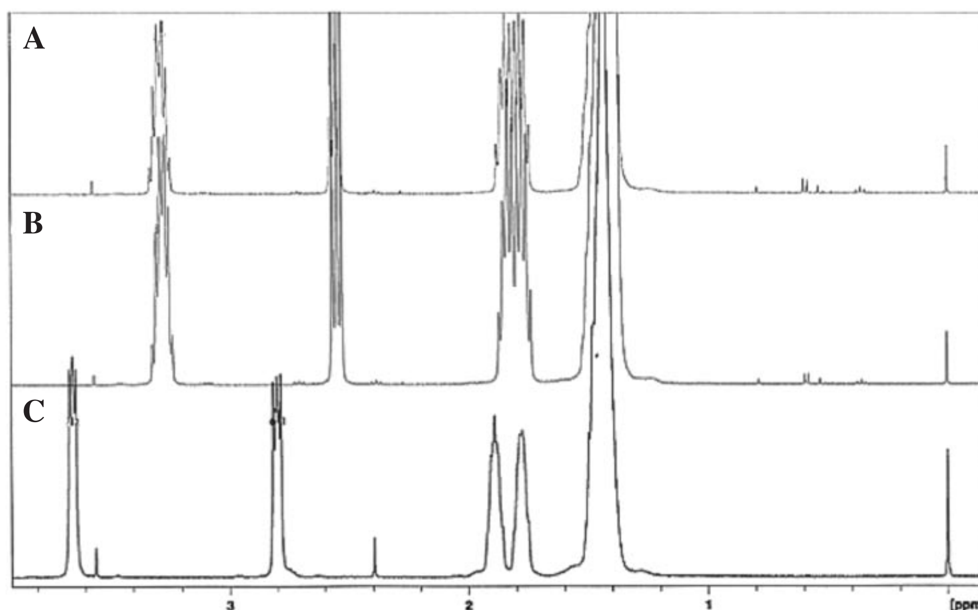

Fig. 5. Purity of the Product of Enzymatic Transcrystallization Using NMR Spectra.
A, product of the reaction; B, authentic 12-aminolauric acid; C, authentic ω-laurolactam.

Table 1. Comparison of Properties of ω-Laurolactam Hydrolase from *Rhodococcus* sp. U224 and 6-Aminohexanoate-Cyclic-Dimer Hydrolase from *Arthrobacter* sp. KI72

Property	Value with purified enzyme from	
	Rhodococcus sp. U224[12)]	*Arthrobacter* sp. KI72[14,25)]
Specific activity (units/mg) at 30 °C		
ω-Laurolactam	6.54	5.88[This study)]
ω-Octalactam	0.44	0.21[This study)]
6-Aminohexanoate-cyclic-dimer	2.31	2.8
Molecular weight		
Gel filtration	79,500	100,000
Gene sequencing	104,390	104,480
Molecular weight of subunit (s)		
SDS–PAGE	47,000	55,000
Gene sequencing	52,195	52,240
No. of subunits	2	2
pH optimum for		
ω-Laurolactam	7.2	—
6-Aminohexanoate-cyclic-dimer	—	7.4
Temperature optimum for		
ω-Laurolactam	45 °C	—
6-Aminohexanoate-cyclic-dimer	—	34 °C
Inhibitor	PCMB,[*)] Hg^{2+}	PCMB, Diisopropylphosphofluoridate
Formation	Non-inducible	Inducible

*) *p*-Chloromercuribenzoate

trial waste containing nylon oligomer. Additionally, all ω-laurolactam degrading microorganisms hydrolyzed ω-laurolactam when the cells were harvested from media containing nutrient broth with and without ω-laurolactam. Our experimental results suggest that ω-laurolactam hydrolase is a constitutive enzyme. This is evidence against the thesis that the enzyme evolved under conditions with nylon oligomer for a long time. However, the biological role of the enzyme is still unknown.

These six genes, include *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* sp. U124, *Rhodococcus* sp. U224, *Sphingomonas* sp. U238, and the Acd hydrolase gene from *Arthrobacter* sp. KI72, were expressed in *E. coli*, and ω-laurolactam hydrolysing activities of the recombinant enzymes were compared. In this paper, we present high-yield synthesis of the enzymatic transcrystallization of ω-laurolactam to 12-aminolauric acid. With the crude enzyme from *E. coli* JM109/pT31, 208 mg/ml of 12-aminolauric acid was produced in 17 h at 35 °C. As an analytical method, NMR using trifluoro acetic acid as a solvent was used to examine the purity of the product. It was synthesized with a conversion of ≧95% without any other by-product (Fig. 5).

In developing the enzymatic process, an important factor, which may prevent bioconversion processes from being practically used, is the fact that often the product concentrations are low. Since an increase in the product concentration can usually not be realized simply by using higher substrate concentrations, the application of the bi-phasic reaction in biocatalysts has been widely studied.[26–29)] In most of these bi-phasic systems, a water-

organic solvent is used as the second phase, and these systems are mainly applied in the conversion of hydrophobic compounds. However, the bi-phasic reaction system has a drawback in leading to inhibition or inactivation of the biocatalyst.

In the case of enzymatic synthesis of 12-aminolauric acid using the bi-phasic system (toluene and water), the conversion ratio was low as compared with the enzymatic transcrystallization method, and it was difficult to develop large-scale synthesis. We attempted dissolving ω-laurolactam in various organic solvents, acetone, methanol, ethanol, 2-propanol, 2-buthanol, hexane, ethylacetate, dimethyl sulfoxide (DMSO), and toluene. However, only DMSO and toluene could be used as solvents of ω-laurolactam, although with low solubility (maximum, approximately 100 mM and 50 mM of ω-laurolactam). Toluene was found to be a better solvent than DMSO for the synthesis of 12-aminolauric acid, showing higher enzyme activity.[12] When partially purified enzyme was used for the synthesis, at most 10 mM 12-aminolauric acid (2.2 mg/ml) was produced in the aqueous phase with precipitation. We solved the problem by enzymatic transcrystallization of non-soluble ω-laurolactam, and by finding that ω-laurolactam was hydrolyzed to crystalline 12-aminolauric acid when the crystalline substrate was added to the enzyme solution directly in water (Fig. 3). Furthermore, the synthesized product could be purified by filtration and washing by water, and thus there is no other purification step such as column chromatography.

Here, we present a new enzymatic process for the synthesis of 12-aminolauric acid by enzymatic transcrystallization without using the organic solvent system. Due to the high yield, this enzymatic transcrystallization process is expected to become an environmentally friendly process, without using organic solvent, acid, or alkaline for the production of 12-aminolauric acid on a larger scale.

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