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Glutathione as an Essential Factor for Chaperon-Mediated Activation of Lactonizing Lipase (LipL) from *Pseudomonas* sp. 109[1]

Junko Tanaka, Takuya Nihira,[2] and Yasuhiro Yamada

Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871

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***Pseudomonas* sp. 109 produces a unique lipase (LipL) which efficiently catalyzes intramolecular transesterification of ω-hydroxyesters to form macrocyclic lactones. The production of the enzymatically active LipL requires a specific molecular chaperon (LimL protein) together with a low-M_r lipase-activation-factor (LAF) of unknown structure. From 50 g of *Pseudomonas* cells, 2.15 mg of LAF was purified as a sulfobenzofurazanyl derivative after methanol extraction, derivatization, and C_{18} reverse-phase HPLC. One-dimensional and two-dimensional 600 MHz ^{1}H-NMR and fast atom bombardment mass spectrometry (FAB-MS) revealed that LAF is glutathione. Because several SH compounds (L-cysteine and mercaptoethanol) were similarly effective to native LAF in the activation of LipL, and because only LipL contains two cysteinyl residues forming an intramolecular disulfide bond, it is concluded that the reduction of and reformation of the intramolecular disulfide bond of LipL is essential to liberate free and fully active LipL.**

Key words: glutathione, lactonizing lipase, lipase modulator protein, *Pseudomonas*.

Lipases [EC 3.1.1.3] are triacylglycerol ester hydrolases that hydrolyze long-chain fatty acid esters of glycerol yielding mono- and diacylglycerol and free fatty acids. In addition to the stereoselective hydrolysis of esters in the presence of water, lipases can catalyze transesterification, esterification, aminolysis (*1*), or oximolysis (*2*) under anhydrous conditions. This makes lipases very useful catalysts in several aspects of organic synthesis (*3*). The lipases used are usually of fungal or bacterial origin, with *Pseudomonas* being the most important bacterial genus (*4*).

We established that an extracellular lipase (LipL) of *Pseudomonas* sp. 109 uniquely catalyzes the intramolecular transesterification of ω-hydroxyesters in an anhydrous organic solvent, thus leading to the efficient formation of 14- to 20-membered lactones (*5*, *6*). Macrocyclic lactones (14-membered or larger) constitute key structures of many useful compounds, such as macrolide antibiotics (*7*), but the chemical cyclization of linear precursors into macrocyclic lactones is not straightforward, usually requiring complicated, often expensive, reagents or else drastic cyclization conditions (*8–10*). Because LipL-mediated cyclization is easy and straightforward and can be done under very mild conditions, LipL offers a good alternative for the synthesis of macrocyclic lactones, provided a sufficient amount of pure LipL is easily available.

The gene (*lipL*) encoding the lactonizing lipase has been cloned in our laboratory from *Pseudomonas* sp. 109, and the amino acid sequence deduced from the nucleotide sequence (*6*). However, the *lipL* gene alone was not sufficient to produce active recombinant LipL (rLipL) in *Escherichia coli*, and the co-existence of a specific chaperon gene (*limL*) either in *trans* or *cis* was found to be essential (*11*). When rLipL alone was over-expressed in *E. coli*, rLipL was obtained as inactive inclusion bodies. *In vitro* rLimL-mediated renaturation experiments of rLipL inclusion bodies in the presence of urea afforded partially active rLipL (6% of the activity observed in native and fully active LipL) due to the tight complex formation between rLipL and rLimL as detected by immunoprecipitation (*12*). This implies that an additional mechanism should exist in *Pseudomonas* sp. 109 to facilitate the dissociation of the complex to form enzymatically active free-LipL. Previously (*13*), we found that *Pseudomonas* sp. 109 possesses a low-M_r (M_r = 330 ± 30) lipase-activation-factor (LAF) which acts on the rLipL-rLimL complex to increase lipase activity by liberating free rLipL from the rLipL-rLimL complex.

