A low- M_r lipase activation factor cooperating with lipase modulator protein LimL in *Pseudomonas* sp. strain 109

Junko Tanaka,¹ Fumio Ihara,² Takuya Nihira¹ and Yasuhiro Yamada¹

Author for correspondence: Takuya Nihira. Tel: +81 6 6879 7433. Fax: +81 6 6879 7432.
e-mail: nihira@biochem.bio.eng.osaka-u.ac.jp

¹ Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

² Department of Plant Protection, National Institute of Fruit Tree Science, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan

***Pseudomonas* sp. strain 109 produces a unique lipase (LipL) which efficiently catalyses intramolecular transesterification of ω -hydroxyesters to form macrocyclic lactones. *In vivo* production of enzymically active LipL requires lipase modulator protein (LimL), which functions as a molecular chaperone for the correct folding of LipL. However, previous work has shown that LipL forms a tight complex with LimL *in vitro* and the resulting LipL–LimL complex is only partially active, suggesting an additional mechanism that facilitates the dissociation of the complex to form enzymically active LipL. In the present work, a low- M_r compound (lipase activation factor, LAF) was found in *Pseudomonas* sp. strain 109 that when added to the LipL–LimL complex resulted in the activation of LipL. Ca^{2+} ions also enhanced lipase activity, but the instantaneous activation by Ca^{2+} was different from the gradual and time-dependent activation by LAF, indicating the novel nature of this compound. LAF passed through an ultrafiltration membrane with an M_r cut-off of 3000 and showed an apparent M_r of 330 ± 30 on Superdex Peptide gel-filtration chromatography. Treatment of the LipL–LimL complex with LAF liberated free active LipL, indicating that LAF was necessary to dissociate the LipL–LimL complex.**

Keywords: *Pseudomonas*, lactonizing lipase, lipase activation factor, lipase modulator protein

INTRODUCTION

Lipase (EC 3.1.1.3), a lipolytic enzyme that catalyses the hydrolysis of triglycerides, is widely distributed in organisms from animals to bacteria. In addition to the stereoselective hydrolysis of esters in the presence of water, lipase can catalyse transesterification, esterification and aminolysis (Zaks & Klivanov, 1984), and also oximolysis (Zaks & Klivanov, 1985), under anhydrous conditions; this makes lipases very useful catalysts in several aspects of organic synthesis (Wong & Whitesides, 1994). Although many lipases of different origins are available, lipases from *Pseudomonas* are very useful for organic synthesis. We previously established that an extracellular lipase (LipL) of *Pseudomonas* sp. strain 109 catalyses the intramolecular transesterification of ω -hydroxyesters in an anhydrous organic solvent, leading to the efficient formation of 14- to 20-membered lactones (Ihara *et al.*, 1991; Makita *et al.*,

1987). Lactones consisting of 13 or more carbons (macrocyclic lactones) are the key structures of many useful compounds, such as macrolide antibiotics (Keller-Schierlein, 1973). However, the chemical cyclization of linear precursors into macrocyclic lactones is not straightforward, and usually requires very complicated, often very expensive, reagents or else drastic cyclization conditions. Because LipL-mediated cyclization is easy and straightforward and can be done under very mild conditions, LipL may become a good alternative for the synthesis of macrocyclic lactones, provided that sufficient pure LipL is easily available. Furthermore, development of an effective procedure for producing large amounts of recombinant LipL is also essential for protein engineering leading to more useful forms of LipL.

The gene (*lipL*) encoding the lactonizing lipase has already been cloned in our laboratory from strain 109, and the amino acid sequence was deduced from the nucleotide sequence (Ihara *et al.*, 1991). However, the *lipL* gene alone was not sufficient to produce active

Abbreviation: LAF, lipase activation factor.

