Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 39 (2005) 97–106
Protein Expression & Purification
www.elsevier.com/locate/yprep

High-level heterologous expression and properties of a novel lipase from *Ralstonia* sp. M1

Dinh Thi Quyen[a], Thi Thu Giang Le[a], Thi Thao Nguyen[a], Tae-Kwang Oh[b], Jung-Kee Lee[b,*]

[a] *Institute of Biotechnology, Vietnamese Academy of Science and Technology 18 Hoang Quoc Viet Road, Distr. Caugiay, 10600 Hanoi, Viet Nam*
[b] *Laboratory of Microbial Genomics, Korea Research Institute of Bioscience and Biotechnology, Yusong, Daejeon 305-333, Republic of Korea*

Received 3 August 2004, and in revised form 30 September 2004
Available online 11 November 2004

Abstract

The mature lipase LipA and its 56aa-truncated chaperone ΔLipBhis (with 6×his-tag) from *Ralstonia* sp. M1 were over-expressed in *Escherichia coli* BL21 under the control of T7 promoter with a high level of 70 and 12 mg protein per gram of wet cells, respectively. The simply purified lipase LipA was effectively refolded by Ni–NTA purified chaperone ΔLipBhis in molar ratio 1:1 at 4 °C for 24 hours in H_2O. The in vitro refolded lipase LipA had an optimal activity in the temperature range of 50–55 °C and was stable up to 45 °C with more than 84% activity retention. The maximal activity was observed at pH 10.75 for hydrolysis of olive oil and found to be stable over alkaline pH range 8.0–10.5 with more than 52% activity retention. The enzyme was found to be highly resistant to many organic solvents especially induced by ethanolamine (remaining activity 137–334%), but inhibited by 1-butanol and acetonitrile (40–86%). Metal ions Cu^{2+}, Sn^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} stimulated the lipase slightly with increase in activity by up to 22%, whereas Zn^{2+} significantly inhibited the enzyme with the residual activity of 30–65% and Fe^{3+} to a lesser degree (activity retention of 77–86%). Tween 80, Tween 60, and Tween 40 induced the activation of the lipase LipA (222–330%) and 0.2–1% (w/v) of Triton X-100, X-45, and SDS increased the lipase activity by up to 52%. However, 5% (w/v) of Triton X-100, X-45, and SDS inhibited strongly the activity by 31–89%. The inhibitors including DEPC, EDTA, PMSF, and 2-mercaptoethanol (0.1–10 mM) inhibited moderately the lipase with remaining activity of 57–105%. The lipase LipA hydrolyzed a wide range of triglycerides, but preferentially short length acyl chains (C4 and C6). In contrast to the triglycerides, medium length acyl chains (C8 and C14) of *p*-nitrophenyl (*p*-NP) esters were preferential substrates of this lipase. The enzyme preferentially catalyzed the hydrolysis of cottonseed oil (317%), cornoil (227%), palm oil (222%), and wheatgerm oil (210%) in comparison to olive oil (100%).

Keywords: Lipase; Chaperone; Over-expression; *Ralstonia* sp. M1; *E. coli*; Properties

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of triglycerides at the interface between the insoluble substrate and water [1] and have been utilized widely in industrial fields including detergents, dairy, diagnostics, oil processing, biotransformations, and chiral due to substrate specificity, regiospecificity, enantiomeric selectivity, thermostability, and alkaline stability [2].

Recently, bacterial lipase family I has been divided into seven subfamilies on basis of amino acid sequence homology [2]. Among microbial lipases, *Pseudomonas* lipases have been extensively studied for their different biotechnological applications. Subfamily I.1 comprises the lipases from *Pseudomonas aeruginosa* [3,4], *Acinetobacter* sp. [5,6], and *Pseudomonas fragi* [7], and subfamily I.2 contains the lipases from *Pseudomonas glumae* [8],

* Corresponding author. Fax: +82 42 860 8595.
E-mail address: jklee@kribb.re.kr (J.-K. Lee).

