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Gene Cloning and Molecular Characterization of an Extracellular Poly(L-Lactic Acid) Depolymerase from *Amycolatopsis* sp. Strain K104-1

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We have isolated a polylactide or poly(L-lactic acid) (PLA)-degrading bacterium, *Amycolatopsis* sp. strain K104-1, and purified PLA depolymerase (PLD) from the culture fluid of the bacterium. Here, we cloned and expressed the *pld* gene encoding PLD in *Streptomyces lividans* 1326 and characterized a recombinant PLD (rPLD) preparation. We also describe the processing mechanism from nascent PLD to mature PLD. The *pld* gene encodes PLD as a 24,225-Da polypeptide consisting of 238 amino acids. Biochemical and Western immunoblot analyses of PLD and its precursors revealed that PLD is synthesized as a precursor (prepro-type), requiring proteolytic cleavage of the N-terminal 35-amino-acid extension including the 26-amino-acid signal sequence and 9-residue prosequence to generate the mature enzyme of 20,904 Da. The cleavage of the prosequence was found to be autocatalytic. PLD showed about 45% similarity to many eukaryotic serine proteases. In addition, three amino acid residues, H^{57}, D^{102}, and S^{195} (chymotrypsin numbering), which are implicated in forming the catalytic triad necessary for cleavage of amide bond of substrates in eukaryotic serine proteases, were conserved in PLD as residues H^{74}, D^{111}, and S^{197}. The G^{193} residue (chymotrypsin numbering), which is implicated in forming an oxyanion hole with residue S^{195} and forms an important hydrogen bond for interaction with the carbonyl group of the scissile peptide bond, was also conserved in PLD. The functional analysis of the PLD mutants H74A, D111A, and S197A revealed that residues H^{74}, D^{111}, and S^{197} are important for the depolymerase and caseinolytic activities of PLD and for cleavage of the prosequence from pro-type PLD to form the mature one. The PLD preparation had elastase activity which was not inhibited by 1 mM elastatinal, which is 10 times higher than needed for complete inhibition of porcine pancreatic elastase. The rPLD preparation degraded PLA with an average molecular mass of 220 kDa into lactic acid dimers through lactic acid oligomers and finally into lactic acid. The PLD preparation bound to high polymers of 3-hydoxybutyrate, ε-caprolacton, and butylene succinate as well as PLA, but it degraded only PLA.

Polylactide, or poly(L-lactic acid) (PLA), is a commercially promising material for use as a renewable and biodegradable plastic. PLA can be synthesized by conventional chemical engineering, and its properties are superior to any of other aliphatic polyesters, for example, higher melting point (170°C), higher glass transition temperature (60°C), and transparency after conversion to a film. Microbial degradation of PLA was implied by the efficient degradation that occurs under composting conditions. Microorganisms able to degrade PLA were described in many reports (1, 9, 17, 19, 28, 33, 34, 36). Pranamuda et al. first isolated PLA-degrading *Amycolatopsis* sp. strain HT (27). Jarerat et al. examined the distribution of PLA degraders among 41 genera (105 strains) of actinomycetes from culture collections and reported that the PLA-degrading strains phylogenetically belong to the *Pseudonocardiaceae* family and related genera, including the genera *Amycolatopsis*, *Lentzea*, *Kibdelosporangium*, *Streptoalloteichus*, and *Saccharothrix* (21). However, PLA-degrading enzymes had not been isolated from the PLA-degrading microbes.

In 2001, we isolated poly(L-lactic acid)-degrading *Amycolatopsis* sp. strain K104-1 and purifying the PLA depolymerase (PLD) to homogeneity from the culture supernatant of the strain (23). The purified enzyme preparation degraded high-molecular-weight PLA in emulsion and in solid film, ultimately forming lactic acid. The PLD preparation also degraded casein and fibrin, but did not hydrolyze collagen type I, triolein, tributyrin, poly(β-hydroxybutyrate), or poly(ε-caprolactone). The PLA-degrading and caseinolytic activities of the enzyme were inhibited by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, but were not significantly affected by trypsin and chymotrypsin inhibitors or *Streptomyces* subtilisin inhibitor. Thus, we concluded that PLD from *Amycolatopsis* sp. strain K104-1 is a unique PLA-degrading serine enzyme (23).

Here, we cloned the PLD gene (*pld*) from the chromosome of *Amycolatopsis* sp. strain K104-1 and determined its nucleotide sequence. We expressed *pld* in *Streptomyces lividans* 1326 carrying plasmid pUPLD with *pld* from *Amycolatopsis* sp. strain K104-1 and obtained a recombinant PLD (rPLD) and mutants for characterization. We also describe the processing

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mechanism by which precursor PLD is converted to mature PLD in *Streptomyces lividans* 1326(pUPLD).

MATERIALS AND METHODS

Bacterial strains and plasmids. *Amycolatopsis* sp. strain K104-1, which was described previously (23), was used as the source of the *pld* gene. *Escherichia coli* DH5α, *E. coli* DH5, and *Streptomyces lividans* strain 1326 were used as host strains for cloning of *pld* and its expression. Plasmids pUC119 (Takara, Kyoto, Japan), Charomid 9-36 (Nippon Gene, Tokyo, Japan), and pUC702 (22), which is a shuttle vector between *E. coli* and *Streptomyces* species, were used as cloning vectors.

Chemicals. PLA with a number-average molecular weight of 220,000 was obtained from Shimadzu Co. (Kyoto, Japan). Poly(3-hydoxybutyrate) (PHB) with an average molecular weight of 1,000,000 and poly(ε-caprolacton) (PCL) with a molecular weights of 40,000 to 70,000, and poly(butylene succinate) (PBS) with an average molecular weight of 300,000 were from Fluka (Buchs, SG, Switzerland), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Showa Highpolymer Corp. Ltd. (Tokyo, Japan), respectively. Chloroform-*d* was from Aldrich Japan Corp. Ltd. (Tokyo, Japan). Porcine pancreatic elastase I, elastatinal, thiostrepton, (3*S*)-*cis*-3,6-dimethyl-1,4-dioxane-2,5-dione, and soybean trypsin inhibitor were from Sigma Chemical (St. Louis, Mo.). Unless otherwise stated, chemicals of analytical grade were used in this study.

