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Lipase Modulator Protein (LimL) of *Pseudomonas* sp. Strain 109

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Plasmids containing a *Pseudomonas* sp. strain 109 extracellular lipase gene (*lipL*) lacking NH_2-terminal sequence and a lipase modulator gene (*limL*) lacking the NH_2-terminal hydrophobic region were constructed and expressed independently in *Escherichia coli* by using the T7 promoter expression vector system. Recombinant LipL (rLipL) was produced as inclusion bodies, whereas recombinant LimL (rLimL) was present as a soluble protein. During in vitro renaturation of the purified rLipL inclusion bodies after they had been dissolved in 8 M urea, addition of rLimL was essential to solubilize and modulate rLipL. The solubility and activity of rLipL were influenced by the rLimL/rLipL molar ratio; the highest level of solubility was obtained at an rLimL/rLipL ratio of 4:5, whereas the highest activity level was obtained at an rLimL/rLipL ratio of 4:1. After renaturation, rLipL and rLimL were coprecipitated with anti-rLipL antibody, indicating the formation of an rLipL-rLimL complex. Activity of the native lipase purified from *Pseudomonas* sp. strain 109 was also inhibited by rLimL. By Western blotting (immunoblotting) with anti-rLimL antibody, native LimL was detected in *Pseudomonas* cells solubilized by sarcosyl treatment. LimL was purified from *Pseudomonas* sp. strain 109, and the NH_2-terminal amino acid sequence was determined to be NH_2-Leu-Glu-Pro-Ser-Pro-Ala-Pro-. We propose that to prevent membrane degradation, LimL weakens lipase activity inside the cell, especially in the periplasm, in addition to modulating lipase folding.

Lipase (EC 3.1.1.3) is a lipid-degrading enzyme and is widely distributed in organisms from animals to bacteria. In addition to hydrolyzing esters, lipase can catalyze transesterification (18), esterification (19), and aminolyis or oximolysis (10) under anhydrous conditions. Recently, we established that extracellular lipase of *Pseudomonas* sp. strain 109 in anhydrous organic solvent catalyzed the intramolecular transesterification of methyl-16-hydroxyhexadecanoate, leading to the efficient formation of cyclohexadecanolide (macrocycliclactone [11]). The gene encoding the lactonizing lipase (*lipL*) of *Pseudomonas* sp. strain 109 was cloned in *Escherichia coli*, and the amino acid sequence was deduced from the nucleotide sequence (6).

Many lipase genes (17 lipases) from members of the genus *Pseudomonas* have been cloned and sequenced, and Gilbert (1) classified them into two groups according to amino acid sequence homology. One group consisted of lipases from *Pseudomonas* sp. strain 109, *P. aeruginosa*, *P. pseudoalcaligenes*, *P. glumae*, *P. cepacia*, and *P. putida*, and the other group consisted of lipases from *P. fluorescens* and *Pseudomonas* sp. strain LS107d2. No similarities between the two lipase groups were found, except for a well-conserved -Gly-X-Ser-X-Gly- sequence which is the active center of lipase (1).

Some but not all lipases require a secondary gene for their activation. These genes are located immediately downstream of the lipase genes and were also classified into two groups according to amino-acid sequence homology. One group consisted of genes from *P. aeruginosa* (15, 17), and the other group consisted of those from *P. cepacia* (9), *P. glumae* (2), and *Pseudomonas* sp. strain KWI-56 (8). The secondary gene (*limL*) of *Pseudomonas* sp. strain 109 falls within the *P. aeruginosa* group, with more than 90% sequence identity. Although the sequence similarity between the two activator groups was approximately 30%, the activation mechanism is estimated to be the same for the two groups. Recent studies of *P. cepacia* (LimA [5]), *P. glumae* (LipB [3]), and *P. aeruginosa* (LipB [4]) suggested that the secondary gene products are required for correct lipase folding.