1046-5928/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2004.10.001

Pseudomonas cepacia [9], *Chromobacterium viscosum* [10], *Pseudomonas luteola* [11], and a novel recently identified lipase from *Ralstonia* sp. M1.[1]

Members of two lipase subfamilies I.1 and I.2 share high amino acid sequence homology (>33%) [12] and most of them have another common property that their lipase genes are clustered with the secondary genes located immediately downstream [3–5,9,13] or upstream of the lipase genes [6]. The secondary genes encode protein products that are so-called lipase-specific foldases (modulator, activator, helper protein, or chaperone) required for formation of active lipases and production of extracellular lipases. These lipases are secreted via the two-step type II secretion pathway, where the lipase chaperones directly participate in the folding and secretion process.

Pseudomonas and *Burkholderia* lipases from subfamilies I.1 and I.2, respectively, have been used broadly as detergent additive and for a variety of biotransformations. These lipases can be over-expressed in *E. coli* using conventional over-expression systems. However, much difficulty has been faced in producing the fully active recombinant lipases in *E. coli*. The main barrier is that *Pseudomonas* and *Burkholderia* lipases require a lipase-specific foldase for fully active formation. Recently, a considerable amount of effort has been directed at improving expression of the fully active lipases in *E. coli*. In a previous study, we have individually over-expressed lipase and its chaperone at levels of 40 and 60% of the total protein in *E. coli*, respectively, and refolded the lipase in vitro with its chaperone to recover 100% activity in comparison with the wild-type lipase [14]. Kim et al. [15] reported that a lipase variant Pro112Gln from *Pseudomonas* species KFCC 10818 formed an active enzyme and displayed 63% of the activity of wild-type lipase expressed in the presence of the chaperone.

In previous study, the lipase-encoding gene *lipA* and its chaperone-encoding gene *lipB* (Accession No. AY364601) from the *Ralstonia* sp. M1 (Accession No. AY376238) were cloned, analyzed, and identified as a member of the subfamily I.2 *Burkholderia* lipase/chaperone.[1] The amino acid sequence of the lipase LipA showed high identities of 49.3–60.3% and 33.9–47.7% to those of subfamily I.2 and I.1 lipases, respectively. Also the chaperone LipB had high identities of 23.7–32.7% and 11.5–28.6% to the subfamily I.1 and I.2 lipase chaperones, respectively. In this report, we have produced the mature lipase LipA and its 56aa-truncated chaperone ΔLipBhis in *E. coli* BL21 under the control of T7 promoter in large quantities. Both proteins were purified and the lipase was activated with its chaperone. Physicochemical properties of the in vitro refolded recombinant lipase were investigated.

[1] D. T. Quyen, T. T. Nguyen, T. T. G. Le, H. K. Kim, T. K. Oh, J. K. Lee, A novel lipase/chaperone pair from *Ralstonia* sp. M1: Analysis of the folding interaction and evidence for gene loss in *Ralstonia solanacearum.* Accepted for Molecular Genetics and Genomics.

Materials and methods

Materials

Triglycerides (C4–C18), triolein, *p*-NP esters (C2, C3, C6, C8, C14, and C16), olive oil, gum arabic, and natural oils (coconut oil, cottonseed oil, corn oil, peanut oil, soybean oil, and wheatgerm oil) were purchased from Sigma (USA). Oil of linseed was from Yakuri Pure Chemical (Osaka, Japan), beef tallow and palm oil were from Nongshim (Korea). Triton X-100 and Triton X-45 were provided by Bio-Rad Laboratories (CA, USA) and Fluka Chemie (AG Buchs, Swiss), respectively. Tween 80, Tween 60, and Tween 40 were from Shinyo pure chemicals (Osaka, Japan). Bacto-tryptone and yeast extract were from Difco (USA). Restriction enzymes, CIAP, T4 ligase were supplied by Roche (Germany). DNA Gel-Extraction Kit and Ni–NTA-matrice were from Qiagen. PCR mix and Minipreps were purchased from Bioneer (Korea). All other reagents were of analytical grade unless otherwise stated.