Culture media. A mineral medium containing 0.1% (wt/vol) emulsified PLA was used for cultivation of *Amycolatopsis* sp. strain K104-1 as described previously (23). ISP medium 1 (23), supplemented with 34% sucrose and 0.5% glycine was used for DNA preparation from strain K104-1. YEME medium (15) or Bacto tryptic soy broth, each supplemented with 50 μg/ml thiostrepton, was used for growth of *Streptomyces lividans* strain 1326(pUC702) and its derivatives. LB medium was used for *E. coli* growth.

Purification of PLD and its peptide fragments after digestion with lysylendopeptidase, and determination of amino acid sequences of the fragments. The purification of PLD from the culture supernatant of *Amycolatopsis* sp. strain K104-1 was done by the method described previously (23). The purified PLD preparation (220 pmol) was completely digested with 2 μg of lysylendoprotease and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The six separated peptides were blotted onto an Immobilon-P membrane (Millipore Corp., Bedford, MA) by the method described previously (7) and N-terminal amino acid sequences of the blotted protein bands were determined using a model 491 protein sequencer (Applied Biosystems, Foster, CA.).

Preparation of chromosomal DNA from *Amycolatopsis* sp. strain K104-1. Chromosomal DNA was prepared from strain K104-1 by the method described by Pospiech and Neumann (26).

Preparation of oligonucleotide primers for cloning of *pld* from *Amycolatopsis* sp. strain K104-1. According to the N-terminal (Ala^{7}-Thr-Ser-Gly-Pro^{11}) amino acid sequence of intact PLD and its lysylendoprotease fragment, NH_2-Lys-Val-Ala-Thr-Val-COOH (Lys residue is a predicted one), the oligonucleotide primers I,F (5′-GCSACSAGCGGBCCSTGGGC-3′), and II,R (5′-ACSCGSACSGT SGCSACCTT-3′), were designed. In the sequences, B represents C, G, and T and S represents C and G. In combination with both primers, a ca. 380-bp fragment was amplified from the chromosomal DNA of strain K104-1 by PCR using Takara Ex *Taq* polymerase. The amino acid sequence predicted from the DNA sequence in the amplified DNA fragment corresponded to that of the 23-, 13-, and 22-residue segments I^{36}-N^{58}, K^{73}-R^{85}, and M^{97}-T^{118}, respectively, of intact PLD. Therefore, we used this 380-bp PCR fragment as a DNA probe for cloning of *pld* from the strain K104-1 chromosomal DNA.

Cloning of *pld* and its DNA sequencing. The chromosomal DNA of strain K104-1 was digested with SacI and its fragments were isolated by agarose (0.8%) electrophoresis. Southern blot hybridization was done using a fluorescein-labeled 380-bp DNA probe (ECL random prime labeling system; Amarsham Bioscience Corp., Poscataway, NJ.). The 4.5-kbp DNA fragment which hybridized was extracted from the gel and inserted in Charomid 9-36 vector DNA. After transduction into *E. coli* DH5, a recombinant *E. coli* strain containing *pld* was selected by colony hybridization using the labeled 380-bp DNA probe. The plasmid containing *pld* was designated p45SacPLD. DNA sequencing was done with a Thermo Sequenase cycle sequencing kit (USB Corp, Cleveland, OH.) with IRD-41 dye-labeled M13 forward and reverse primers (Aloka, Ltd., Tokyo, Japan) using a model 4000 DNA sequencing system (Li-Cor, Lincoln, Neb.).

ORF identification, homology search, and alignment of multiple nucleotide and amino acid sequences. Protein and nucleotide sequences were compared with those on databases using FASTA (version 3.0) and BLAST (version 1.49) programs implemented at the EMBL/GenBank/DDBJ nucleotide sequence databases and the SWISSPROT/NBRF-PIR protein sequence databases. ORF identification was done by FramePlot analysis (18). Multiple-sequence alignment was done using a GENETYX program (Software Development Co., Tokyo, Japan).

TABLE 1. Substrate specificity of rPLD

Substrate[a]	*p*NA and AMC released from the substrate (mM/min/mg of protein)[b]	
	rPLD	PPE I
1 Suc-Ala-Ala-Ala-*p*NA	34	10
2 Suc-Ala-Pro-Ala-*p*NA	134	123
3 Glt-Ala-Ala-Pro-Leu-*p*NA	0.4	31
4 Suc(OMe)-Ala-Ala-Pro-Val-MCA	1.9	0.74
5 Bz-Arg-*p*NA	0	NT
6 Bz-Tyr-*p*NA	0	NT

[a] Substrates 1 to 4 are for elastase and substrates 5 and 6 are for trypsin and chemotrypsin, respectively.
[b] NT, not tested. PPE I, porcine pancreatic elastase I.

Construction of plasmid pUPLD for PLD expression in *S. lividans* strain 1326. For preparation of rPLD, a DNA fragment containing the *pld* structural gene and its predicted promoter region was amplified from p45SacPLD by PCR. We designed primers which combined HindIII site (underlining) in the forward primer 1, (5′-GCGAAGCTTAACGCCGACAAGCCCTGAGACAT-3′) and XbaI site (underlining) in reverse primer 2, (5′-GACTCTAGAATTTCGACG TCCTGACCGCAAAAA-3′) upstream and downstream of *pld*, respectively. Using primers 1 and 2, a fragment of about 1.2-kbp was amplified. The 1.2-kbp fragment was digested by HindIII and XbaI, and inserted in pUC702 (22) to construct plasmid pUPLD. Plasmid pUPLD was introduced into a protoplast of *S. lividans* 1326 which was prepared by the method of Hopwood et al. (15). Thiostrepton resistant transformants of *S. lividans* 1326 were selected and confirmed the presence of pUPLD by the method of Chater et al. (6). The transformant, *S. lividans* 1326(pUPLD) thus obtained was incubated in YEME medium containing 50 μg/ml thiostrepton at 30°C for 5 days. The culture supernatant was used for purification of rPLD.

Site-directed mutagenesis of *pld*. Site-directed mutagenesis was done by overlapping extension method (14, 16). The mutation of each mutant was confirmed by DNA sequencing.