In this paper, we have purified LAF from cells of *Pseudomonas* sp. 109. The purified LAF was identified as glutathione by means of one-dimensional and two-dimensional 600 MHz ^{1}H-NMR, fast atom bombardment mass (FAB-MS) and CD spectroscopy, which suggested that the reduction of the intramolecular disulfide bond of rLipL is required for rLimL-mediated activation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media—Pseudomonas sp. 109, a LipL producer (*6*) (strain FERM-P no. 3025, the Fermentation Research Institute, Agency of Industrial Science and Technology), was used as the source of lipase-activation factor (LAF). *E. coli* BL21(DE3)pLysS [*hsdS gal*

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[2] To whom correspondence shoud be addressed. Tel: +81-6-6879-7433, Fax: +81-6-6879-7432, E-mail: nihira@biochem.bio.eng.osaka-u.ac.jp

Abbreviations: LAF, lipase-activation-factor; SBD-F, 4-fluoro-7-sulfobenzofurazan ammonium salt; SBD-LAF, sulfobenzofrazanyl lipase-activation-factor; FAB-MS, fast atom bombardment mass spectrometry; CD, circular dichroism.

(λ*cIts-857 ind-1 Sam-7 nin-5 lacUV5*-T7 gene 1) pLysS] was used as the host for the overexpression of *lipL* and *limL* genes from the pET-3d (*14*) based-vectors, pETY402 and pLIM413 (*12*), respectively.

Renaturation of Recombinant LipL (rLipL)—rLipL and recombinant LimL (rLimL) were overexpressed from plasmids pETY402 and pLIM413 in *E. coli*, respectively, using an LB medium containing 1% (W/V) glucose, 10 μg/ml ampicillin, and 2 μg/ml chloramphenicol. They were purified as described previously (*12*) and stored at –80°C until use. To obtain the rLipL-rLimL complex, purified inclusion bodies of rLipL (0.5 mg) were solubilized in 1 ml of 6 M urea. After 1 h of incubation at room temperature, rLimL (1.15 mg in 50–100 μl, two equivalents of rLipL) was added to the solubilized rLipL, and the solution was made up to 5 ml with 6 M urea. The solubilized mixture of rLipL and rLimL was renatured by decreasing the urea concentration in a stepwise manner from 4 to 0 M by successive dialyses against 500 ml of 50 mM potassium phosphate buffer (pH 6.5) containing 2 M urea for 3 h at 4°C and two changes of 500 ml of 50 mM potassium phosphate buffer (pH 6.5) for 3 h.

Purification of LAF from Pseudomonas sp. 109—(i) Preparation of crude extract: *Pseudomonas* sp. 109 was grown in 250 ml of LB medium in a 500-ml Sakaguchi flask at 30°C and at 120 spm for about 12 h until OD_{600} of 2.5. The cells were harvested by centrifugation (3,000 ×*g*, 10 min, 4°C). The harvested cells (50 g from 10 liter of culture) were frozen at –80°C and ground in a 10-fold volume of methanol in a mortar. Supernatant was obtained by decantation and concentrated by evaporation to remove methanol.

(ii) First C_{18} reverse-phase HPLC: The crude extract (1,000 μl) was directly applied at room temperature onto a Mightysil RP-18 column (1.0 × 25 cm, Kanto Kagaku, Tokyo) preequilibrated with 20 mM ammonium formate buffer (pH 4.0) at a flow rate of 3.0 ml/min with detection at 210 nm. LAF activity appeared at the elution volume of 18 ml, and fractions corresponding to elution volumes of from 15 to 21 ml were pooled and concentrated by lyophilization.

(iii) Derivatization with 4-fluoro-7-sulfobenzofurazan ammonium salt (SBD-F): Derivatization with SBD-F was carried out by a modification of Toyooka's method (*15*) as follows: a freeze-dried sample was dissolved in 100 μl of water and 200 μl of 2.5 M potassium borate buffer (pH 9.5) containing 4 mM disodium EDTA, then 100 μl of SBD-F solution (2.0 mg SBD-F per ml of 2.5 M potassium borate buffer, pH 9.5) was added. After vigorous mixing, the solution was kept at 60°C for 1 h and at room temperature for 2 h.