Plasmids, bacterial strains, and culture conditions

Two plasmids pELipAB and pELipB (Fig. 1) constructed previously[1] derived from the pET22b+ vector under the control of the T7 promoter were used to over-express the lipase LipA and its 56aa-truncated chaperone ΔLipBhis in *E. coli* BL21 (DE3), respectively. *E. coli* BL21 was grown routinely in LB medium with ampicillin (final concentration of 100 μg/ml) at 37 °C. Two hundred milliliters of LB medium containing 200 μl ampicillin (100 mg/ml) was inoculated with 2 ml of the overnight culture and cultivated at 37 °C, 220 rpm for more 3 h until $OD_{600\,nm}$ reached 0.6, then induced with 200 μl IPTG (100 mM). The cultures were incubated at 37 °C, 220 rpm for 3 h of induction. Wet cells harvested by centrifugation at 6000 rpm for 10 min at 4 °C can be used immediately for protein purification or stored at −20 °C for later purification.

Enzyme purification and estimation of protein concentrations

The 56aa-truncated chaperone ΔLipBhis was expressed in *E. coli* pELipB as soluble form containing a 6-his-tag. To purify the chaperone ΔLipBhis, the cell pellet (~200 mg wet weight) of 50 ml LB medium culture was harvested by centrifugation and dissolved in 1 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0). After ultrasonic cell disintegration (3 × 1 min with 1 min pause), the cell suspension was

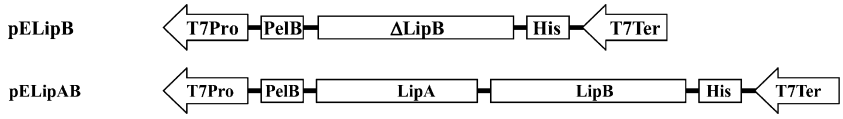

Fig. 1. Different expression vectors pELipAB and pELipB derived from the expression vector pET22b+ for *E. coli* BL21 system. The inserts and restriction sites used for cloning are given. T7Pro, T7 promoter; PelB, PelB signal peptide; LipA, mature lipase; His, 6-histidine tag; T7Ter, T7 terminator; ΔLipB, 56aa truncated chaperone; and LipB, the complete chaperone.

centrifuged at 12,000 rpm at 4 °C for 15 min. During that one milliliter of the Ni–NTA resins (Qiagen) was transferred to a 15-ml column and centrifuged at 2000 rpm for one minute at 4 °C. After equilibration of the column with 3 ml lysis buffer, the cell supernatant was applied to the Ni–NTA column, mixed well, and incubated at room temperature for 15 min. Then the column was centrifuged at 2000 rpm for one minute at 4 °C to remove all nontagged contaminating proteins and washed with two bed volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 50 mM imidazole, pH 8.0). The tagged protein was eluted three times with 3 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 200 mM imidazole, pH 8.0). Flow-throughs, wash fractions and eluates were collected for SDS–PAGE analysis.

In contrast to the chaperone, the mature lipase LipA was expressed as inclusion bodies. To purify LipA, the pellet from 50 ml culture was sonified (3 × 1 min with 1 min pause) in 1 ml of 5 mM imidazole buffer (pH 8.0) and the cell lysate was centrifuged at 12,000 rpm at 4 °C for 15 min. The process was repeated at least 5 times until the pellet was homogeneous with only one white phase. The homogeneous white pellet was resuspended in 1 ml of 8 M urea buffer (pH 8.0) and agitated for at least 1 h at room temperature. The lipase LipA solution was used for in vitro refolding study.

Protein concentrations were determined by Bradford method with Bio-Rad protein assay kit.

Gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli [16] with Bio-Rad equipment. SDS–PAGE was usually performed with gels of 12.5% (w/v) of acrylamide according to the manufacturer's recommendations.

In vitro refolding

In vitro refolding was performed as described previously.[1] Two hundred micrograms of lipase LipA obtained after purification was refolded in 10 ml distilled water in the presence of 220 μg (in molar ratio 1 to 1) of the 56aa-truncated chaperone ΔLipBhis. After 24 h at 4 °C, the lipase in the supernatant was used for characterization without removing the chaperone because no lipase activity was detected in the chaperone-only solution.[1]

Lipase activity estimation

pH-stat assay. Lipase activity was estimated using olive oil as a substrate in a pH-stat 718 (Metrohm) as described previously [17]. One per cent (v/v) of olive oil (or other natural oils and fats for substrate specificity) was emulsified in distilled water containing 0.5% (w/v) of gum arabic as stabilizer using a homogenizer (blendor, USA) for 3 × 1 min at maximum speed. A 20 ml volume of the triglyceride solution was heated to 55 °C and adjusted to pH 9.0. Autohydrolysis was measured in 4 min without addition of enzyme. After addition of 5–50 μl of the lipase solution the activity was measured for 4 min. One unit of lipase activity was defined as the amount of enzyme which released 1 μmol of fatty acid per min.

Effect of pH, temperature, organic solvents, detergents, and metal ions on lipase activity and stability

The optimum temperature for the enzyme activity was measured at pH 8.0 with olive oil as a substrate. The assay was carried out by incubating the reaction mixture at various temperatures from 25 to 75 °C. The effect of temperature on the lipase stability was determined by incubating aliquots of pure lipase solution for 30 min in 0.1 M Tris buffer, pH 8.0, at various temperatures from 25 to 80 °C. Residual activity was determined by pH-stat assay at pH 9.0 and 55 °C using olive oil as a substrate.

The optimum pH for the enzyme activity was measured at 55 °C by pH-stat assay using olive oil as a substrate. The assays were carried out by incubating the reaction mixtures at 55 °C, and at various pH values from 7.0 to 11.5. The effect of pH on the lipase stability was determined by incubating aliquots of pure lipase solutions for 30 min at 30 °C in 0.1 M (acetate, phosphate, Tris–HCl, glycine/HCl, or phosphate/KOH) buffers at different pH values. Residual activity was measured by pH-stat assay at pH 9.0 and 55 °C using olive oil as a substrate.

The effect of detergents on the lipase activity was determined by incubating the enzyme for 30 min at 30 °C in 0.1 M phosphate buffer (pH 8.0) containing 0.2, 1, and 5% (w/v) of detergents. The lipase activity was measured only at the end of the incubation time by pH-stat assay at pH 9.0 and 55 °C using olive oil as a substrate.

The effect of 10 and 30% (v/v) of organic solvents on the lipase activity was determined in a similar way as that of detergents. The enzyme mixtures were incubated

for 30 min at 30 °C; the control contained no organic solvent. The lipase activity was measured only at the end of the incubation time by pH-stat assay at pH 9.0 and 55 °C using olive oil as a substrate.

The effect of metal ions and inhibitors on the lipase activity was determined by incubating the enzyme for 30 min at 30 °C in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1, 1, and 10 mM of metal ions or inhibitors. The residual activity was determined by pH-stat assay at pH 9.0 and 55 °C using olive oil as a substrate.

All measurements were carried out three times and from these values the average value was taken.

Determination of substrate specificity

To determine the substrate specificity of the lipase LipA, the triglycerides tributyrin (C4), tricaproin (C6), tricaprylin (C8), tricaprin (C10), trilaurin (C12), trimyristin (C14), tripalmitin (C16), triolein (C18:1), and tristearin (C18) at a final concentration of 10 mM were each emulsified in distilled water containing 0.5% (w/v) of gum arabic and used as a substrate solution. The lipase activity of the purified enzyme towards triacylglycerides of fatty acids of different chain length was determined by pH-stat assay at 55 °C and pH 9.0.