In this study, lipase modulator protein (LimL) of *Pseudomonas* sp. strain 109 lacking its NH_2-terminal hydrophobic region was used for in vivo and in vitro lipase modulation assays. Recombinant LipL (rLipL)-rLimL complex formation was detected by immunoprecipitation in the renatured lipase solution, and the binding of rLimL was shown to reduce lipase activity. Native LimL in *Pseudomonas* sp. strain 109 was found as a membrane-bound protein. Native LimL was purified by affinity chromatography with anti-rLimL antibody, and the NH_2-terminal amino acid sequence was determined. Finally, the function of LimL in *Pseudomonas* cells was determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Pseudomonas* sp. strain 109 has been described previously (6). *E. coli* JM105 [Δ(*lac-proAB*) *thi rpsL endA sbcB15 hsdR4*/F′ *proAB lacI*^{q}*ZΔM15 traD36*] was used as the host for recombinant plasmids (14). *E. coli* BL21(DE3)pLysS [*hsdS gal* (λc*Its*857 *ind*1 *sam*7 *nin*5 *lacUV5*-T7 gene 1) pLysS] was used for overexpression of *lipL* and *limL* genes in the pET-3d vector (16). Plasmids used in this study are listed in Table 1. *E. coli* plasmids pUC19, pKK233-2, pHSG399, and pMW119 were described previously (7). pHSG399 and pMW119 were used as compatible vectors, and pET-3d was used for overexpression of protein. Bacteriophages M13mp18 and M13mp19 (14) were used to confirm the nucleotide sequence around the junction between vector and insert.

LB medium and 2 × YT medium (14) were used for culture of *E. coli*, and ampicillin (10 μg/ml) and/or chloramphenicol (2 μg/ml) was used as a supplement as necessary. A medium containing 1.0% polypeptone (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.2% yeast extract (Wako Pure Chemical Industries, Ltd.), 0.1% KH_2PO_4, 0.2% $MgSO_4$, 0.25% soybean oil (Yoshihara Seiyu, Osaka, Japan), and 0.1% NoigenHC (pH 7.0) was used for culture of *Pseudomonas* sp. strain 109 for lipase production.

DNA manipulations. Plasmid DNA preparations, isolation and purification of DNA fragments, DNA ligation, and gel electrophoresis were carried out as described elsewhere (14). Restriction enzymes and DNA modification enzymes were obtained from Toyobo Co., Ltd., Osaka, Japan, except for T4 DNA ligase (Takara Shuzo Co., Shiga, Japan). Synthetic oligonucleotides, 5′-CATGAAGA AGATCCTGCTGCTG-3′ and 5′-AATCAGCAGCAGGATCTTCTT-3′, were purchased from Vekkusu Co. (Tokyo, Japan).

The nucleotide sequence was determined by the dideoxynucleotide chain termination method (14) by using [α-^{32}P]dCTP (>3,000 Ci/mmol; ICN Biomedicals

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TABLE 1. Plasmids used in this study

Plasmid	Properties and construction information	Origin or reference
pUC19	Amp^r	14
pKK233-2	Amp^r, *trc* promoter	Pharmacia LKB
pET-3d	Amp^r, T7 promoter	16
pMW119	Amp^r, compatible vector	Nippon Gene Co.
pHSG399	Cr^r, compatible vector	Takara Shuzo Co.
pUY45	pUC19::2.2-kbp *lipL-limL* genes	7
pUY49	pUC19::1.1-kbp *lipL* gene	7
pCMY402	pHSG399::1.0-kbp *rlipL* gene	7
pCMY403	pHSG399::1.1-kbp *lipL* gene	7
pKM423	pKK233-2::1.0-kbp *lipL* gene	7
pETY402	pET-3d::pKM423 *Nco*I-*Bam*HI fragment	This study
pLIM402	pMW119::1.2-kbp *limL* gene	7
pLIM403	pUC19::pUY45 *Ava*I fragment	This study
pLIM410	pUC19::pLIM403 (*Sal*I fragment) + pUY45 *Sal*I fragment	This study
pLIM411	pKK233-2::pLIM410 *Ava*I-*Eco*RI fragment + linker	This study
pLIM412	pMW119::pLIM411 *Nco*I-*Kpn*I fragment	This study
pLIM413	pET-3d::pLIM411 *Nco*I-*Sac*I fragment	This study

Inc.) and phage M13 single-stranded DNAs as templates with Sequenase (United States Biochemical Corp.).

Assay. Substrates used for determination of lipase activity were tributyrin for the plate assay and *p*-nitrophenyl acetate for the liquid assay, as described previously (7). Protein concentration was determined by the dye-binding assay (Protein Assay Kit; Bio-Rad) with bovine serum albumin (BSA) used as the standard.

SDS-PAGE and amino acid sequence analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a commercially available gradient gel as described previously (7).