(iv) Second C_{18} reverse-phase HPLC: The derivatized sample (100 μl) was applied at room temperature onto a CAPCELL PAK C_{18} SG 120 column (1.0 × 25 cm, Shiseido, Tokyo) preequilibrated with 100 mM ammonium acetate buffer (pH 4.0) at a flow rate of 5.0 ml/min. Elution was monitored by UV absorbance at 230 nm and fluorescence (excitation 385 nm, emission 515 nm). The fractions showing a characteristic sulfobenzofurazanyl moiety (elution volume 40 ml to 46 ml) were pooled and concentrated by lyophilization.

(v) Third C_{18} reverse-phase HPLC: A concentrated sample (10 μl) from step IV was applied onto a CAPCELL PAK C_{18} SG 120 column (1.0 × 25 cm, Shiseido) preequilibrated with 20 mM ammonium acetate buffer (pH 7.0) containing 2% CH_3CN at room temperature at a flow rate of 5.0 ml/min with detection by UV absorbance at 210 nm and fluorescence (excitation 385 nm, emission 515 nm). The sulfobenzofurazanyl LAF (SBD-LAF) was eluted at the elution volume of 58 ml.

Analytical Methods—HPLC was performed with a JASCO model PU980 equipped with a UV detector (JASCO UV-975) and a fluorescence detector (JASCO FP-20). ^{1}H-NMR spectra were recorded on a Varian model Unity Inova 600 spectrometer at 600 MHz using H_2O δ_H 4.78 as an internal reference in the D_2O solution. Fast-atom-bombardment (FAB) mass spectra were obtained on a JEOL JMS-DX-303HF spectrometer using α-thioglycerol as a matrix. CD spectra were obtained on a JASCO J-725 spectropolarimeter.

Lipase Activation Assay for LAF—Lipase activation assay was assayed as described previously (*13*) by measuring the increase of lipase activity during the incubation of rLipL-rLimL complex with or without LAF. Each experiment was repeated at least three times, and the data are expressed as the means of triplicate experiments. Deviation in the LAF-mediated activation was within ±10%. The <3,000 cell lysate of *Pseudomonas* sp. 109 was prepared as described previously (*13*) and used as the positive control for LAF activity.

Enzyme and Protein Assay—Lipase activity was measured as described previously (*13*) using *p*-nitrophenyl caproate as a substrate. One unit of lipase activity was defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min. Protein concentration was determined by a dye-binding assay (Protein Assay Kit; Bio-Rad) with bovine serum albumin as the standard.

Dissociation Analysis of rLipL-rLimL Complex—Gel-filtration of rLipL-rLimL complexes was performed on a SMART system (Pharmacia Biotech) as described previously (*13*). A fractionated sample (5 μl) from the SMART system was directly applied to SDS-PAGE after boiling for 10 min in 1 × SDS buffer (0.0625 M Tris-HCl, pH 6.8, 4.0% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.001% bromophenol blue). Western blot analysis was carried out by using the ECL™ Western blotting detection kit (Amersham Life Science) under conditions recommended by the manufacturer. Polyclonal anti-rLipL or anti-rLimL antibodies have been described previously (*12*). Immunoreactive proteins on Western blots were visualized by the use of horseradish peroxidase-conjugated anti–rabbit IgG (Amersham Life Science).

Detection of Disulfide Bond in LipL—Disulfide bonds in LipL were detected by a modification of Jaeger's method (*16*) as follows: the rLipL-rLimL complex activated by LAF or 0.1 mM glutathione was boiled for 2 min with or without 20 mM dithiothreitol in the presence of 1% (w/v) SDS. The sample was mixed with an equivalent volume of the 2 × SDS buffer without β-mercaptoethanol, and a 10-μl portion containing 0.1 μg of protein was analyzed by SDS-PAGE in the absence of β-mercaptoethanol using a ready-made 10–20% polyacrylamide-gradient gel (Daiichi Pure Chemicals, Tokyo). The native and fully active LipL, as well as inactive rLipL inclusion body, were analyzed similarly.