To determine the substrate specificity of the lipase LipA towards *p*-NP esters, 10 μl of substrates (10 mM of *p*-NP acetate, propionate, caproate, and caprylate in acetonitrile) were incubated with 40 μl ethanol, 930 μl of 100 mM Tris–HCl buffer (pH 8.0) at 55 °C for 1 min. Then the reaction mixture was added with 20 μl of the enzyme solution and incubated at 55 °C for 3 min. Cleavage of pNPP was measured at $OD_{405\,nm}$ and 55 °C in a spectrophotometer (Sumizu). One unit was defined as the amount of enzyme, which cleaved 1 μmol pNPP to *p*-nitrophenol and palmitate per min under the assay conditions.

To measure the activity toward *p*-NP myristate and palmitate, 20 μl of the enzyme solution was incubated with 880 μl of the reaction mixture containing 100 mM Tirs–HCl buffer (pH 8.0), 0.1% gum arabic, and 0.2% deoxycholate at 55 °C for 3 min. The reaction mixture was then added with 100 μl of 8 mM substrate solution in isopropanol and incubated further for 3 min. The reaction was stopped with 0.5 ml of 3 N HCl and centrifuged. 333 μl of the supernatant was added with 1 ml of 2 M NaOH and measured at 420 nm.

Results and discussion

Expression, purification, and in vitro refolding of the lipase and chaperone

To over-expess the recombinant lipase in *E. coli*, the expression plasmid pELipAB containing the mature lipase gene with the chaperone gene under the control of T7 promoter was used (Fig. 1). The mature lipase LipA was expressed in *E. coli* BL21 (pELipAB) with a high level of 70 mg protein per one gram of wet weight cells (Fig. 2, lane 3). The mature lipase LipA was purified by the simple method of many steps ultrasonification-centrifugation. The SDS–PAGE pictures showed only one band after purifications (Fig. 2, lane 4).

For expression of the recombinant chaperone, the expression plasmid pELipB containing the 56aa-truncated chaperone gene under the control of T7 promoter was used (Fig. 1). The 56aa-truncated chaperone ΔLipB-his was expressed in *E. coli* BL21 (pELipB) with a high level of 12 mg protein per gram of wet weight cells (Fig. 2, lane 1). The purified ΔLipABhis exhibited only one protein band on SDS–PAGE picture (Fig. 2, lane 2). The yield from the Ni–NTA purification could be estimated as high as 70–80%. On SDS–PAGE picture (data not shown), most of the chaperone ΔLipABhis was recovered in the eluate in comparison to the flow-throughs and wash fractions.

Two hundred micrograms of the denatured lipase LipA purified from inclusion bodies from *E. coli* pELipAB was subjected to refolding procedures with 220 μg of the Ni–NTA purified truncated chaperone ΔLipBhis (in molar ratio 1:1) in 10 ml of distilled water at 4 °C for 24 h. This refolded lipase was used to characterize some physicochemical properties.

Effect of temperature on lipase activity and stability

One of important characteristics required for applications in detergent formulations and biotransformations is that the enzyme should be active at high temperature.

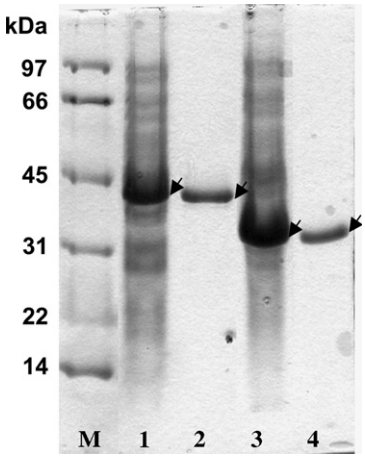

Fig. 2. SDS–PAGE of the over-expressed and Ni–NTA purified lipase LipA and its chaperone ΔLipB in *E. coli* BL21. Proteins are stained with Coomassie brilliant blue. Lane M, molecular standards indicated in kDa; lane 1, *E. coli* pELipB cell lysate after IPTG induction; lane 2, Ni–NTA purified chaperone ΔLipBhis (from *E. coli* pELipB lysate); lane 3, *E. coli* pELipAB cell lysate after IPTG induction; and lane 4, purified lipase LipA (from *E. coli* pELipAB cell lysate).