Determination of the NH_2-terminal sequence was performed as follows. Purified protein was subjected to SDS-PAGE and electroblotted to an Immobilon-P^{SQ} polyvinylidene difluoride membrane (Millipore Corp.) by using Trans-Blot SD (Bio-Rad) as recommended by the manufacturer. The membrane was stained with Coomassie brilliant blue R-250, and the corresponding protein band was excised. For rLimL, to remove the formyl group from the NH_2 terminus of methionine, the excised membranes were treated with 6 M HCl for 4 h at 37°C. The amino acid sequence was analyzed by using a pulsed-liquid protein sequencer, model 477A (Applied Biosystems), equipped with an on-line phenylthiohydantoin-amino acid analyzer, model 120A (Applied Biosystems).

Expression and purification of rLipL. The *E. coli* BL21 transformant was grown in 250 ml of LB medium containing 1.0% glucose and 10 μg of ampicillin per ml at 37°C to achieve an optical density of about 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture broth to obtain a final concentration of 0.5 mM, and the culture broth was incubated at 37°C for 2 h with shaking. Cells were harvested by centrifugation (3,000 × *g*, 10 min, 4°C) and suspended in 10 ml of 30 mM Tris buffer (pH 7.5) containing 30 mM NaCl. Cells were disrupted by sonication, and inclusion bodies were harvested by centrifugation (8,000 × *g*, 20 min, 4°C). The resulting pellets were suspended in 30 ml of 1 M sucrose solution and centrifuged at 8,000 × *g* at 4°C for 20 min. The precipitate was resuspended in 2% Triton X-100 solution containing 10 mM EDTA and kept on ice overnight. After centrifugation (8,000 × *g*, 20 min, 4°C), inclusion bodies of rLipL with more than 90% purity were obtained.

Expression and purification of rLimL. The method for expression of rLimL in *E. coli* was the same as that for rLipL expression except for the induction temperature (30°C). Overexpressed rLimL did not form inclusion bodies; therefore, rLimL was isolated from cell lysate of *E. coli*. The cell pellets were suspended in 10 ml of 50 mM Tris buffer (pH 7.5) containing 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF; Wako Pure Chemical Industries, Ltd.) and subjected to sonication. Cell debris were removed by centrifugation (10,000 × *g*, 20 min, 4°C), and the supernatant was used as cell extract. Insoluble fraction obtained from the cell extract upon addition of 60% saturated $(NH_4)_2SO_4$ was collected by centrifugation (8,000 × *g*, 10 min, 4°C). The pellet was dissolved in a small volume of the above-described buffer and was applied to a Sepharose CL-6B column (2.8 by 50 cm; Pharmacia LKB) preequilibrated with the same buffer. The sample was developed with the same buffer, and 10-ml fractions were collected. Since detection of the LimL activity was difficult (as shown in Results and Discussion), to detect the LimL protein in the fractions, small amounts of the collected fractions were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250. The rLimL-containing fractions were then adsorbed on a DEAE-Sephacel column (1.8 by 20 cm; Pharmacia LKB) preequilibrated with the above-described buffer and eluted with a linear gradient of NaCl at concentrations from 0 to 0.5 M in 1 liter of the same buffer. Fractions containing rLimL were collected and concentrated by ultrafiltration (UP-20; M_r cutoff, 20,000; Advantec Toyo, Tokyo, Japan). Purified rLimL was equilibrated with 50 mM potassium phosphate buffer (pH 6.5) containing 1 mM $MgCl_2$ by dialysis and stored at −80°C until use.

Immunoassay. Polyclonal rabbit serum anti-rLipL and anti-rLimL antibodies were raised against the purified rLipL inclusion bodies and rLimL, respectively. Protein A-Sepharose 4 Fast Flow (Pharmacia LKB) was used for immunoprecipitation of immunoglobulin G (IgG).

Western blotting (immunoblotting) was carried out as described elsewhere (14). Visualization of the immunoreactive proteins on Western blots was accomplished by the use of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham); chemiluminescence of proteins was detected by using electrochemiluminescence detection reagent (Amersham).