RESULTS AND DISCUSSION

Purification of Lipase Activation Factor (LAF)—In our previous paper, we established that LAF is a low-M_r compound (M_r of 330 ± 30) (*13*). However, its hydrophilic and thermolabile nature prevented us from purifying a sufficient amount of LAF for structural analysis. To overcome this problem and to obtain more insight into the structure of LAF, we investigated adsorption-elution profiles of LAF using several kind of resins, *i.e.*, anion-exchange resins [diethylaminoethyl-Sephacel, Amersham-Pharmacia; -CO-NH$(CH_2)_3$N$(CH_3)_3^+$, Accell Plus QMA, Waters; or trimethyl benzylammonium styrene divinylbenzene, Dowex 1, DOW], a cation-exchange resin (-COO^-, CM; Waters), active charcoal, C_{18} reverse-phase resin, and thiol affinity resin (Affigel 501; Bio-Rad). LAF was sufficiently retained only on strong anion-exchange resins and thiol affinity resin (data not shown). However, its recovery from strong anion-exchange columns was very low, probably due to its degradation in the strong basic environment of the resins. Also, the high unit price of the thiol affinity resin hindered its use in large-scale purification. The fact that LAF was retained on the thiol affinity column suggested that it contains thiol group(s), and its heat-labile nature may arise from the oxidation of the thiol group(s). Treatment of the crude cell lysate with SH-modifying reagents [ammonium 4-fluoro-7-sulfobenzofurazan, SBD-F; 5,5′-dithiobis(2-nitrobenzoic acid), DTNB; or *N*-(9-acridinyl)maleimide, NAM] resulted in complete loss of LAF activity, confirming that LAF contains thiol group(s). To increase the stability and the detection sensitivity and also to facilitate purification by changing LAF into a more hydrophobic compound, we attempted to modify LAF with a fluorescent thiol-modifying reagent, SBD-F.

After MeOH extraction, *Pseudomonas* crude cell extract

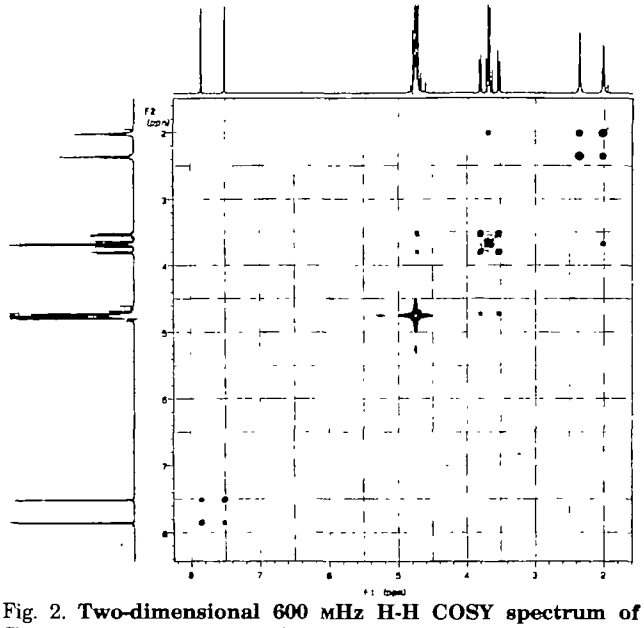

Fig. 2. **Two-dimensional 600 MHz H-H COSY spectrum of SBD-LAF.** Experimental conditions were similar to those described in the legend to Fig. 1. The two signals at 7.85 and 7.51 ppm arose from the SBD moiety, and the three coupling units ranging from 4.72 to 2.02 ppm arose from LAF. Signals arising from Gly-α and Glu-α overlapped at 3.68 ppm.

TABLE I. **Characterization of LAF.** The <3,000 lysate is the filtrate obtained by passing the crude cell-free extract of *Pseudomonas* sp. 109 through a M_r >3,000 ultrafiltration membrane as described previously (*13*), and it contains 100 nmol of thiols per ml. Five microliters of the <3,000 lysate was used per 3 μg of rLipL-rLimL complex. Each thiol (0.1 mM) was assayed for the ability to activate 3 μg of rLipL-rLimL complex after 2 h of incubation at 30°C. The + Ca^{2+} indicates that $CaCl_2$ was added to the activation mixture at a final concentration of 1 mM.