Purification of rPLD and its mutants. The purification procedures of the rPLD and its mutants were essentially same to for intact PLD (23). The molecular masses of the intact rPLD and its mutants were measured by TSK gel G3000SW gel filtration column (Tosoh; diameter, 0.75 cm; height, 30 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.0) containing 200 mM NaCl, using albumin (64.7 kDa), ovalbumin (45.8 kDa), chymotrypsinogen (19.9 kDa), and RNase A (15.4 kDa) as molecular standards.

Detection of PLD precursor and mature PLD, and their caseinolytic activities. To detect PLD precursor in culture supernatant of *S. lividans* 1326(pUPLD), 20 μl of culture supernatants were obtained from 3-, 5-, 7-days cultures after removing the cells. Each sample was mixed with a same volume of 100 mM Tris-HCl buffer (pH 8.0) containing 2% SDS and 0.5% mercaptoethanol, and boiled for 10 min. The samples were then subjected to SDS-PAGE. Zymography to detected protease was done according to the method described by Rice et al. (29).

PLD and protease activities of the purified rPLD preparation. PLD assay was done according to the method described previously (23). L-Lactic acid was measured using L-lactic acid dehydrogenase. The reaction mixture was also analyzed by a silica gel chromatography using silica gel plate (no. 1.05715.0009; Merck, Darmstadt, Germany) for detection of degradation products by spraying with 5% phosphomolybdate and heating at 120°C for 1 h. Proteolytic activity of the rPLD preparation was assayed using synthetic oligopeptides, listed in Table 1, as substrates. One ml of the reaction mixture contained 2 pmol of enzyme and 250 μM substrate in 20 mM Tris-HCl buffer (pH 8.0). The reaction mixture was incubated at 25°C for 5 min. The amount of *p*-nitroaniline released was measured at 410 nm. With methoxysuccinyl [Suc(OMe)]-Ala-Ala-Pro-Val-MCA [MCA is (7-methoxycoumarin-4-yl) acetyl] as a substrate, the 7-amino-4-methyl-coumarin (AMC) concentration was monitored fluorometrically at λ_{ex} = 380 nm and λ_{em} = 460 nm, using a Hitachi fluorescence spectrophotometer F-2500 (Tokyo, Japan).

Kinetic study. Kinetic parameters, k_{cat} and K_m were calculated from initial rate for release of *p*-nitroaniline from the *p*-nitroanilide substrates, by fitting to the Michaelis-Menten equation using a nonlinear regression algorithm. The range of substrate concentrations was 0.5 to 20 mM.

Binding study. PHB, PCL, PBS, and PLA as adsorbents were emulsified to prepare 0.5% (wt/vol) of solution. The rPLD preparation (2 μg) was incubated in 1 ml of 50 mM Tris-HCl (pH 8.0) containing each 5 mg of polymer granules for 10 min at room temperature. The granules were collected by centrifugation and washed twice with 50 mM Tris-HCl buffer (pH 8.0). The adsorbed rPLD was eluted from the granules with 1% SDS and analyzed by SDS-PAGE.

Identification of L-lactic acid oligomers. Identification of lactic acid dimer isolated from the enzymatic degradation products of PLA was done by ^{1}H nuclear magnetic resonance and mass spectrometer. Emulsified PLA of 220 kDa (0.1%, wt/vol) was incubated with 1 μg of the purified rPLD preparation in 2 ml of 20 mM Tris-HCl buffer (pH 8.0) at 37°C. Two hundred μl of the reaction mixture was withdrawn at time intervals and centrifuged. Supernatant and precipitate fractions were put onto the silica gel plate and developed twice by a solvent system of ethylacetate:toluene:water:formic acid (2:3:1.2:0.9 [vol/vol]). Lithium lactate and (3*S*)-*cis*-3,6-dimethyl-1,4-dioxane-2,5-dione solutions were used as the standards for lactic acid and lactyl lactate (lactic acid dimer), respectively. The band corresponding to the lactic acid dimer was eluted from the silica gel plate with chloroform. The sample was dried, dissolved in chloroform-*d*, and analyzed by a Varian Inova 500 nuclear magnetic resonance spectrophotometer at 500 MHz.

Other analytical procedures. Protein was assayed by the method of Bradford (5) using bovine serum albumin as the standard. SDS-PAGE was done as described previously (23). Circular dichroism analysis of PLD was done with a Jasco J-720 spectropolarimeter at room temperature in a 1-mm path length cell containing 5 μM enzyme in 5 mM potassium phosphate buffer (pH 6.5).

Nucleotide sequence accession number. The nucleotide sequence of *pld* has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB126228.

RESULTS

Cloning and nucleotide sequence of the *pld* gene of *Amycolatopsis* sp. strain K104-1. The screening of a SacI library from the chromosomal DNA of *Amycolatopsis* sp. strain K104-1 gave seven clones positive for *pld* from approximately 10,000 colonies. A positive clone contained an insert of 4,446 bp whose sequence included an open reading frame (ORF) which encoded a 238-amino-acid protein of 24,224 Da, whose N-terminal sequence matched that determined for the purified PLD preparation and its fragments; later functional testing (described below) verified the assignment of this ORF as *pld*. *pld*, which started with a GTG codon and ended with a TGA stop codon, consisted of 714 nucleotides, encoding a protein of 238 amino acids with a predicted molecular mass of 24,224 Da. Putative −35 (CGGACA) and −10 (GACAAT) sequences (32) were found 86 and 65 bp upstream of the translation initiation codon of *pld*, respectively. A potential Shine-Dalgarno sequence (AAGGAG) was present 6 bp upstream of the gene. An inverted repeat sequence consisting of a stem-loop with a 14-bp arm was found downstream of the TGA stop codon. The nucleotide sequence corresponding to a signal peptide (13), consisting of 26 residues (M^1KFGKFVLLAASTA LAVVGLGGPAAA^{26}), was present at the N-terminal end of the ORF. The percentage of G+C content in the *pld* gene was high (69.5%), which is reflected in the preferential use of codons with G or C in the third position as is common in actinomycetes.

