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The *Escherichia coli pldC* Gene Encoding Lysophospholipase L_1 Is Identical to the *apeA* and *tesA* Genes Encoding Protease I and Thioesterase I, Respectively[1]

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We deduced the amino acid sequence of *Escherichia coli* lysophospholipase L_1 by determining the nucleotide sequence of the *pldC* gene encoding this enzyme. The translated protein was found to contain 208 amino acid residues with a hydrophobic leader sequence of 26 amino acid residues. The molecular weight of the purified enzyme (20,500) was in good agreement with the predicted size (20,399) of the processed protein. A search involving a data bank showed that the nucleotide sequence of the *pldC* gene was identical to those of the *apeA* and *tesA* genes encoding protease I and thioesterase I, respectively. Consistent with the identity of the *pldC* gene with these two genes, the enzyme purified from *E. coli* overexpressing the *pldC* gene showed both protease I and thioesterase I activities.

Key words: *ApeA* gene, *Escherichia coli*, lysophospholipase L_1, *PldC* gene, *TesA* gene.

Two kinds of lysophospholipases termed lysophospholipase L_1 and lysophospholipase L_2 are found in *Escherichia coli*. Each enzyme preferentially hydrolyzes an acyl ester bond of 1-acyl or 2-acyl lysophospholipids, respectively. Lysophospholipase L_1 and L_2 activities were found to be mainly distributed in the soluble fraction and inner membrane, respectively (*1*). Gene cloning of the *pldB* gene encoding lysophospholipase L_2 showed that lysophospholipase L_2 consisted of 340 amino acids with a molecular weight of 38,934 (*2, 3*). On the other hand, the *pldC* gene encoding lysophospholipase L_1 was isolated by screening Clarke and Carbon's collection (*4*) based on the increase in enzyme activity (*5*). Moreover, lysophospholipase L_1 was purified from the strain overproducing lysophospholipase L_1 and the apparent molecular weight of the purified lysophospholipase L_1 was estimated to be 20,500 by SDS-PAGE (*5*).

To clarify the relationship between lysophospholipases L_1 and L_2, we sequenced the *pldC* gene and showed that it was identical to the *apeA* and *tesA* genes which were found in the data base. The *apeA* gene was characterized as the gene encoding protease I in *E. coli* (*6, 7*) and *Salmonella typhimurium* (*8*). In addition, its molecular cloning, sequencing and mapping have been described by Ichihara *et al.* (*9*). Protease I activity was assayed using a synthetic substrate of chymotrypsin including *N*-acetyl phenylalanine naphthyl ester (APNE) and *N*-benzyloxycarbonyl phenylalanine *p*-nitrophenyl ester (Cbz-Phe-ONp), although this enzyme did not appear to contribute to protein degradation (*10*). On the other hand, the *tesA* gene was a structural gene responsible for *E. coli* thioesterase I activity (*11, 12*). Thioesterase I was assayed as the ability to hydrolyze a thioester of fatty acyl-coenzyme A (CoA). The *tesA* gene cloned by Cho and Cronan had the same DNA sequence and genomic location as the *apeA* gene (*12*). In addition, both protease I and thioesterase I activities were found to utilize the same active site through substrate competition assays (*13*). In this study, we showed not only that the *pldC* gene has the same DNA sequence as the *tesA*/*apeA* genes but also that the purified lysophospholipase L_1 has protease I/thioesterase I activities.

MATERIALS AND METHODS

DNA Techniques—Plasmid pC124 was constructed by subcloning a 5.5-kbp DNA fragment containing the *pldC* gene into the vector DNA, pUC8 (*5*). In order to generate a series of deletion mutants, pC124 was digested with restriction endonucleases, *Eco*RI and *Hind*III, and then the 5.5-kbp DNA fragment was subcloned into the vector DNA, pBluescript II sk(−), which was digested with the same restriction endonucleases. A series of deletion clones were obtained by deleting the plasmid unidirectionally away from the *Eco*RI into the insert sequence by treatment with exonuclease III and mung bean nuclease for varying lengths of time (Fig. 1). The DNA sequence was determined by the dideoxychain-termination method using a DNA sequencing system, Model 373S (Perkin Elmer).