LipL. The co-existence of a downstream *limL* gene, either on the same plasmid or on a separate plasmid, was found to be essential for the functional expression of LipL (Ihara *et al.*, 1992). A similar requirement of a secondary gene has been observed for several lipases (Frenken *et al.*, 1993a; Iizumi *et al.*, 1991; Jørgensen *et al.*, 1991; Kok *et al.*, 1995; Ogierman *et al.*, 1997; Siomi *et al.*, 1992; Wohlfarth & Winkler, 1992), and studies of *Burkholderia cepacia* (LimA; Hobson *et al.*, 1993), *Pseudomonas glumae* (LipB; Frenken *et al.*, 1993b), *Pseudomonas aeruginosa* (LipB; Hirayama *et al.*, 1993) and *Pseudomonas* sp. strain KWI-56 (Act; Iizumi & Fukase, 1994) suggested that the secondary gene product is required for correct lipase folding.

When recombinant LipL (rLipL) and recombinant LimL (rLimL) were overexpressed in *Escherichia coli*, rLimL was formed as a soluble protein but rLipL was obtained as inactive inclusion bodies. *In vitro* renaturation experiments with rLipL inclusion bodies in the presence of urea revealed that rLimL was essential to obtain soluble and active rLipL (Ihara *et al.*, 1995). However, the renatured rLipL showed only 6% of the activity observed in native and fully active LipL, due to the formation of a tight complex between rLipL and rLimL as detected by immunoprecipitation (Ihara *et al.*, 1995). This suggests that bound rLimL inhibits hydrolytic activity of rLipL and that some additional mechanism exists that facilitates LimL dissociation. To obtain fully active LipL in large amounts, it is essential to devise an efficient reactivation procedure for the inclusion bodies of rLipL, because the amount of native LipL that can be obtained in *Pseudomonas* sp. strain 109 is low.

In this study, we found a novel lipase-activating factor (LAF) in *Pseudomonas* sp. strain 109. This factor synergistically acted with Ca^{2+} on the rLipL–rLimL complex to increase lipase activity 1.8-fold, and was found to be a novel low- M_r compound (M_r 330 ± 30). The increase of lipase activity was confirmed to be associated with the release of free rLipL from the rLipL–rLimL complex.

METHODS

Bacterial strains, plasmids and media. *Pseudomonas* sp. strain 109, a LipL producer (Ihara *et al.*, 1991) (strain FERM-P no. 3025, the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan), was used as the source of lipase activation factor throughout this study. *E. coli* BL21(DE3)pLysS [*hsdS gal* (λ clts-857 *ind-1 Sam-7 nin-5 lacUV5-T7* gene 1) pLysS] was used as the host for overexpression of *lipL* and *limL* genes from the pET-3d (Studier *et al.*, 1990) based vectors pETY402 (Ihara *et al.*, 1995) and pLIM413 (Ihara *et al.*, 1995), respectively.

Preparation of crude cell-free lysate of *Pseudomonas* sp. strain 109 and fractionation by ultrafiltration. *Pseudomonas* sp. strain 109 was grown in 250 ml LB medium in a 500 ml Sakaguchi flask at 30 °C and 120 r.p.m. for about 12 h to an OD₆₀₀ of 2.5. Cells were harvested by centrifugation (3000 g, 10 min, 4 °C), suspended in 5 ml 50 mM potassium phosphate buffer (pH 6.5) and sonicated (4 × 30 s, 4 °C). Cell debris was removed by centrifugation (10000 g, 20 min, 4 °C), and the

supernatant was used as crude cell-free lysate and stored at –80 °C until use.

To fractionate the crude cell-free lysate by M_r , the lysate was either dialysed with a cellulose dialysis membrane (Wako Pure Chemical) against a 100-fold volume of 50 mM potassium phosphate buffer (pH 6.5) for 12 h at 4 °C, or filtered through a Centrprep 3 (M_r cut ≥ 3000) or Centrprep 30 (M_r cut ≥ 30000 , Amicon). The filtrates from the ultrafiltrations were referred to as <3000 lysate and <30000 lysate, respectively.