Functional analysis of the *pld* gene products. To prove that *pld* is the gene encoding PLD from *Amycolatopsis* sp. strain K104-1, the gene was expressed in *Streptomyces lividans* 1326. rPLD was purified to homogeneity with about 15% yield using chromatography on CM-Toyopearl 650 M, TSKgel CM-5PW, and TSKgel Phenyl-5PW from *S. lividans* 1326(pUPLD) supernatant. The purified rPLD preparation had characteristics identical to those of the earlier *Amycolatopsis* sp. strain K104-1 PLD preparation (23). Hence, we concluded that the ORF is the *pld* gene of *Amycolatopsis* sp. strain K104-1. The correct processing of premature to mature PLD occurs in *S. lividans* 1326.

Amino acid sequence homologies of PLD with eukaryotic serine proteases. Comparison of the amino acid sequence of PLD and other serine proteases from prokaryotes and eukaryotes revealed that PLD exhibits 67% identity and 74% similarity to a putative hydrolase from *Streptomyces coelicolor* A3(2) (2), 26% identity and 41% similarity to prepro-protein for kallilrein from *Mastomys natalensis* (African soft-furred rat) (8), 29% identity and 45% similarity to chymotrypsin B1 from *Litopenaeus vannamei* (Pacific white shrimp) (31), 30% identity and 45% similarity to trypsin-like precursor from *Culex pipiens quinquefasciatus* (southern house mosquito) (12), and 29% identity and 44% similarity to brachyurin from atrantic san fiddler crab (10).

In addition, absolute conservation of amino acid residues implicated in catalytic domains was found in PLD and all chymotrypsins as follows. (i) Three amino acid residues, H^{57}, D^{102}, and S^{195} (chymotrypsin numbering), of the catalytic triad are known to be essential for peptide bond cleavage in eukaryotic chymotrypsins. They are located between two β-barrel domains of the enzyme, and their arrangement is conservative in chymotrypsin family (24). These three residues were also conserved in *Amycolatopsis* sp. strain K104-1 PLD as residues H^{74}, D^{111}, and S^{197}. (ii) The S^{214} residue (chymotrypsin numbering) is hydrogen bonded to D^{102} and the nitrogen atom of the scissile bond in the substrate, and this residue contributes significantly to the polar environment for stabilizing the charge of the buried D^{102} residue. This residue was also conserved in PLD as S^{212}. (iii) The G^{193} residue (chymotrypsin numbering) forms an oxyanion hole with the S^{195} residue and forms a hydrogen bond for interaction with the carbonyl group of the scissile peptide bond. It too was conserved in PLD as G^{195}. Thus, we concluded that PLD belongs to the chymotrypsin family of serine proteases, which includes chymotrypsin, trypsin, and elastase.

Characteristics of PLD mutants. Three mutants, H74A, D111A, and S197A, of *Amycolatopsis* sp. strain K104-1 PLD were created, expressed in *S. lividans*, and purified from the culture media after their full growth. The semifinal step of purification of the H74A mutant using CM-5PW column chromatography gave two protein peaks, I and II, both which were cross-reacted with anti-PLD antiserum. N-terminal amino acid sequences of peaks I and II were D^1STP^4 and I^1IGGS^5, respectively, indicating that peaks I and II are pro- and mature types, respectively (Fig. 1A). No peak I was detected in the wild type. The purified peak II had about 1/20,000 protease activity, compared to that of the wild-type one, while the peak I preparation had no detectable caseinolytic activity. When the purified peak I protein itself was incubated in 50 mM Tris-HCl buffer (pH 8.0) at 37°C for 24 h or 36 h and analyzed by SDS-PAGE, it was found that peak I protein, which corresponds to band I protein in panel B, was converted to the mature type with the N-terminal sequence of I^1IGGS^5 at 36 h of incubation (band II in panel B). We obtained the same

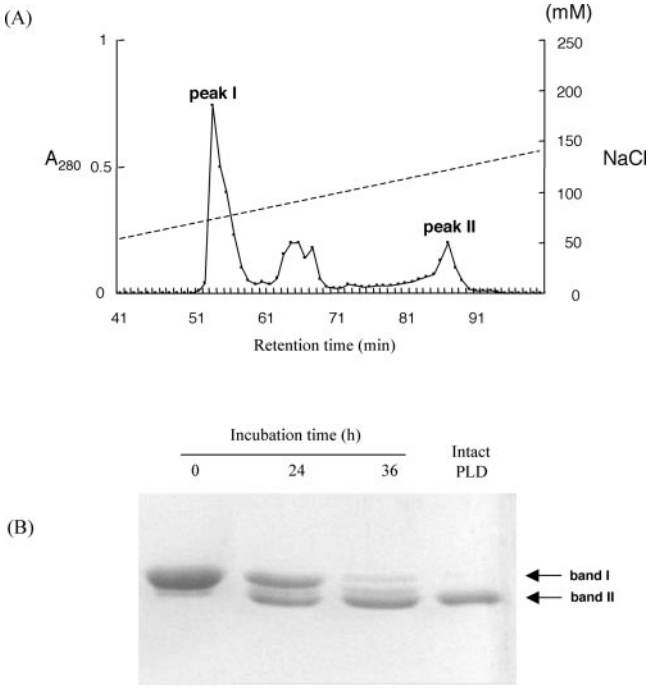

FIG. 1. Elution profile of the PLD mutant H74A from the CM-5PW cation-exchange column (A), and cleavage of prosequence from the pro-type of the mutant PLD (B). Panel A: Two liters of culture supernatant from *S. lividans* 1326 carrying a plasmid pUPLD-H74A, which expressed mutant PLD, H74A, was added 30 ml of a CM Toyopearl 650 M resins (Tosoh Corporation, Tokyo Japan) and mixed for 1 h at 4°C. The resins were washed with 20 mM potassium phosphate buffer (pH 6.0). Adsorbed proteins were eluted from the resins with 20 mM potassium phosphate buffer (pH 6.0) containing 250 mM NaCl, dialyzed against 20 mM potassium phosphate buffer (pH 6.0), and put onto a Tosoh TSK gel CM-5PW column (diameter, 0.75 cm; height, 7.5 cm) equilibrated with the same buffer. A desorbed proteins were eluted with a linear gradient of NaCl (0 to 250 mM). The other PLD mutants, D111A and S197A gave similar protein profiles as that in H74A on CM-5PW column. Panel B: The purified peak I preparation of H74A (60 μg) was incubated at 37°C in 300 μl of 50 mM Tris-HCl buffer (pH 8.0) at 37°C for 24 h and 36 h. The samples were analyzed by SDS-PAGE.