Purification of Lysophospholipase L_1—Lysophospholipase L_1 was purified from the soluble fraction of the overproducing strain, KL16-99, carrying pC 124-85, by the method described in our previous paper (*5*). The homogeneity of the purified lysophospholipase L_1 was judged by SDS-PAGE and CBB-staining.

Lysophospholipase L_1 Assay—The purified enzyme (10 μl) was mixed with 10 μl of 3 mM 1-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (200 dpm/nmol), 25 μl of 100 mM Tris-HCl (pH 7.0), and 5 μl of 5 mM EDTA. After

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incubation at 37°C for 5 min, the released [^{14}C]fatty acid was measured according to the method of Dole and Meinertz (*14*).

Protease I Assay—Protease I activity was measured according to the method of Ichihara *et al.* (*4*). Briefly, the purified enzyme (10 μl) was mixed with 800 μl of 12.5 mM Tris-HCl (pH 8.0) containing 0.25% Triton X-100 and 10 μl of Cbz-Phe-ONp dissolved in DMSO. After incubation at 37°C for 30 min, the reaction was stopped by adding 10 μl of soybean trypsin inhibitor (5 mg/ml). The released *p*-nitrophenol was quantitated by measuring the absorbance at 405 nm.

Thioesterase I Assay—Thioesterase I activity was measured according to the method of Barnes (*11*). The assay mixture contained 0–2000 μM palmitoyl-CoA, 60 mM potassium phosphate (pH 7.4), 0.8 mg/ml bovine serum albumin, and 100 μM 5,5′-dithiobis (2-nitrobenzoic acid) in a total volume of 500 μl. The reaction was carried out at 25°C for 30 min and the absorbance at 412 nm was measured. A molar extinction coefficient of 13,600 was used to estimate 5,5′-dithiobis (2-nitrobenzoic acid) reduction.

RESULTS AND DISCUSSION

Cloning and Sequencing of the pldC Gene Encoding Lysophospholipase L_1—We previously cloned the *pldC* gene encoding lysophospholipase L_1 by screening Clarke and Carbon's collection based on the increase in lysophospholipase L_1 activity (*4*), and constructed plasmid pC124 by subcloning a 5.5 kbp DNA fragment into the vector DNA, pUC8 (*5*). To determine the location of the *pldC* gene, in this study, we further re-cloned this 5.5 kbp DNA fragment digested with restriction endonucleases, *Eco*RI and *Hind*III, into the vector DNA, pBluescript II sk(−), and obtained a series of mutants with various extents of deletion. The approximately 1.0-kbp fragment cloned on pC 124-85 was the minimum length of DNA that gave the enzyme activity, suggesting that the *pldC* gene was located within a region of this 1.0-kbp fragment (Fig. 1). Sequence analysis revealed three possible open reading frames. Among them, the second ATG appeared to be a favorable initiation codon, because the sequence, CGGA, preceding the second ATG exhibits good homology with the ribosomal binding site, the Shine-Dalgano sequence. The putative promoter elements were found to be TTGACAACC for the −35 region and TGAGGAT for the −10 region. We had already determined the amino acid sequence of the NH_2-terminus of the purified lysophospholipase L_1 to be Ala-Xaa-Thr-Xaa-Leu-Ile-Leu-Gly-Xaa-Xaa-Leu (*5*), which was found at 27 to 37 in the amino acid sequence, suggesting that the preceding 26 residues are cleaved as a signal peptide. The mature lysophospholipase L_1 was deduced to contain 182 amino acid residues with a molecular weight of 20,399. The molecular weight of the mature enzyme was in good agreement with that (20,500) of the purified lysophospholipase L_1 (*5*). *E. coli* lysophospholipase L_1 activity is inhibited by diisopropylfluorophosphate (DFP), which reacts with active serine (*1, 5*). A pentapeptide, G-D-S-L-S, was included in the deduced amino acid sequence as the putative active site at residues 34–38.