The optimum temperature of the lipase LipA was investigated using olive oil as a substrate at pH 8.0. The maximum temperature was 55 °C for hydrolysis of olive oil (Fig. 3). The lipase activity increased very gradually from 20% at 25 °C to the maximum of 100% at 55 °C and then decreased steeply to 69% at 60 °C. From 60 to 75 °C, the lipase activity decreased very slightly from 69 to 57%.

The various incubation temperatures from 25 to 80 °C showed an obvious temperature effect on the lipase stability with olive oil as the substrate incubated in 0.1 M Tris–HCl buffer, pH 8.0, for 30 min. The lipase activity was determined using pH-stat at 55 °C and pH of 9.0. The relative activity of the lipase LipA not treated with temperature (control sample) was fixed as 100%. The relative residual lipase activity did not increase monotonically with temperature. It increased up to 117% when incubated at the lower temperature (25–40 °C) (Fig. 3). The lipase LipA was stable up to 45 °C when incubated for 30 min at pH 8.0 in 0.1 M Tris buffer. Its relative residual lipase activity remained 84% at the incubation temperature of 45 °C. Above this temperature, the residual activity decreased steeply to 3% at 60 °C. Most of the lipase was inactivated (<3%) in the temperature range from 60 to 80 °C (Fig. 3).

The effect of temperature on the activity and stability of the recombinant lipase LipA was little a bit different to that of the wild-type lipase from *Ralstonia* sp. M1. The wild-type lipase had a maximum activity at 55–60 °C and was stable up to 75 °C (Table 4). At the temperature interval 25–75 °C, the residual activity was up to 133%.[2] The wild-type lipase was much more stable than the recombinant lipase LipA. Why was there a big difference in the thermostability between both enzymes? One explanation might be that the wild-type lipase was more correctly refolded than the recombinant lipase LipA, which was in vitro refolded, thus it had higher thermostability than the recombinant lipase. The optimal reaction temperature reported here is lower than that of the lipase from *Bacillus thermocatenulatus* (60–70 °C) [17], the same of the lipases from *Acinetobacter* sp. RAG-1 [18], and higher than that of the lipases from *Acinetobacter* sp. SY-01 (50 °C) [19]. The lipase LipA from *Ralstonia* sp. M1 showed lower thermostability (up to 45 °C) than the lipase from *B. thermocatenulatus* (50 °C). However, the data of the optimal reaction temperature and thermostability supported that the lipase LipA is thermophilic.

Effect of pH on lipase activity and stability

The optimum activity of the lipase LipA was investigated at 55 °C using olive oil as a substrate with a pH

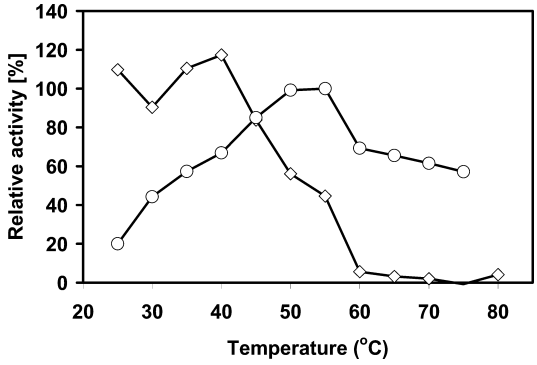

Fig. 3. Effect of temperature on the lipase activity (○) and stability (□). For temperature optimum, the activity of 1 μg of the refolded lipase was measured by pH-stat assay with 1% olive oil as a substrate at pH 8.0 and various temperature 25–75 °C. For thermostability, 1 μg of the lipase was incubated in 0.1 M Tris–HCl buffer, pH 8.0, for 30 min, at various temperature 25–80 °C, and activity was determined by pH-stat assay with 1% olive oil as a substrate at pH 9.0 and 55 °C.