Renaturation of recombinant LipL (rLipL). rLipL and recombinant LimL (rLimL) were overexpressed from plasmids pETY402 and pLIM413, respectively, in *E. coli*, using an LB medium containing 1% (w/v) glucose, 10 µg ampicillin ml⁻¹ and 2 µg chloramphenicol ml⁻¹, purified as described previously (Ihara *et al.*, 1995), and stored at –80 °C until use. For obtaining the rLipL–rLimL complex, purified inclusion bodies of rLipL (0.5 mg) were solubilized in 1 ml 6 M urea. After 1 h 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 50 mM potassium phosphate buffer (pH 6.5) containing 2 M urea for 3 h at 4 °C and two changes of 500 ml 50 mM potassium phosphate buffer (pH 6.5) for 3 h.

Gel-filtration HPLC. HPLC was performed with a JASCO model Tri Rotor-V equipped with a UV detector (JASCO UVIDEC-100-V). The <3000 lysate (250 µl) was applied at room temperature onto a Superdex Peptide HR 10/30 column (1.0 × 30 cm, Pharmacia Biotech) pre-equilibrated with 50 mM potassium phosphate buffer (pH 6.5) containing 0.2 M NaCl. The <3000 lysate was then fractionated from the column with the same buffer at a flow rate of 0.5 ml min⁻¹ and collected in 200 µl fractions.

Gel-filtration on a SMART system. Gel-filtration of rLipL–rLimL complexes was performed using a SMART system (Pharmacia Biotech). The rLipL–rLimL complex, either without treatment or activated by LAF, was injected into a Superose12 PC 3.2/30 (0.32 × 30 cm, Pharmacia Biotech) gel-filtration column equilibrated with 50 mM potassium phosphate buffer (pH 6.5) containing 0.2 M NaCl and 0.1% (w/v) NoigenHC at a flow rate of 25 µl min⁻¹. Protein elution was monitored by absorbance at 280 nm and the eluate was fractionated at room temperature into portions of 25 µl.

Western blot analysis. The fractions (5 µl samples) from gel-filtration on the SMART system were directly applied to SDS-PAGE after boiling in the presence of 1% SDS, and 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 (Ihara *et al.*, 1995). Immunoreactive proteins on Western blots were visualized by the use of horseradish-peroxidase-conjugated anti-rabbit IgG (Amersham Life Science).

Lipase activation assay for LAF. A typical activation mixture (100 µl) contained 0.05 M potassium phosphate (pH 6.5), 3 µg of the solubilized rLipL–rLimL complex, *Pseudomonas* cell-free lysate, and/or other factors as indicated. Reactions were initiated by the addition of cell-free lysate containing LAF. Samples (10 µl) were withdrawn at appropriate times, mixed with 90 µl potassium phosphate buffer (pH 6.5), and assayed for lipase activity. Each experiment was repeated at least three times, and data are expressed as means of triplicate experi-

ments with all samples. Deviation in the activation by LAF was within $\pm 10\%$.

Enzyme and protein assay. Lipase activity was routinely assayed by measuring the amount of *p*-nitrophenol formed from *p*-nitrophenyl hexanoate. The substrate (5 mM *p*-nitrophenyl hexanoate) was emulsified completely by sonication in the presence of 0.5% (w/v) Triton X-100 in 50 mM potassium phosphate buffer (pH 6.5) containing 1 mM MgCl₂. After preincubation of the enzyme solution (1.5 ml) in the same buffer at 37 °C for 1 min, reactions were initiated by the addition of an equal volume of the substrate solution, followed by further incubation at 37 °C for 10 min. Reactions were terminated by the addition of ethanol (3 ml), and the absorbance at 400 nm was measured. One unit of lipase activity was defined as the amount of enzyme which liberates 1 μ mol *p*-nitrophenol min⁻¹. Protein concentration was determined by a dye-binding assay (Protein Assay Kit; Bio-Rad) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Effect of various factors on the activation of rLipL-rLimL complex