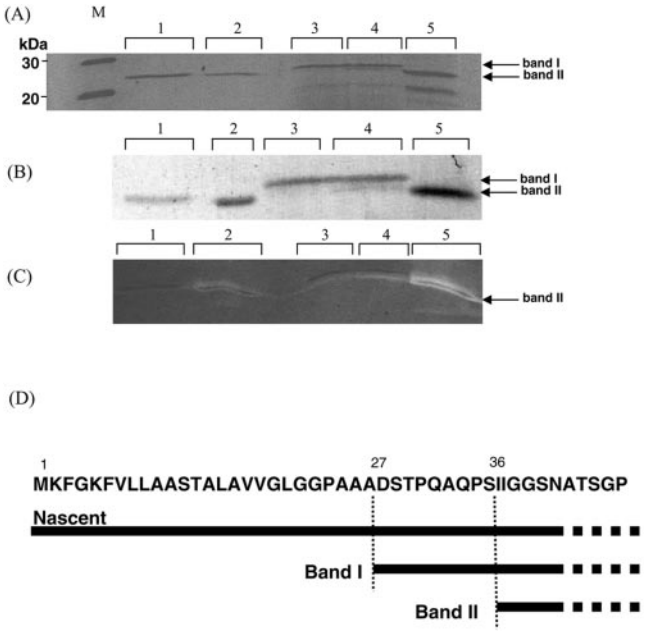

FIG. 2. SDS-PAGE (A), Western blotting (B), and zymography (C) of mature PLD and its precursor forms in culture supernatants from 3-, 5-, and 7-day cultures of *S. lividans* 1326(pUPLD), and N-terminal sequences of nascent PLD, and bands I and II preparations (D). Lanes 1 and 2 represent boiled and intact PLD preparations, respectively, from *Amycolatopsis* sp. strain K104-1. Lanes 3, 4, and 5 represent samples from 3-, 5-, and 7-day cultures. Lane M represents carbonic anhydrase (30.0 kDa) and trypsin inhibitor (20.1 kDa) as molecular size markers. SDS-PAGE, Western blotting, and zymographical analyses were done according to the methods described in Materials and Methods. The N-terminal amino acid sequencing of the mature rPLD and its precursor bands on the gel was done as described in the text. Western immunoblot analysis for detection of rPLD and its precursor was done using anti-rPLD antibodies as the primary antibody and alkaline phosphate-conjugated anti-mouse (heavy and light chain) immunoglobulin G (Promega Corp., Madison, WI) as the second antibody.

results as in Fig. 1A and B with D111A and S197A. Hence, the H^{74}, D^{111}, and S^{197} residues in PLD are important for both protease and PLD activities and for processing of the prosequence of pro-type PLD. The molecular masses of native forms of the mature type of the three mutants as well as wild-type PLD were 19 kDa as determined by gel filtration. Thus, we conclude that PLD functions as a monomer. The far-UV circular dichroism spectra of the three mutants were similar to that of wild-type PLD (data not shown), suggesting that no major conformational alterations occurred in the mutant enzymes.

Processing mechanism of premature to mature PLD. Proteins in the culture fluids after 3-, 5-, and 7-day cultures of *S. lividans*(pUPLD) were analyzed by SDS-PAGE (Fig. 2). In a sample of the 3-day culture, a major protein band of about 25 kDa (band I in lane 3 of panel A) was strongly stained with both Coomassie brilliant blue and anti-PLD antibodies (lane 3 of panels A and B) but lacked detectable caseinolytic activity (lane 3 of panel C). In the sample of the 5-day culture, band II, whose molecular mass coincides with that of the intact PLD preparation in SDS-PAGE, was stained by anti-PLD antibodies (lane 4 in panel B) and exhibited caseinolytic activity (lane 4 in panels A, B, and C). In the 7-day culture, band I disappeared completely and band II became major with strong caseinolytic activity (lane 5 in panels A, B, and C).

The N-terminal amino acid sequence of band I was determined to be D^{1}STPQAQPSIIGG^{13}, which corresponds to the 27th to 39th amino acid residues from the first Met residue of the nascent PLD, while the N-terminal sequence of band II was I^{1}IGGSNA^{7} which is identical with that of the intact PLD preparation (23). As band I area on the SDS gel was cut and soaked for 4 h on ice in 50 mM Tris-HCl buffer (pH 8.0) containing 2.5% (vol/vol) Triton X-100, and then subjected to Western blot analysis using anti-PLD antiserum after SDS-PAGE, band I was converted to band II. Taken together with the aforementioned data for the three mutants of PLD, it was indicated that cleavage of the pro-sequence of pro-type PLD is autocatalytic. Hence, it is concluded that nascent PLD (pre-pro-type) is first processed by signal peptidase in the cell membrane between A^{26} and D^{27} to form pro-type precursor (band I) with undetectable caseinolytic activity and is secreted out of

TABLE 2. Effect of inhibitors on peptidase activity of rPLD and pancreatic elastase[a]

Inhibitor	Concentration	Residual activity (%)[b]	
		rPLD	PPE I
Diisopropyl fluorophosphate	1 mM	0	0
	10 mM	0	0
Phenylmethyl sulfonyl fluoride	1 mM	94	114
	10 mM	19	90
Elastatinal	0.1 mM	114	0
	1 mM	106	0
Trypsin inhibitor	5 μg/ml	98	NT
Chymostatin	0.15 μg/ml	93	NT

[a] The reaction mixture contained 250 μM Suc-Ala-Ala-Ala-*p*NA and 2 pmol of either rPLD preparation or pancreatic elastase, in 1 ml of 20 mM Tris-HCl buffer (pH 8.0) with or without inhibitors. The reaction was done at 25C°C for 10 min. *p*NA released from the substrate was measured at 410 nm. The activity without inhibitor was taken as 100%.

[b] PPE 1, porcine pancreatic elastase 1; NT, not tested.

the cells, and then processed by autocatalysis between S^{35} and I^{36} to form the mature PLD with both depolymerase activities towards PLA and protein (Fig. 2D). The calculated molecular mass (20,904 Da) of the predicted mature PLD consisting of 203 amino acid residues coincided with the accurate molecular mass of the intact PLD preparation (20,904 Da) which was measured by a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer.