Purification of native LipL. Purification of native lipase from culture broth of *Pseudomonas* sp. strain 109 was achieved as follows. All purification procedures were done at 4°C unless otherwise specified. *Pseudomonas* sp. strain 109 was grown in 50 ml of lipase-producing medium in a 500-ml shaking flask at 30°C for 42 h with reciprocal shaking. 6,9-Diamino-2-ethoxy-acridine lactate (Acrinol; Nacalai Tesque, Inc., Kyoto, Japan) was added to the culture to a final concentration of 0.4%. After thorough mixing, insoluble materials including cells were removed by centrifugation (5,000 × *g*, 20 min). Excess Acrinol was removed by addition of NaCl to a final concentration of 0.5 M and centrifugation (5,000 × *g*, 20 min). The supernatant was concentrated 10-fold by ultrafiltration and equilibrated with 30% saturated $(NH_4)_2SO_4$ in 20 mM sodium phosphate buffer (pH 6.5) by dialysis. The sample was applied to an Octyl Sepharose CL-4B column (1.3 by 11 cm; Pharmacia LKB) preequilibrated with 20 mM sodium phosphate buffer (pH 6.5), and the column was washed with 200 ml of the same buffer. Lipase was eluted with 0.1% Triton X-100 dissolved in the same buffer, and 5-ml fractions were collected. After collection of the fractions containing lipase, $(NH_4)_2SO_4$ was added to the lipase fractions until 30% saturation was attained. A second Octyl Sepharose CL-4B chromatography step was carried out with a small column (1.0 by 8 cm), and 2-ml fractions were collected. The lipase-containing fractions were applied to a DEAE-Sephacel column (1.3 by 11 cm) preequilibrated with 0.2% Triton X-100 in 50 mM potassium phosphate (pH 6.5) and eluted with a linear gradient of NaCl at concentrations from 0 to 0.5 M dissolved in 200 ml of the same buffer. To remove Triton X-100, the collected lipase fractions were dialyzed with a detergent absorber gel (1 ml; Boehringer Mannheim) in 50 mM potassium phosphate buffer. Finally, the lipase solution was concentrated by ultrafiltration (UP-20; M_r cutoff, 20,000) and stored at −80°C until use.

Purification of native LimL. Purification of native LimL was achieved by using an immunoaffinity column. To purify anti-LimL rabbit IgG, an Econo-Pac DEAE Blue cartridge (Bio-Rad) and a Protein A-Sepharose (Pharmacia LKB) column (1 g) were used. The LimL affinity column was prepared by using the Affi-Gel Hz Immunoaffinity Kit (Bio-Rad) under conditions recommended by the manufacturer. All purification procedures were performed at 4°C unless otherwise specified. In each purification step, native LimL was detected by Western blotting (see above).

Pseudomonas sp. strain 109 cells (70 g [wet weight]) were harvested by centrifugation (3,000 × *g*, 20 min) from the 4-liter culture by a method similar to that used for purification of native LipL. The cell pellets were suspended in a small volume of 50 mM Tris buffer (pH 7.5) containing 0.1 mM APMSF and 0.1 M EDTA and subjected to sonication. Cell debris were removed by centrifugation (6,000 × *g*, 10 min), and the supernatant was used as the cell extract. The insoluble fraction obtained from the cell extract upon addition of 50% saturated $(NH_4)_2SO_4$ was collected by centrifugation (10,000 × *g*, 10 min, 4°C). The pellet was dissolved in a small volume of the above-described buffer, and $(NH_4)_2SO_4$ was removed by dialysis against the same buffer. The sample was applied to a DEAE-Sephacel column (1.8 by 25 cm), preequilibrated with the above-described buffer, and eluted with a linear gradient of NaCl at concentrations from 0 to 0.5 M in 1 liter of the same buffer. The fractions containing native LimL were adsorbed to an anti-LimL immunoaffinity column and eluted with 6 M urea. After dialysis, native LimL was concentrated by lyophilization.

RESULTS AND DISCUSSION

In vivo lipase activation. In a previous report (7), we showed that LimL could activate the lipase (*lipL*) in *E. coli* in *trans* and that the NH_2-terminal signal sequence of LipL was not necessary for the activation process. The deduced amino acid sequence of LimL showed the NH_2-terminal 20 amino acids to be highly hydrophobic, suggesting that the region was either a signal sequence or a membrane-associated region. To confirm whether the NH_2-terminal sequence of LimL is necessary for activation, plasmids having a truncated *limL* gene were con-