The presence of rLimL was essential to obtain lipase activity during the *in vitro* renaturation of purified rLipL inclusion bodies. However, the specific activity was only 6% of that of the corresponding amount of native lipase, and the renatured rLipL was found to be bound to rLimL (Ihara *et al.*, 1995). Therefore, we suspect that dissociation of rLimL is necessary to obtain a fully active enzyme. We found that native LipL lost its activity by binding with rLimL, reaching a plateau of 40% residual activity by binding 2 mol rLimL per 1 mol LipL. However, because the complex was very tight, it was not possible to liberate LipL from the complex under simple *in vitro* conditions (data not shown). This difficulty of spontaneous dissociation suggests the presence of additional component(s) in *Pseudomonas* sp. strain 109 that facilitate(s) the dissociation. By liberating fully active rLipL, the dissociation should theoretically result in a 2.5-fold increase of lipase activity from the rLipL-rLimL complex.

To investigate the possibility that another factor, of either high or low *M_r*, was required for the activation of the rLipL-rLimL complex, we tested several compounds for their ability to enhance lipase activity from the solubilized rLipL-rLimL complex. Immediately after the addition of 1 mM CaCl₂, lipase activity was enhanced 1.7-fold, while 1 mM ATP or other divalent metal ions (1 mM MgCl₂, 1 mM ZnCl₂, 1 mM FeCl₃) were either inhibitory or had no effect. Because Ca(NO₃)₂ was similarly effective in enhancing lipase activity (data not shown), Ca²⁺ was concluded to be an effective component. Addition of *Pseudomonas* crude cell-free extract resulted in a small increase in activity at 0 h, but after incubation at 4 °C for 24 h, lipase activity increased about 1.8-fold, suggesting that some novel component(s) were present in the lysate. A similar level of activation (1.4-fold) was observed when native LipL was treated similarly after denaturation with 6 M urea. To discriminate between the activation by Ca²⁺ and that by the lysate, the time-course of activation was com-

Fig. 1. Increase of lipase activity during incubation of rLipL-rLimL complex at 4 °C in the presence of (a) CaCl₂ at 0 mM (○), 1 mM (□) or 5 mM (△), and (b) *Pseudomonas* cell lysate at 0 μ l (●), 5 μ l (■) or 25 μ l (▲). One microlitre of the cell lysate contained 60 μ g protein.

pared in the presence of Ca²⁺ or the lysate (Fig. 1). The effect of Ca²⁺ was instantaneous and concentration dependent, but the enhanced activity gradually decreased after a 24 h incubation, reaching 47–83% of that at 0 h (Fig. 1a). The effect of the lysate was also concentration dependent, but in this case the activation proceeded gradually with time until 24 h (Fig. 1b), indicating clearly that the action of the LAF in the lysate was different from that of Ca²⁺. Atomic absorption spectroscopy indicated that the crude cell-free lysate contained only 70 μ M Ca²⁺. Because at least 0.5 mM Ca²⁺ was necessary to see the activation, 70 μ M Ca²⁺ could not account for the activation by the lysate; this suggested that the LAF is not Ca²⁺. Activation by the lysate was faster at higher temperatures (1.8-fold increase after 2 h at 30 °C or after 24 h at 4 °C), although longer incubation at higher temperatures caused gradual loss of lipase activity due to thermal inactivation. To facilitate the reaction, we chose to perform activation by LAF at 30 °C in the following studies.

Characteristics of LAF

To gain insight into the structure of LAF, the crude cell-free extract was dialysed using a cellulose membrane. The dialysed sample did not show any activation, suggesting that LAF is a low-*M_r* compound that passes through the dialysis membrane. No lipase-activation activity was detected in the outer solution, although at least 20-fold volume of dialysis buffer was necessary to

Table 1. Characterization of LAF

Filtrate from $M_r \geq 3000$ ultrafiltration (<3000 lysate) was treated as indicated and assayed for its ability to activate the rLipL-rLimL complex after 2 h incubation at 30 °C. One unit of each enzyme was used to treat the <3000 lysate at 37 °C for 30 min. For pH stability, the <3000 lysate was mixed with an equal volume of 2 M NaOH or 2 M HCl. The solution was left for 60 min at 37 °C, neutralized, and then added to the rLipL-rLimL complex. Similarly diluted lysate was used as a control. Heat treatment of the lysate was done in a boiling water bath for 10 min. All samples containing lysate contained 5 μ l cell-free lysate per 3 μ g rLipL-rLimL complex.