The pldC Gene Encoding Lysophospholipase L_1 Is Identical to the apeA and tesA Genes Encoding Protease I and Thioesterase I, Respectively—Sequence analysis revealed that the *pldC* gene was completely identical to the *apeA* (*9*) and *tesA* (*12*) genes encoding protease I and thioesterase I, respectively. In addition, lysophospholipase L_1 exhibited 49.4% amino acid identity with *Vivrio mimicus* arylesterase, which shows a preferential substrate specificity for aromatic esters (*15*), as shown in Fig. 2. There was no sequence similarity between *E. coli* lysophospholipases L_1 and L_2 at all. The *pldB* gene is located near the *pldA* gene encoding *E. coli* detergent-resistant phospholipase A at about 85 minutes on the *E. coli* genetic map (*16*). Since the *tesA/apeA* genes, which have the same DNA sequence as *pldC*, are mapped at 11.6 minutes, the *pldC* gene is located away from the *pldA/pldB* region (*9, 12*). This fact suggests that the *pldC* gene would be regulated independently of the *pldA/pldB* genes, of which the close location is suspected to take part in the sequential degradation of glycerophospholipids (*16*). *E. coli* lysophospholipase L_1 does not exhibit significant homology with other bacterial and mammalian lysophospholipases from bovine pancreas, *Vibrio parahaemolyticus*, human eosino-

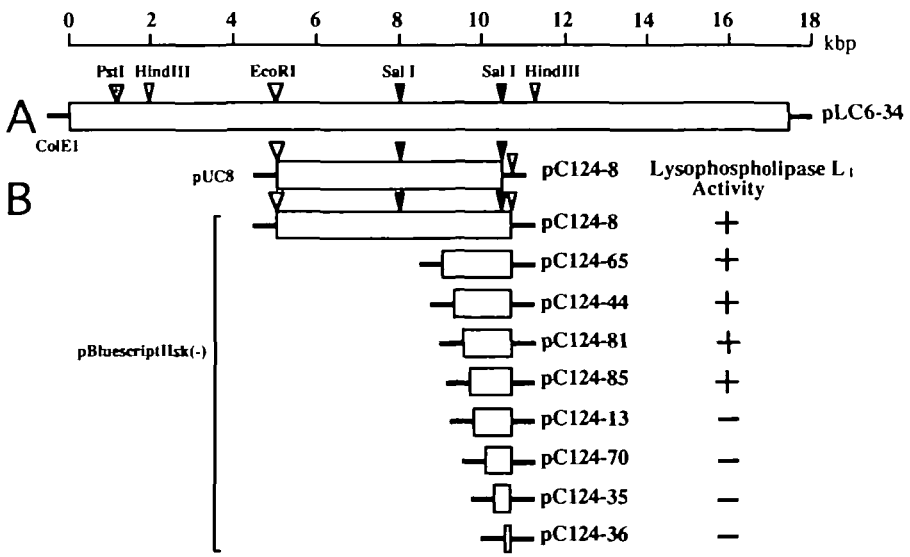

Fig. 1. **Subcloning of the *pldC* gene.** (A) The restiction sites of the pLC6-34 plasmid are shown. (B) The DNA fragment digested with restriction endonucleases, *Eco*RI and *Sal*I was subcloned into pUC8. The fragment digested with restriction endonucleases, *Eco*RI and *Hind*III was then subcloned into pBluescript sk(−). Various mutants generated by deleting the plasmid unidirectionally away from *Eco*RI into the insert sequence were obtained, and lysophospholipase L_1 activity in the transfected *E. coli* was determined.

phil, *Penicillium notatum*, and *Saccharomyces cerevisiae*.