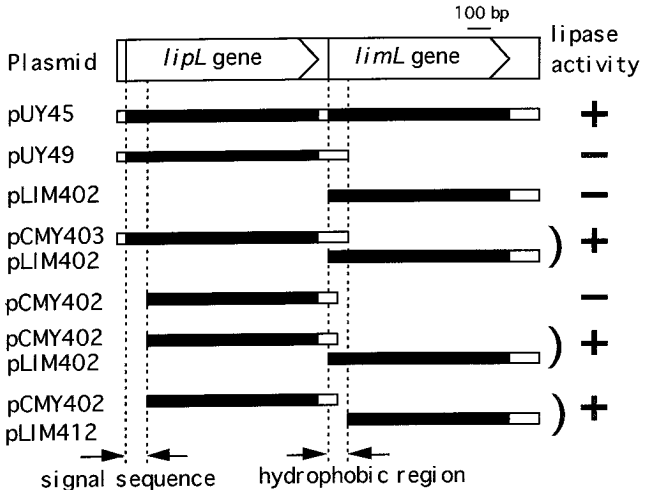

FIG. 1. The NH_2-terminal amino-acid sequence of LimL was not necessary for lipase modulation in *E. coli*. The restriction map and locations of the *lipL* and *limL* genes in the 2.2-kbp fragment are shown at the top. Several *lipL* and/or *limL* fragments were expressed from the *lac* or *trc* promoter (indicated from left to right). Lipase activity was assayed qualitatively on the basis of clear-zone formation on a tributyrin agar plate. A ")" shows that both plasmids are present in the same *E. coli* cell. +, lipase activity present; −, lipase activity absent.

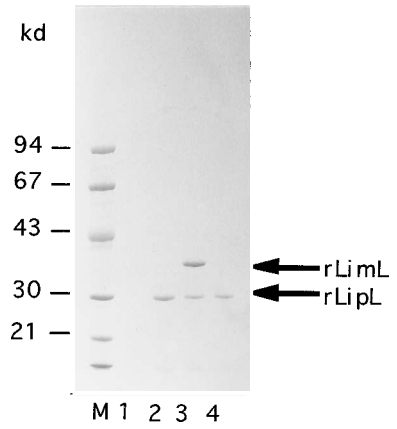

FIG. 2. Solubilization of rLipL inclusion bodies by addition of rLimL during the renaturation process. Inclusion bodies of rLipL (0.28 mg) were denatured with 8 M urea and renatured by decreasing the urea concentration in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of rLimL (0.34 mg). After centrifugation (8,000 × *g*, 5 min), soluble fractions (lanes 1 and 3) and precipitates (lanes 2 and 4) were separated on an SDS-polyacrylamide gel and stained with Coomassie brilliant blue G-250. Lane M, marker proteins (phosphorylase *b*, M_r = 94,000; BSA, M_r = 67,000; ovalbumin, M_r = 43,000; carbonic anhydrase, M_r = 30,000; soybean trypsin inhibitor, M_r = 20,000).

structed as follows. A restriction endonuclease *Ava*I-treated *limL* gene fragment was ligated into the *Nco*I-*Eco*RI site of plasmid pKK233-2 via a synthetic oligonucleotide linker (Table 1). From the resulting plasmid, pLIM411, the *Bam*HI-*Sac*I fragment was excised and ligated into the same site of pMW119 (pLIM412; Table 1). pLIM412 was transformed into *E. coli* JM105 having the *lipL* gene on a compatible vector (pCMY402). On a tributyrin agar plate, an *E. coli* JM105 double transformant harboring both pLIM412 and pCMY402 produced active LimL, as evident from a clear zone around the colony (Fig. 1), indicating that the NH_2-terminal hydrophobic sequence of LimL is not necessary for lipase activation and could be a signal sequence. Thus, we used mature LipL (rLipL) and truncated LimL (rLimL) in the succeeding experiments.

Expression and purification of rLipL and rLimL. To study the interaction between LipL and LimL, an in vitro system for LimL-mediated lipase activation should be constructed. However, with the *trc* promoter vector (pKK233-2) the level of LipL production in *E. coli* was very low, although efficient transcription in *E. coli* was confirmed by Northern (RNA) hybridization analysis (data not shown). Therefore, to obtain large enough amounts of rLipL and rLimL, the T7 promoter vector was used for overexpressing *lipL* and *limL* genes.

(i) Expression of lipase. The method for construction of the lipase gene without signal sequence was described previously (7). The gene (r*lipL*) was ligated into the *Nco*I site of the T7 promoter vector (pET-3d). The resulting plasmid, pETY402 (Table 1), was transformed into *E. coli* BL21(DE3)pLysS, and expression was induced by addition of IPTG. Expressed rLipL was purified as inclusion bodies (7). The soluble lipase was not obtained at all, although many culture conditions were examined (data not shown).