Addition	Lipase activity (units mg ⁻¹)	Activation (-fold)
No lysate	14	(1.0)
Crude cell-free lysate	27	1.9
Dialysed lysate	16	1.2
<30000 lysate	28	2.0
<3000 lysate	29	2.1
<3000 lysate (proteinase treated)	27	1.9
<3000 lysate (amylase treated)	28	2.0
<3000 lysate (glucoamylase treated)	26	1.9
<3000 lysate (phosphatase treated)	26	1.9
<3000 lysate (lipase treated)	30	2.1
<3000 lysate (37 °C)	20	1.4
<3000 lysate (1 M HCl treated)	27	1.9
<3000 lysate (1 M NaOH treated)	26	1.9
<3000 lysate (boiled)	15	1.1

Fig. 2. M_r determination of LAF by gel-filtration HPLC. Filtrate (250 μ l) from $M_r \geq 3000$ ultrafiltration (<3000 lysate) was separated on a Superdex Peptide column (1.0 cm $\times$ 30 cm, Pharmacia-Biotech) into fractions of 200 μ l, and each fraction was assayed for lipase activation (vertical bars, right scale). Substance P (M_r 1347.6), glutathione (oxidized form, M_r 612.6), glutathione (reduced form, M_r 307.3) and cysteine (M_r 121.2) were used as M_r markers. The M_r of LAF was estimated from a $\log(M_r)$ versus retention time plot ($\bullet$, left scale).

sufficiently remove low- M_r compounds from the lysate. Using centrifugation-forced ultrafiltration, both filtrates (<3000 lysate and <30000 lysate) showed the same level of lipase activation as that of the whole cell-lysate (Table 1), indicating that LAF is a low- M_r compound.

Fig. 3. Additive effect of LAF and Ca^{2+} ions on the activation of the rLipL-rLimL complex. The rLipL-rLimL complex was incubated with no additions ($\bullet$), with 1 mM CaCl_2 ($\blacklozenge$), with <3000 lysate ($\blacksquare$), or with 1 mM CaCl_2 and <3000 lysate ($\blacktriangle$).

LAF in the <3000 lysate was resistant to proteinase, amylase, glucoamylase, phosphatase and lipase (Table 1). LAF was stable under acid or alkaline conditions, but was heat labile. The hydrophilic nature of LAF is indicated by the fact that it was not extracted by organic solvents (hexane, CH_2Cl_2 , ethyl acetate), nor was it retained on a C_{18} reverse-phase column with water as mobile phase. Although LAF was weakly retained on a strong anion-exchange column (such as Dowex 1), recovery of LAF activity was very low (less than 10%), which prevented further purification.

Fig. 4. Effects of LAF and Ca^{2+} ions on the dissociation of the rLipL-rLimL complex. (a) Filtrate (10 μl) from 10-fold concentrated rLipL-rLimL complex, incubated with or without <3000 lysate or 1 mM CaCl_2 for 2 h, was separated on a Superose 12 column (0.32 $\times$ 30 cm, Pharmacia Biotech). Each fraction (25 μl) was assayed for lipase activity. (b) Western blot analysis of fractions 12–20 from the Superose 12 column.

To determine its M_r more precisely, the <3000 lysate was separated by gel-filtration HPLC using a Superdex Peptide column (Fig. 2). Only factors eluting from 32.8 min to 33.2 min corresponding to M_r 330 ± 30 from a $\log(M_r)$ versus retention time plot showed lipase-activation activity. Therefore, it can be concluded that the LAF of *Pseudomonas* sp. strain 109 is a negatively charged, hydrophilic compound(s) of M_r 330 ± 30 .