Supplementary agent	Lipase activity	
	(units/mg)	Activation (-fold)
No lysate	16	(1.0)
<3,000 lysate	33	2.1
Reduced glutathione	32	2.0
Oxidized glutathione	17	1.1
Dithiothreitol	35	2.2
Mercaptoethanol	34	2.1
L-Cysteine	35	2.2
Ascorbic acid	15	0.9
<3,000 lysate + Ca^{2+}	53	3.3
Reduced glutathione + Ca^{2+}	59	3.7

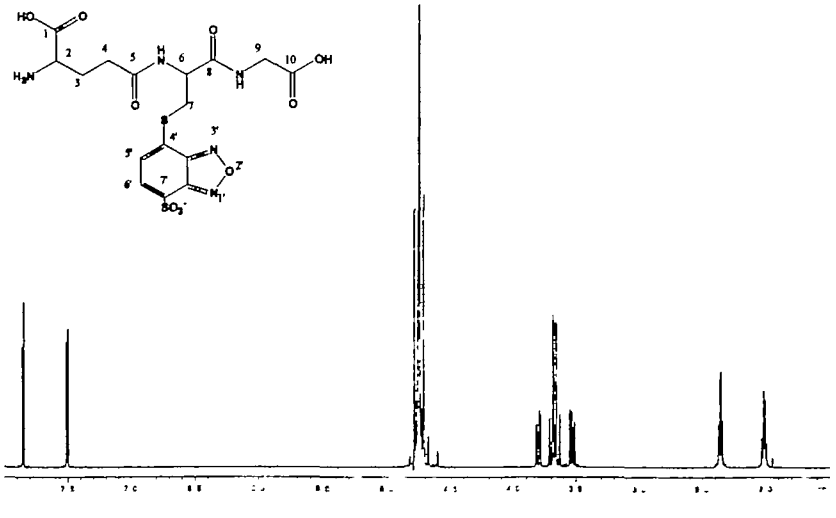

Fig. 1. **600 MHz ^{1}H-NMR spectrum of SBD-LAF.** SBD-LAF (0.8 mg) was dissolved in D_2O, and NMR spectrum was measured at ambient temperature using a Varian model Unity Inova 600 spectrometer at 600 MHz. The numbers above each signal correspond to those in the structure. H_2O δ_H 4.78 was used as an internal reference in the D_2O solution.

was first applied to C_{18} reverse-phase chromatography to remove hydrophobic substances that tend to interfere with the separation of derivatized LAF. Fractions were assayed for LAF activity as described in "MATERIALS AND METHODS." LAF activity was observed only at the elution volume of 18 ml, and fractions with elution volumes ranging from 15 to 21 ml were pooled, freeze-dried, and derivatized with SBD-F. The derivatized sample became hydrophobic in the acidic HPLC buffer (pH 4.0), and the fluorometric detection (excitation, 385 nm; emission, 515 nm) for the sulfobenzofurazanyl group gave only one peak at the elution volume of 42 ml. To ensure purity, the collected fractions corresponding to the SBD-derivatized LAF (SBD-LAF) were further separated on a C_{18} reverse-phase column using a neutral buffer (pH 7.0) with fluorometric detection at 515 nm, as well as UV detection at 210 nm. Under the separation conditions, the SBD-LAF was very hydrophobic, eluting at the elution volume of 58 ml. Minor contaminants showing UV absorption but no fluorescence were readily removed as more hydrophilic compounds. The total yield of SBD-LAF from 50 g of wet cells of *Pseudomonas* sp. 109 was 2.15 mg.

Structure Elucidation of LAF—A one-dimensional 600 MHz ^{1}H-NMR spectrum of the purified SBD-LAF is shown in Fig. 1. From the two-dimensional J-correlated ^{1}H-NMR (H-H COSY) spectrum, it became evident that LAF has three coupling units (signals of 3.70–3.65 ppm; signals of 4.72-3.81-3.53 ppm; signals of 3.68-2.36-2.36-2.02 ppm) in addition to those arising from the sulfobenzofurazanyl moiety (7.85 and 7.51 ppm). This suggested that LAF may be a tripeptide (Fig. 2). Because glutathione is the most probable candidate as a natural SH-containing tripeptide, a sulfobenzofurazanyl derivative of glutathione was synthesized and compared with SBD-LAF. All the physico-chemical properties (^{1}H-NMR, FAB-MS and CD spectrum) of SBD-LAF and sulfobenzofurazanyl glutathione were identical, confirming that LAF is glutathione.