(ii) Expression and purification of rLimL. The truncated *limL* gene was constructed as described above (Table 1). The *Nco*I-*Eco*RI fragment from pLIM412 was ligated into the *Nco*I-*Eco*RI site of plasmid pET-3d (Table 1). The resulting plasmid, pLIM413, was transformed and expressed in the manner described above. The expressed rLimL did not form inclusion bodies and was recovered in the supernatant of the cell extract. Purification of rLimL was achieved by three steps, i.e., $(NH_4)_2SO_4$ precipitation, gel filtration, and anion-exchange chromatography, as described in Materials and Methods. Purified rLimL was subjected to SDS-PAGE, and a single band appeared on the Coomassie brilliant blue G-250-stained gel (data not shown).

The NH_2-terminal sequences of the first 6 amino acids of both the purified rLipL and rLimL were determined. The sequences of rLipL and rLimL were identical to the amino acid sequences deduced from the nucleotide sequences of *lipL* and *limL*, respectively. Thus, the expressed and purified recombinant proteins were confirmed to be rLipL and rLimL.

Refolding of rLipL modulated by rLimL. Simple mixing of rLimL with inactive inclusion bodies of rLipL did not give lipase activity, suggesting that the insolubility of rLipL in the form of inclusion bodies may prevent the action of LimL. Therefore, in order to obtain soluble rLipL, 0.28 mg (10 nmol) of rLipL inclusion bodies was solubilized with 1 ml of 8 M urea solution and renatured by decreasing the urea concentration in a stepwise manner from 6 to 0 M during dialysis. When no LimL was present during the renaturation step, insoluble inclusion bodies appeared again at urea concentrations of 4 M or lower, and neither soluble rLipL nor lipase activity was recovered at 0 M urea (Fig. 2 and Table 2). When an equal molar ratio (10 nmol, 0.36 mg) of rLimL to rLipL was added at 4 M urea during the renaturation, the soluble protein having the lipase activity appeared (Table 2). In the soluble fraction, rLipL and rLimL were detected on the SDS-PAGE gel; in the insoluble fraction, on the other hand, no rLimL protein was detected (Fig. 2). The amount of soluble rLipL was calculated as the difference between the total soluble protein and the amount of rLimL added. By LimL-dependent renaturation, 52% of rLipL was converted into a soluble form, suggesting the possibility that LimL acts like a chaperone in modulating the folding of LipL. BSA could not substitute for rLimL (Table 2), which confirmed the rLimL-specific nature of the LipL renaturation and the fact that rLimL is not merely working to prevent the aggregation. Studies of cytoplasmic chaperones such as Gro proteins have shown that chaperone-mediated protein folding requires binding of a nucleotide such as ATP

TABLE 2. Effects of various factors on rLipL renaturation[a]

Supplementary agent(s)	Amt (μg) of soluble lipase (%)	Amt (U) of lipase activity (%)
None	0 (0)	>0.05 (—)[b]
rLimL	164 (100)	0.35 (100)
BSA	ND[c] (—)[d]	>0.05 (—)[b]
rLimL, 1 mM ATP	222 (135)	0.25 (71)
rLimL, 1 mM $MgCl_2$	121 (74)	0.25 (71)

[a] Inclusion bodies of rLipL (10 nmol) were dissolved in 1 ml of 8 M urea and renatured by dialysis. When the urea concentration was 4 M, supplementary agents were added during dialysis. ATP and $MgCl_2$ were added in the buffer, and BSA was added in the rLipL solution. Supplemented BSA was adjusted to the same molarity as that of rLipL.

[b] —, lower than the limits set for this assay.

[c] ND, not determined.

[d] —, determination of soluble lipase concentration impossible since the insoluble fraction contained BSA.

without hydrolysis (12). However, in the recovery of lipase activity as a measure of the LimL-mediated lipase modulation process, ATP or Mg^{2+} was not required (Table 2).

The solubilized fraction gave only 0.35 U of activity against *p*-nitrophenyl acetate used as the substrate (Table 2). The specific activity (2.4 U/mg) was much lower than that of the native lipase (40 U/mg [6]), showing that LipL solubilized by LimL possessed only 6% of its original activity.

In the renaturation step, the rLimL/rLipL molar ratio was varied from 1:4 to 8:4. LipL solubilization is shown in Fig. 3. While the largest amount of soluble LipL was obtained at an rLimL/rLipL ratio of 5:4, lipase activity increased as the rLimL ratio decreased and was at a maximum at an rLimL/rLipL ratio of 1:5 (data not shown). After the renaturation of rLipL at various rLimL/rLipL ratios, soluble fractions were immunoprecipitated with anti-rLipL antibody and analyzed by SDS-PAGE (Fig. 4). Two bands corresponding to rLimL and rLipL appeared from the precipitates, showing directly for the first time that rLimL binds with rLipL to form a stable rLipL-rLimL complex.