Synergistic effect of LAF and Ca^{2+}

Although the activation by LAF and that by Ca^{2+} were observed to be different, activation by LAF was synergistic with Ca^{2+} (Fig. 3). When the rLipL-rLimL complex was incubated with <3000 lysate in the presence or absence of Ca^{2+} , the activity observed in the presence of Ca^{2+} was 1.8 fold higher than that in the absence of Ca^{2+} . Addition of 1 mM CaCl_2 was sufficient for maximum activation in these experiments: no additional increase was observed with higher Ca^{2+} concentrations, such as 5 mM (data not shown). Although the mechanism is unclear at present, LAF seems to stabilize the Ca^{2+} -activated form of lipase.

Dissociation of free rLipL from the rLipL-rLimL complex by LAF

We propose that LAF enhances lipase activity from the rLipL-rLimL complex by dissociation of free rLipL from the complex. To confirm this, the LAF-activated rLipL-rLimL complex was analysed 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. 4). After a 2 h activation at 30 °C with or without <3000 lysate, 30 μg of the complex was injected and separated into 25 μl fractions. Although the elution profiles of proteins at 280 nm looked very similar, the elution profiles of lipase activities were different. Lipase activity from the sample without <3000 lysate eluted from 43.4 min to 59.4 min (fractions 11–21) with a maximum at 53.4 min (fraction 16), and followed the A_{280} profile. Western blot analysis of each fraction revealed that these lipase-active fractions contained both rLipL and rLimL in a ratio of 1:1, indicating that they represented the rLipL-rLimL complex. Under the gel-filtration conditions, native LipL elutes in fractions 14–15 (i.e. with a higher M_r than expected, probably because the protein is in complex

with the detergent present in the column buffer) and rLipL elutes in fractions 18–19 (data not shown). In the sample incubated with < 3000 lysate, peak lipase activity eluted earlier, in fraction 14, before the main protein peak. These fractions (fractions 14 and 15) contained a higher LipL:LimL ratio. Incubation of the rLipL–rLimL complex with Ca^{2+} alone resulted in peak lipase activity in fractions 14–15 but showed no dissociation of rLipL when the fractions were assayed with antibodies, while incubation with both < 3000 lysate and Ca^{2+} resulted in 2.0-fold higher absorption area in fractions 13–15 than that of the sample with < 3000 lysate alone. The 2.0-fold higher absorption area agreed well with the 1.8-fold increase of lipase activity and suggested the increase of free rLipL, as confirmed by Western blot analysis. These results indicate that coexistence of Ca^{2+} stimulated the LAF-dependent dissociation of free LipL from the rLipL–rLimL complex, although the mechanism by which Ca^{2+} has its effect is not clear at present.

Conclusions

We have demonstrated that two low- M_r factors (Ca^{2+} ions and natural LAF from *Pseudomonas* sp. strain 109) act on a rLipL–rLimL complex to increase lipase activity. Although the structure of LAF has not been identified, it is a hydrophilic and proteinase-resistant compound(s) of M_r 330 ± 30 , and its major role in lipase activation is as a stimulator of rLipL dissociation. Ca^{2+} alone did not cause rLipL dissociation but it stimulated the LAF-dependent rLipL dissociation.

ACKNOWLEDGEMENTS

This research was supported in part by the grant from the Nisshin Seifun Foundation.