The Purified Lysophospholipase L_1 Has Thioesterase I and Protease I Activities—To determine whether or not the lysophospholipase L_1 has thioesterase I and protease I activities, we purified lysophospholipase to homogeneity from the *E. coli* strain carrying pC124-85 according to the previously described method (*5*), and measured protease I and thioesterase activities using Cbz-Phe-ONp and palmitoyl-CoA, respectively (Fig. 3). The purified lysophospholipase L_1 caused the hydrolysis of Cbz-Phe-ONp and palmitoyl-CoA. However, the kinetics of hydrolysis were different between these enzyme activities. The hydrolysis of 1-palmitoyl-*sn*-glycero-3-phosphocholine exhibited typical Michaelis-Menten kinetics, while negative feedback was observed in the hydrolysis of palmitoyl-CoA. A detailed kinetic study of protease I was not possible due to the low solubility of a substrate, Cbz-Phe-ONp. In our previous work, a serine residue was thought to be involved in the active sites of lysophospholipase L_1 based on the inhibitory effect of DFP. On the other hand, similar inhibition of protease I (*6*) and thioesterase I (*12*) by DFP has been reported. Therefore, the same active sites would be responsible for three kinds of enzyme activity. These results indicate that the three kinds of enzyme activity are due to the same protein. Besides the above three kinds of enzyme activity, arylesterase activity has been reported to be catalyzed by this enzyme (*17*), although arylesterase, which hydrolyzes the aromatic thiol-ester bond of *N*-carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester, could be classified as the same enzyme activity as protease I. In addition, *V. mimicus* arylesterase has been shown to have thioesterase I activity (*18*).

What Is the Substrate for the Enzyme Encoded by the apeA/tesA/pldC Genes?—The fact that three enzyme activities originate from the same protein raises the question of which activity(s) is implicated in the physiological function. Among these three kinds of enzyme activity, the protease I activity is not likely to be involved in protein degradation (*10*). This enzyme cleaves the aromatic ester bond in model chymotrypsin substrates and proteolytic activity against casein is extremely low.