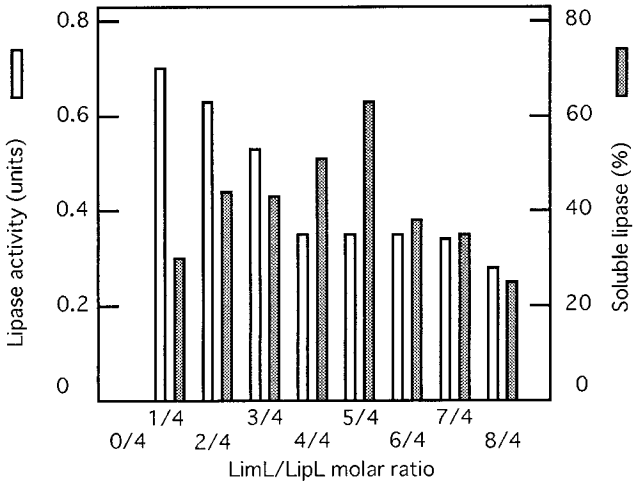

FIG. 3. Effect of rLipL/rLimL molar ratio on in vitro lipase renaturation. LipL inclusion bodies (0.32 mg) were dissolved and denatured with 4 ml of 8 M urea. During lipase renaturation, when the urea concentration was 4 M, LimL was added to the LipL solution at various molar ratios and the mixture was incubated for 24 h at 4°C. Dialysis was continued until the urea concentration reached 0 M, and the insoluble protein was removed by centrifugation. Total lipase activity in the soluble fractions and the percentage of soluble LipL were analyzed.

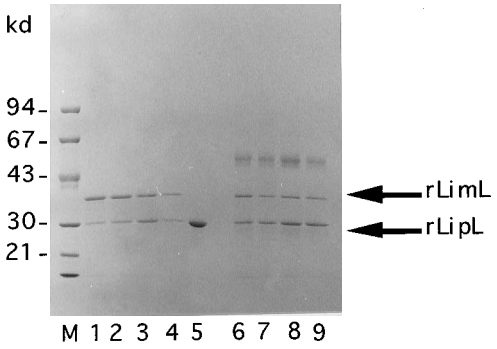

FIG. 4. Detection of the rLipL-rLimL complex by immunoprecipitation. Soluble fractions obtained from renaturation at various rLipL/rLimL ratios (Lanes 1 to 4) were immunoprecipitated with anti-rLipL antibody on a protein A-Sepharose 4 Fast Flow column (lanes 6 to 9). The samples were separated on an SDS-polyacrylamide gel and stained with Coomassie brilliant blue G-250. Lane 5 contains rLipL inclusion bodies, Lane M contains marker proteins as described in the legend to Fig. 2, lane 1 shows 0 to 50% saturated $(NH_4)_2SO_4$ precipitation, lane 2 shows Sepharose CL-6B gel filtration, and lane 3 shows DEAE-Sephacel ion-exchange chromatography.

rLimL interacts with native lipase. In order to further confirm the interaction between LimL and LipL, native LipL was purified from the culture of *Pseudomonas* sp. strain 109 (see Materials and Methods). Native LipL (167 U), appearing as a single band on an SDS-PAGE gel, was obtained from the 500-ml culture (845 U) of *Pseudomonas* sp. strain 109. The native LipL and rLimL were mixed at various molar ratios and incubated for 1 h at 4°C. Lipase activity decreased as the amounts of rLimL increased until the LipL/rLimL ratio reached 1:1 (Fig. 5) and remained constant at higher ratios. Complex formation between the native LipL and rLimL was also verified by immunoprecipitation with anti-rLipL antibody (data not shown). We therefore conclude that lipase modulator (LimL) acts like a chaperone to modulate lipase folding. LimL is necessary to convert LipL into an active form, but its association with active LipL renders it partially inactive.