Substrate preference and effect of various inhibitors of the purified enzyme. Previously, we reported that PLD degrades casein and fibrin as well as PLA, and the degrading activities are inhibited by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride only at very high concentration but not by specific inhibitors for trypsin-type, chymotrypsin-type and subtilisin-type proteases (23). In this study, the substrate specificity of the rPLD preparation was examined using several synthetic substrates in Table 1. The rPLD preparation released *p*-nitroaniline (*p*NA) from all synthetic substrates for elastase, but not from *N*-benzoyl (Bz)-L-Arg-*p*NA and Bz-Tyr-*p*NA, which are substrates for trypsin and chymotrypsin, respectively (Table 1).

The data showed that the S1 subsite of PLD is rather specific for P1 = Ala. The rPLD preparation also digested bovine elastin. In contrast, porcine pancreatic elastase I did not degrade PLA. The *p*NA-releasing activity of the rPLD preparation from Suc-Ala-Ala-Ala-*p*NA was not inhibited by 1.0 mM elastatinal, which is 10 times higher than the concentration needed for complete inhibition of porcine pancreatic elastase I (Table 2). The kinetic parameters for *p*NA-releasing activity from the substrates Suc-Ala-*p*NA, Suc-Ala-Ala-*p*NA, and Suc-Ala-Ala-Ala-*p*NA by the rPLD preparation at concentrations ranging from 0.5 to 20 m, were determined and the results are listed in Table 3. When the catalytic efficiency of the enzyme was defined as the k_{cat}/K_m ratio obtained with the three substrates, the rPLD preparation had about 1,570 times higher efficiency towards Suc-Ala-Ala-Ala-pNA than Suc-Ala-pNA (Table 3).

Mode of action of rPLD on PLA. Treatment of PLA (220,000Da) emulsion with the purified rPLD preparation at 37°C for 3 h decreased turbidity of the emulsion. The reaction

TABLE 3. Effect of peptide length on *p*NA-releasing activity from the substrates in the rPLD preparation[a]

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m	Catalytic efficiency
Suc-Ala-*p*NA	0.2	17.3	13	1.0
Suc-Ala-Ala-*p*NA	1.2	10.5	110	8.46
Suc-Ala-Ala-Ala-*p*NA	97.7	5.2	19,000	1,461

[a] Reaction mixture contained 0.5 to 20 mM substrate, 2 pmol of the purified enzyme, and 20 mM Tris-HCl (pH 8.0) in a total volume of 1 ml. The reaction was done at 25°C for 5 min. *p*NA released from each substrate was measured at 410 nm.

mixtures after 3- and 6-h incubations were analyzed for degradation products by silica gel thin-layer chromatography (Fig. 3). As the incubation mixtures after a 3-h incubation was still cloudy, it was separated into supernatant and pellet fractions by centrifugation and both fractions were subjected to silica gel chromatography. Substances I and II and III and residual PLA were observed in supernatant and pellet fractions, respectively. On the other hand, after a 6-h incubation, the incubation mixture became clear. The PLA spot disappeared and substances I, II, and III were detected.

Substances II were eluted from the plate with chloroform and subjected to nuclear magnetic resonance and mass spectrometric analyses. Both ^{1}H-nuclear magnetic resonance and mass spectra of substance II were identical to those of authentic L-lactic acid dimer. In ^{1}H-nuclear magnetic resonance, the chemical shifts of substance II were 1.5 (3H, de, J = 7.0 Hz, H-3′), 1.59 (3H, d, J = 7.0 Hz, H-3), 4.37 (1H, q, J = 7.0 Hz, H-2′), and 5.24 (1H, q, J = 7.0 Hz, H-2). The molecular mass of substance II was determined to be 162 Da by mass spec-

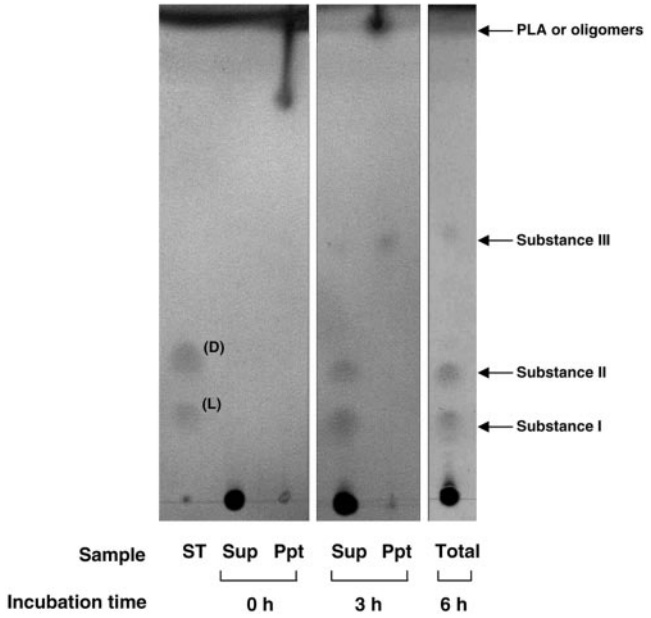

FIG. 3. Thin-layer chromatogram of the degradation products from PLA of 220 kDa by the purified rPLD preparation. Enzyme reaction and detection of the degradation products were carried out as described in Materials and Methods. ST: mixture of authentic lactic acid (L) and lactic acid dimer (D). The black spot at the origin of each supernatant sample on the plate and 6-h incubation sample was Trisma base in the incubation mixtures.