Physicochemical Data of SBD-LAF—FAB-MS *m/z* 506 $(M+H)^+$, 528 $(M+Na)^+$, 550 $(M+K)^+$; ^{1}H-NMR δ (D_2O, 600 MHz) 7.85 (1H, d, J = 7.2 Hz, H-6′), 7.51 (1H, d, J = 6.0 Hz, H-5′), 4.72 (1H, m, H-6), 3.81 (1H, dd, J = 15.0 and 4.8 Hz, H-7a), 3.70 (1H, d, J = 17.4 Hz, H-9a), 3.68 (1H, t, J = 6.0 Hz, H-2), 3.65 (1H, d, J = 17.4 Hz, H-9b), 3.53 (1H, dd, J = 14.4 and 9.0 Hz, H-7b), 2.36 (1H, t, J = 7.8 Hz, H-4a), 2.36 (1H, t, J = 7.2 Hz, H-4b), 2.02 (2H, ddd, J = 14.4, 7.2 and 1.2 Hz, H-3); CD(H_2O) $\Delta\varepsilon_{261}$ = +15.8, $\Delta\varepsilon_{236}$ = +16.0.

Effects of Low-Molecular-Mass Thiols on the Activation of rLipL-rLimL Complex—Since LAF from *Pseudomonas* sp. 109 was identified as glutathione, we investigated the effects of other low-M_r thiols on the activation of the rLipL-rLimL complex (Table I). Each thiol at 0.1 mM (dithiothrei-