REFERENCES

- Frenken, L. G. J., Bos, J. W., Visser, C., Muller, W., Tommassen, J. & Verrips, C. T. (1993a). An accessory gene, *lipB*, required for the secreted lipase of *Pseudomonas glumae* lipase. *Mol Microbiol* **9**, 579–589.
- Frenken, L. G. J., Groot, A., Tommassen, J. & Verrips, C. T. (1993b). Role of the *lipB* gene product in the folding of the secreted lipase of *Pseudomonas glumae*. *Mol Microbiol* **9**, 591–599.
- Hirayama, N. O., Yoshikawa, K., Nishioka, T. & Oda, J. (1993). Lipase from *Pseudomonas aeruginosa*. *Eur J Biochem* **215**, 239–246.
- Hobson, A. H., Buchley, C. M., Aamand, J. L., Jørgensen, S. T., Diderichsen, B. & McConnell, D. J. (1993). Activation of bacterial lipase by its chaperone. *Proc Natl Acad Sci USA* **90**, 5682–5686.
- Ihara, F., Kageyama, Y., Hirata, M., Nihira, T. & Yamada, Y. (1991). Purification, characterization, and molecular cloning of lactonizing lipase from *Pseudomonas* species. *J Biol Chem* **266**, 18135–18140.
- Ihara, F., Okamoto, I., Nihira, T. & Yamada, Y. (1992). Requirement in *trans* of the downstream *limL* gene for activation of lactonizing lipase from *Pseudomonas* sp. 109. *J Ferment Bioeng* **73**, 337–342.
- Ihara, F., Okamoto, I., Akao, K., Nihira, T. & Yamada, Y. (1995). Lipase modulator protein (LimL) of *Pseudomonas* sp. strain 109. *J Bacteriol* **177**, 1254–1258.
- Iizumi, T., Nakamura, K., Shimada, Y., Sugihara, A., Tominaga, Y. & Fukase, T. (1991). Cloning, nucleotide sequencing, and expression in *Escherichia coli* of a lipase and its activator genes from *Pseudomonas* sp. KWI-56. *Agric Biol Chem* **55**, 2349–2357.
- Iizumi, T. & Fukase, T. (1994). Role of the gene encoding lipase activator from *Pseudomonas* sp. strain KWI-56 in *in vitro* activation of lipase. *Biosci Biotechnol Biochem* **58**, 1023–1027.
- Jørgensen, S. T., Skov, K. W. & Diderichsen, B. (1991). Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes. *J Bacteriol* **173**, 559–567.
- Keller-Schierlein, W. (1973). Chemie der Makrolid-Antibiotica. *Fortschr Chem Org Naturst* **30**, 313–460.
- Kok, R. G., van Thor, J. J., Nugteren-Roodzant, I. M., Vosman, B. & Hellingwerf, K. J. (1995). Characterization of lipase-deficient mutants of *Acinetobacter calcoaceticus* BD413: identification of a periplasmic lipase chaperone essential for the production of extracellular lipase. *J Bacteriol* **177**, 3295–3307.
- Makita, A., Nihira, T. & Yamada, Y. (1987). Lipase catalysed synthesis of macrocyclic lactones in organic solvent. *Tetrahedron Lett* **28**, 805–808.
- Ogierman, M. A., Failarimo, A., Riess, T., Williams, S. G., Attridge, S. R. & Manning, P. A. (1997). Characterization of the *Vibrio cholerae* El Tor lipase operon *lipAB* and a protease gene downstream of the *bly* region. *J Bacteriol* **179**, 7072–7080.
- Siomi, M. C., Yoshikawa, K., Hirayama, N. O., Yamamoto, K., Sogabe, Y., Nakatani, T., Nishioka, T. & Oda, J. (1992). Purification, molecular cloning, and expression of lipase from *Pseudomonas aeruginosa*. *Arch Biochem Biophys* **296**, 505–513.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). Use of RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60–89.
- Wohlfarth, S. & Winkler, U. K. (1992). Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. *J Gen Microbiol* **138**, 1325–1335.
- Wong, C. & Whitesides, G. M. (1994). *Enzymes in Synthetic Organic Chemistry*, pp. 70–108. Amsterdam: Elsevier.
- Zaks, A. & Klibanov, A. M. (1984). Enzymatic catalysis in organic media at 100 °C. *Science* **224**, 1249–1251.
- Zaks, A. & Klibanov, A. M. (1985). Enzyme-catalyzed processes in organic solvents. *Proc Natl Acad Sci USA* **82**, 3192–3196.

Received 29 January 1999; revised 19 April 1999; accepted 7 June 1999.