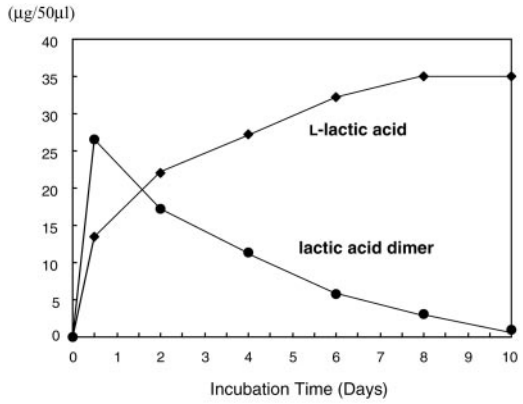

FIG. 4. Time course of the degradation of lactic acid dimer into lactic acid monomer. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 0.1% PLA emulsion, and 5 μg of the purified rPLD preparation in a total volume of 2 ml. The reaction mixture was incubated at 37°C. At indicated time intervals, 50 μl of the reaction mixture was withdrawn and centrifuged to remove insoluble PLA, if necessary, and loaded onto a Shiseido Capcelpack C_{18} AQ (Tokyo, Japan) (4.6 mm inner diameter by 250 mm) which was equilibrated with 50 mM ammonium phosphate buffer (pH 2.4). Eluate was monitored at 210 nm. Lactic acid and lactic acid dimer fractions were pooled and quantitatively analyzed using L-lactic dehydrogenase as described in Materials and Methods. The lactic acid dimer fraction was hydrolyzed by 1 N NaOH at 100°C for 5 h and the hydrolysate was assayed for L-lactic acid. Solid circles and diamonds represent lactic acid and lactic acid dimer, respectively.

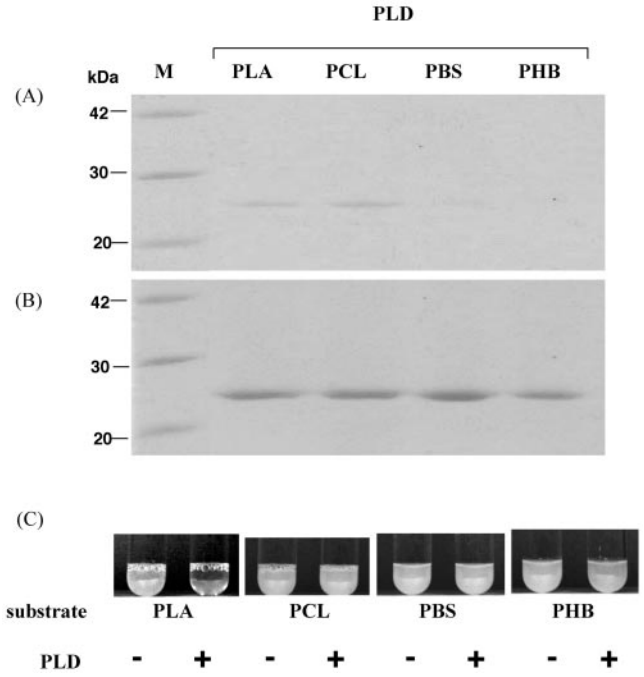

FIG. 5. Adsorption (panels A and B) and degradation (panel C) activities of rPLD preparation to PLA, PCL, PBS, and PHB. The purified rPLD preparation was incubated with biodegradable plastic granules under the conditions described in Materials and Methods. After centrifugation, supernatant (panel A) and plastic granules were separated. The adsorbed enzyme on the granules (panel B) was eluted as described in Materials and Methods. Both fractions were analyzed by SDS-PAGE. Proteins on the gel was stained with Coomassie brilliant blue R-250. Lane M represents aldolase from rabbit muscle (42.4 kDa), carbonic anhydrase (30.0 kDa), and trypsin inhibitor (20.1 kDa) as molecular size markers. For degradation of each biodegradable plastic by rPLD, the incubation mixture (2 ml) contained 2 μg PLD, each substrate (0.1% [wt/vol]), and 50 mM Tris-HCl (pH 8.0). The degradation activity of the rPLD preparation for PHB, PCL, and PBS (panel C) was measured by decrease of turbidity of the reaction mixture with and without rPLD after 5 h of incubation at 37°C.

trometer. Thus, it was concluded that substances II is lactic acid dimmer. The substance I was eluted with distilled water and identified to be L-lactic acid using L-lactic acid dehydrogenase. We failed to identify substance III.

Figure 4 shows fluctuation of the amounts of L-lactic acid and lactic acid dimer in 50 μl of reaction mixture which contains 50 μg of emulsified PLA and 5 μg of the purified rPLD preparation in a long-term incubation at 37°C. The reaction mixture at various times was analyzed for lactic acid and its dimer by high-pressure liquid chromatography with octyldecylsilane (ODS) column. In the ODS column used in this study, the recoveries of L-lactic acid and its dimer were 80% and 81%, respectively. As shown in this figure, the accumulation of lactic acid dimer reached approximately 27 μg/50 μl of the reaction mixture at 12 h-incubation. Thereafter, the amount of lactic acid dimer decreased between 0.5 and 10 days, accompanying with an increase of L-lactic acid. After 10-days of incubation, no more than 1 μg of lactic acid dimer was detected in the reaction mixture. On the other hand, accumulation of L-lactic acid in the reaction mixture proceeded up to 6 days and reached approximately 35 μg/50 μl of the reaction mixture. Taken together with the data in Fig. 3, it was strongly suggested that PLD starts to attack within the PLA polymer of an average molecular mass of 220 kDa and subsequent release of shorter esters including dimer and unidentified oligomer (substance III), and the reaction culminates in the formation of lactic acid from lactic acid dimer.

Thus, we concluded that the PLD preparation is capable of degrading PLA of an average molecular mass of 220 kDa into lactic acid oligomers and L-lactic acid dimer, and finally L-lactic acid.

Binding of rPLD preparation to biodegradable plastics. The rPLD preparation showed binding to PHB with an average molecular weight of 1,000,000, PCL with a molecular weights of 40,000 to 70,000, and PBS as well as PLA (Fig. 5A and B). Treatment of PLA, PHB, PCL, and PBP emulsions with the purified rPLD preparation at 37°C for 5 h decreased no turbidity of the emulsions except PLA (Fig. 5, panel C). Proteinase K, which is known to be a PLA-degrading protease, showed less than 1/10 binding activity to them compared with that of rPLD preparation (data not shown). As negative control, bovine serum albumin, and RNase A were used for their binding to PHB, PLC, PBS, or PLA. Neither bovine serum albumin, nor RNase I binds to the four biodegradable plastics (data not shown). Thus, we conclude that the rPLD preparation has binding activity for four bioplastics but can degrade only PLA.