A rationale for the presence of thioesterase I activity in

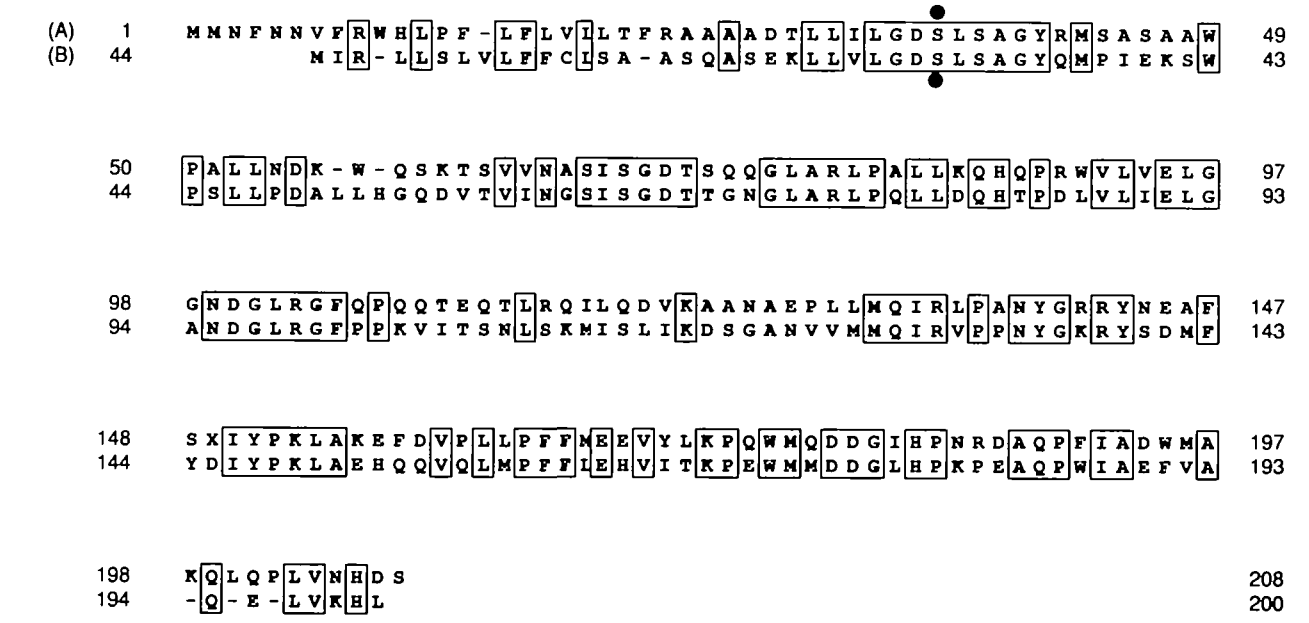

Fig. 2. **Comparison of the amino acid sequences of *E. coli* lysophospholipase L_1/protease I/thioesterase I (A) and *Vibrio arylesterase* (B).** Identical amino acid residues are boxed. Possible active serines are denoted by closed circles.

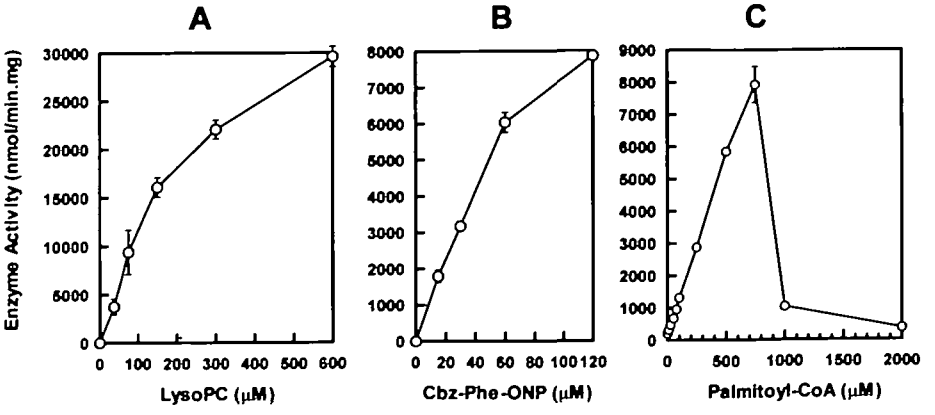

Fig. 3. **Lysophospholipase L_1 (A), protease I (B), and thioesterase I (C) activities of the purified *pldC* gene product.** The lysophospholipase L_1, protease I and thioesterase I activities of the purified enzyme were examined using 1-[^{14}C]palmitoyl-*sn*-glycerophosphocholine, Cbz-Phe-ONP and palmitoyl-CoA, as described under "MATERIALS AND METHODS." The Cbz-Phe-ONP hydrolyzing activity was not determined at concentrations of higher than 120 μM because of the insolubility of the substrate. The data presented are the means for duplicate determinations.

E. coli is obscure. Cho and Cronan (*13*) showed that *E. coli* overproducing the *tesA* gene accumulated free fatty acids and concluded that this enzyme acted as a thioesterase *in vivo*. In addition, they supposed that the produced fatty acids were due to the cleavage of the thio-ester bond of acyl-ACPs (*13, 19*). However, thioesterase I located in the periplasm might have difficulty in gaining access to possible substrates, acyl-ACPs in the cytoplasm, although a portion of the enzyme activity remained in the cytoplasm in the overproducing strains. They reported that the tesA/apeA proteins fail to cleave nonactivated oxygen esters except activated oxygen esters like chymotrypsin substrates in which the ester bond is unusually labile because of the attached chromatophores. However, in this study, the oxygen ester bond was shown to be cleaved by this enzyme as well as the thio-ester and activated oxygen ester bonds. Lysophospholipids generated from the *E. coli* membrane could be a possible source of free fatty acids, as well as acyl-ACPs.

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