The fact that only 6% of the lipase activity was observed in renatured rLipL (Table 2) but native LipL showed 40% activity, even in the presence of excess rLimL (Fig. 4), suggests that rLimL alone is not sufficient for full activation of LipL. Some

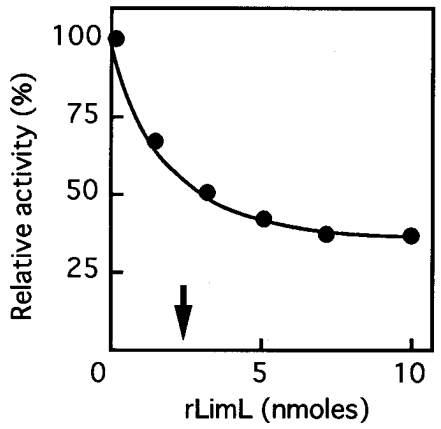

FIG. 5. Inhibition effect of rLimL on native lipase. Native lipase (3 nmol) and various amounts of LimL (0 to 10 nmol) were mixed and incubated at 4°C for 60 min. Lipase activity was assayed by using *p*-nitrophenyl acetate as a substrate. An arrowhead indicates the point at which the LipL/LimL ratio reached 1:1.

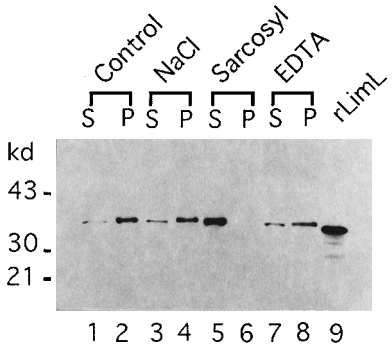

FIG. 6. Location of native LimL in *Pseudomonas* sp. strain 109. Cell extract of *Pseudomonas* sp. strain 109 was incubated with various agents for 30 min at 4°C. Lanes: 1 and 2, no added agents; 3 and 4, 500 mM NaCl; 5 and 6, 1% sarcosyl; 7 and 8, 5 mM EDTA. Membrane fraction was separated by ultracentrifugation (330,000 × *g*, 60 min). Supernatants (S) (lanes 1, 3, 5, and 7) and precipitates (P) (lanes 2, 4, 6, and 8) were subjected to SDS-PAGE. Detection of LimL is discussed in Materials and Methods. Lane 9 contains LimL.

other factor(s) (i.e., membrane components) or hydrophobic region of LimL might be necessary for full activation.

Location of native LimL. By Western blotting with the anti-rLimL antibodies, native LimL of *Pseudomonas* sp. strain 109 was detected from the cells but was not detected from the culture (data not shown), suggesting that native LimL was not a secreted protein. To determine the location of native LimL in *Pseudomonas* sp. strain 109, cell lysate was separated into soluble and insoluble fractions by ultracentrifugation (100,000 × *g*, 60 min). The supernatant and the precipitate were subjected to SDS-PAGE, and the native LimL was detected by immunoblot analysis with the anti-rLimL antibodies. A strong signal appeared in the precipitate and the signal in the supernatant was weak, although the two bands showed almost the same molecular weight (Fig. 6). When the cell extract was treated with NaCl, sarcosyl, or EDTA, LimL protein appeared in the supernatant only with sarcosyl treatment (Fig. 6).

As shown in Fig. 6, LimL in the crude cell extracts had a molecular size similar to that of rLimL which was lacking the NH_2-terminal hydrophobic region (21 amino acids). To confirm the NH_2-terminal amino acid sequence of native LimL, LimL was purified from *Pseudomonas* sp. strain 109. Purification of LimL from the cell extract is described in Materials and Methods. Since the amount of LimL in *Pseudomonas* sp. strain 109 was very small, LimL protein in each purification step was detected only by Western blotting. From 70 g of the wet cells used as starting material, 10 μg of LimL with 60 to 70% purity was obtained. After SDS-PAGE and transfer of LimL to a polyvinylidene difluoride membrane, NH_2-terminal amino acid sequences were determined as NH_2-Leu-Glu-Pro-Ser-Pro-Ara-Pro-. Surprisingly, the cleavage site was between Trp and Leu, suggesting that the usual *Pseudomonas* signal peptidase does not participate in the cleavage of precursor LimL (13).

The hypothetical function of LimL in LipL modulation in *Pseudomonas* spp. is summarized as follows. LimL is present in the periplasm and is peripherally associated with the cytoplasmic membrane (or outer membrane). Newly synthesized and secreted LipL will form a complex with LimL and will be converted to achieve correct folding. At this point, LimL suppresses lipase activity to prevent hydrolysis of the membranes. Finally, by an unknown mechanism, LipL is released from LimL and becomes fully activated. The fact that LimL is a membrane-associated protein suggests the possibility that Xcp-like proteins (13) may interact with LimL for the activation of LipL.

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