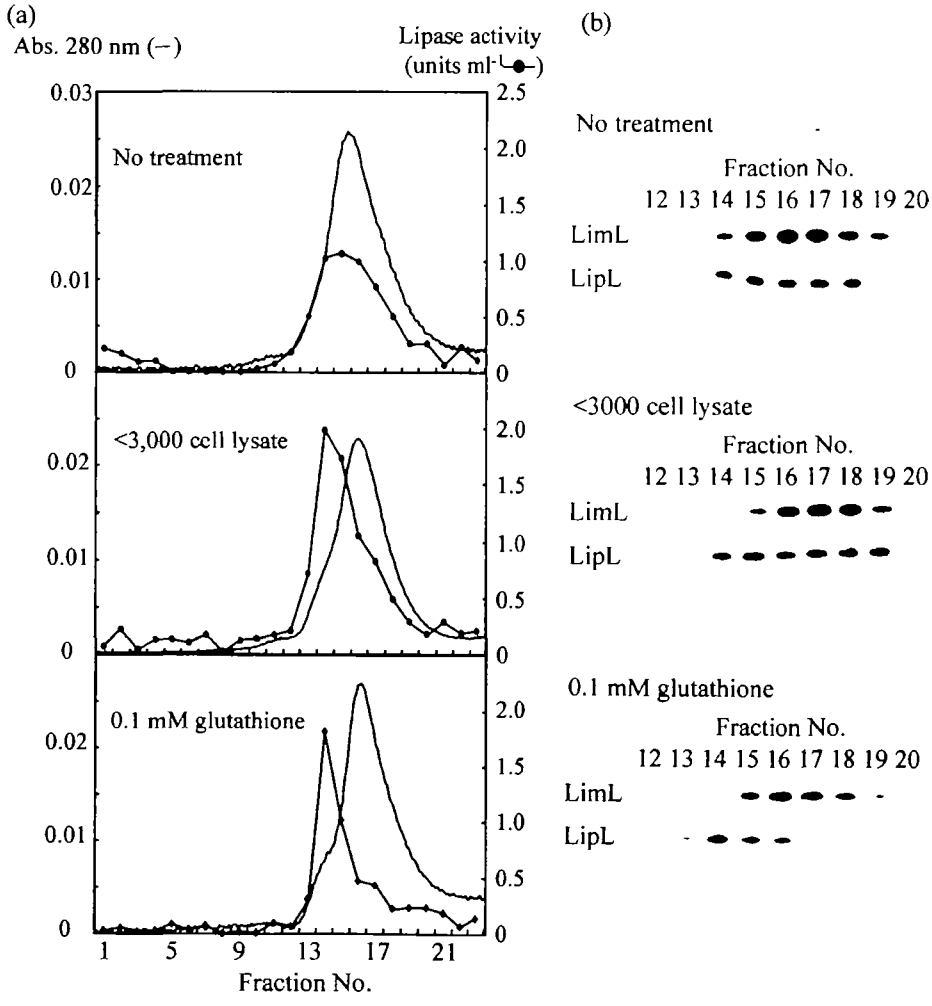

Fig. 3. **Effects of LAF and glutathione on the dissociation of the rLipL-rLimL complex.** (a) The filtrate (10 μl) from the 10-fold concentrated rLipL-rLimL complex, incubated with or without <3,000 lysate or 0.1 mM glutathione for 2 h, was separated on a Superose 12 column (0.32 × 30 cm, Pharmacia Biotech). Each fraction (25 μl) was assayed for lipase activity. (b) Western blot analysis of each fraction separated on the Superose 12 column.

tol, mercaptoethanol, L-cysteine) was similarly effective to native LAF or 0.1 mM glutathione, while neither oxidized glutathione nor ascorbic acid was effective, indicating that not glutathione but the free SH group is necessary for the activation of rLipL.

As reported previously, in addition to the increase of lipase activity during co-incubation with the rLipL-rLimL complex, native LAF showed two additional phenomena: (i) a synergistic increase of lipase activation in the presence of Ca^{2+}, and (ii) dissociation of rLipL from the rLipL-rLimL complex. To exclude the possibility that any additional compounds other than glutathione might be necessary for these phenomena, synergy with Ca^{2+} and the dissociation of rLipL were investigated. When the rLipL-rLimL complex was incubated with glutathione in the presence of Ca^{2+}, the activity in the presence of Ca^{2+} was 1.8-fold higher than that in the absence of Ca^{2+}. This was comparable to the value (1.6-fold) observed with the <3,000 lysate as native LAF (Table I) in the presence and absence of Ca^{2+}. Next, the rLipL-rLimL complex activated by glutathione was analyzed by molecular-sieve HPLC using a Superose 12 column on a SMART system that was designed to separate and recover microgram quantities of protein (Fig. 3a). After a 2-h activation at 30°C with <3,000 lysate or glutathione, 30 μg of the complex was injected and separated into 25-μl fractions. The elution profiles of lipase activity and of proteins at 280 nm agreed well between the <3,000 lysate-treated and the glutathione-treated sample. In both cases, lipase activity eluted earlier than the main protein peak, and these fractions (fractions 14 and 15) contained a higher ratio of rLipL/rLimL, as confirmed by Western blot analysis (Fig. 3b). These results indicate that glutathione liberated enzymatically active free-rLipL from the rLipL-rLimL complex, in the same manner as native LAF.

Because a free SH-group such as that in glutathione was found to be necessary for the activation of the rLipL-rLimL complex, and because rLimL contains no cysteinyl residues, we investigated whether two cysteinyl residues of rLipL are present as free SH or S-S before and after activation by 0.1 mM glutathione, which can be detected by SDS-PAGE with or without a 20 mM dithiothreitol pretreatment. Without dithiothreitol pretreatment, rLipL (before and after glutathione activation) as well as native and fully-active LipL migrated slightly faster than with dithiothreitol pretreatment with an estimated M_r of 30.9 k and 29.5 k, respectively, indicating that rLipL as well as native LipL have an intramolecular disulfide bond. When the rLipL-rLimL complex was treated with a high concentration of glutathione or dithiothreitol, all the lipase activity was lost because LipL was degraded. In *Pseudomonas aeruginosa*, DsbA-mediated formation of disulfide bonds of LipA in periplasm is a prerequisite for the production of the active LipA (*17*). These results suggested that not only the reduction of the preformed disulfide bond but also the re-formation of the intramolecular disulfide bond were necessary for the rLimL-mediated LipL activation process.

In conclusion, we have purified low-M_r lipase-activation-factor (LAF) as a sulfobenzofurazanyl derivative and elucidated that LAF is a reduced form of glutathione. Since several thiol-compounds were also effective as lipase activation factors, we concluded that the thiol group(s) is required for the LimL-mediated refolding process to generate enzymatically active LipL.

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