DISCUSSION

In this study, we cloned *pld* from *Amycolatopsis* sp. strain K-104-1 and expressed it in *S. lividans* strain 1326. The molecular mass of PLD estimated by SDS-PAGE (23) was approx-

imately 3.0 kDa higher than the value measured using the MALDI-TOF-mass spectrometer. The difference might be due to the high pI (≥10) of PLD. Proteinase K of 28 kDa with a high isoelectric point also shows apparent molecular mass of 30 kDa on SDS-PAGE.

Expression studies of *pld* clearly showed that the prepro-type of PLD is processed by typical prokaryotic signal peptidase 1 to form the pro-type one (nonactive type), and further processed into the mature (active type) PLD. The presence of the pro-type of rPLD in culture broth of *S. livisans*1326(pUPLD) indicates that the same processing mechanism of a signal sequence (M^1 to A^{26} residues) and prosequence ($D^{27}STPQAQPS^{35}$) is present in both *Streptomyces* species and *Amycolatopsis* species. From the conversion experiment of the isolated pro-type PLD mutant to the mature one, the processing of the prosequence from pro-PLD was found to be autocatalytic. It is different from other chymotrypsin family members because the prosequence of trypsinogen (pro-type of trypsin) is first processed by enteropeptidase and mature trypsin is responsible for the activation of the proenzymes of all the other digestible enzymes such as chymotrypsin, elastase, and trypsin itself in animals.

PLD has homology with many eukaryotic serine proteases of the chymotrypsin family. All peptidases in this family are endopeptidase, and have a catalytic triad consisting of His, Asp, and Ser residues in this order in the sequence and their proteolytic activity is inhibited by DFP. Elastases, which are also classified in the chymotorypsin family, are defined by their ability to release soluble peptides from insoluble elastin fibers by a proteolytic process. Deliberating on our results, it is considered that PLD belongs in the chymotrypsin family and is a kind of elastase-like protease. Because the aforementioned PLD mutants lose both protease activity and PLA depolymerase activity, PLD probably depolymerizes protein and PLA with the same mechanism as that of the chymotrypsin family.

The depolymerization pathway might begin with the initial reversible formation of an adsorption (Michaelis) complex between the enzyme and its substrate, a nucleophilic attack by the γ-oxygen of the Ser195 residue (chymotrypsin numbering) on the peptide bond or ester bond, the subsequent formation of an acyl enzyme intermediate, and the release of the first product, the C-terminal part of the substrate, and culminating in hydrolysis of the covalent intermediate in the deacylation step, which regenerates active enzyme and releases the carboxyl group of the second product. It is known that such acylation and deacylation steps need the charge relay among three amino acid residues, His, Asp and Ser. Replacement of either His, Asp, or Ser with Ala results in a protease which is 1/20,000 as active as the wild-type enzyme.

The residual activity in H74A, D111A, and S197A might be due to other structural features in the protease which help to stabilize the tetrahedral intermediate, as shown by S. Halfon and C. S. Craik (11). Based on the findings of tertiary structure of several proteases including *Fusarium oxysporum* trypsin (30), porcine pancreatic elastase (35), and fiddler crab collagenase (25), PLD might consist of two β-barrel domains and its active site might be located in the cleft between two β-barrel domains as in other enzymes belonging to chymotrypsin family. However, the following dissimilarities were evident between PLD and elastase. (i) Residue V^{216} in the S1 site of porcine pancreatic elastase 1 (chymotrypsin numbering), which has been known to hinder the binding of bulky P1 residues with T^{226}, resulting in substrate specificity for nonbulky residues such as Ala, Ser, Gly, and Val in P1, was replaced by S^{216} in PLD. Pancreatic elastase II in pig, rat, and bovine pancreas has Gly^{216} and Ser^{226} residues (chymotrypsin numbering) in the S1 site, which gives them the ability to hydrolyze substrates with bulky P1 residues, such as Leu, Met, Phe, or Tyr residues. (ii) Residues C^{191} and C^{220} (chymotrypsin numbering) are known to form disulfide linkage bonds for the structure of the P1 pocket. This disulfide bridges the two walls of the S1 site and likely provides a degree of structural rigidity to the cavity (24). These two residues are absent in PLD. (iii) It is known that porcine pancreatic elastase I has E^{70} and E^{80} residues (chymotrypsin numbering) which consist of calcium-binding site. But these residues are absent in PLD. Thus, the structure of the PLD of strain K104-1 might be different from that of elastase or other serine proteases, resulting in different substrate specificity.

It has been known that the catalytic activity of porcine pancreatic elastase 1 increases considerably with the chain length of the synthetic substrates. For example *k*cat/km value of porcine pancreatic elastase I for the hydrolysis of Suc-$(Ala)_n$-*p*NA is 4, 12,000, and 170,000 M^{-1} s^{-1}, respectively, for the series $n = 2$, 3, and 4 (3). Occupancy of subsite S4 thus appears to be of critical importance for efficient catalysis. The sensitivity of porcine pancreatic elastase 1 to peptide chain elongation is much more pronounced than that of any other related proteinase. PLD also have same sensitivity (Table 3). This sensitivity might be explained by secondary enzyme-substrate interactions (i.e., by interactions remote from the S1-P1 binding) as explained in porcine pancreatic elastase 1. For instance, occupancy of subsite S4 by a succinyl group very strongly increases the catalytic rate constant *k*cat (4). The increase in *k*cat in PLD may result either from an indirect, P4-induced tightening of the S1-P1 binding, with resultant optimal closeness of the scissile bond of the substrate to the serine residue of the catalytic triad, or from a P4-induced enhancement of the nucleophilicity of γ-oxygen of Ser^{197} residue of the catalytic site in PLD.

In this paper, we showed that PLD degrade PLA of an average molecular mass of 220 kDa and lactic acid dimers temporarily converges through lactic acid oligomers and finally into lactic acid. The data suggest that occupancy of subsite S2 is important to degrade PLA of high molecular weight.

PHB depolymerases are well studied. Most PHB depolymerases have a substrate binding domain consisting of 40 to 60 amino acids in the C-terminal end and bind to PHB granules (20). PLD also has an ability to bind to PHB. However, a similar substrate binding domain for PHB could not be found in our PLD preparation. Therefore, it is expected that PLD binding to PHB is due to hydrophobic interactions between PLD and the substrate.

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