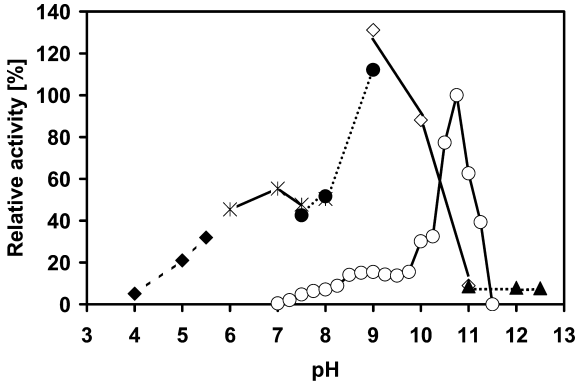

Fig. 4. Effect of pH on the lipase activity (○) and stability (□). For pH optimum, the activity of 1 μg of the lipase was estimated by pH-stat assay with 1% olive oil as a substrate at 55 °C and various pH 7.0–11.0. For pH stability, 1 μg of the enzyme was treated in 0.1 M buffer with various pH 4.0–12.5 at 30 °C for 30 min and the lipase activity was determined by pH-stat assay with 1% olive oil as a substrate at 55 °C and pH 9.0.

range from 7.0 to 11.5 (Fig. 4). The lipase showed maximum activity at pH 10.75. The relative activity increased very slowly and gradually from 0.4% at pH 7.0 to 14.1% at pH 8.5 then it remained constantly 13.7–15.5% in a pH range from 8.5 to 9.75. The residual activity increased steeply from 15.5% at pH 9.75 to the maximum activity (100%) at 10.75 as well as decreased steeply after the maximum. At pH 11.5, no lipase activity with olive oil was observed. This lipase belongs to alkaline lipases.

Different pH values of incubation buffers of 0.1 M (sodium acetate pH 4.0–5.5, potassium phosphate pH 6.0–8.0, Tris–HCl pH 7.5–9.0, glycine/NaOH pH 9.0–11.0, and phosphate/KOH pH 11.0–12.5) showed an obvious effect on the lipase stability with olive oil after 30 min of incubation at 30 °C. The relative activity of the lipase which was not incubated as control was fixed as 100%. The lipase LipA stability was low at the pH range

[2] D. T. Quyen, T.-k. Oh, J.-K. Lee, Production and characterization of an extracellular thermophilic, alkaline, highly organic-solvent-resistant and detergent-inducible lipase from *Ralstonia* sp. M1, Submittion to Enzyme Microb. Technol., a manuscript in process.

from 4.0 to 6.0 and the residual lipase activity increased gradually from 5% at pH 4.0 to 45% at pH 6.0. The residual lipase activity remained one half (45–55%) in the pH range 6.0–8.0 and increased steeply to the maximum at pH 9.0 (112 or 131% for Tris–HCl buffer and glycine/KOH buffer, respectively). When the incubation pH increased to 10, the lipase remained stable with a relative lipase activity of 88%. Then the residual lipase activity decreased steeply to 9% at the incubation pH of 11.0. Within the high pH range from 11.0 to 12.5, the lipase LipA showed the low relatively constant stability (8–9%) after 30 min incubation at 30 °C (Fig. 4).

The wild-type lipase from *Ralstonia* sp. M1 had an optimal pH 10 and was stable over a wider pH range from 7.5 to 11[2] (Table 4). The optimal pH (10.75) of the recombinant lipase LipA was higher than that (pH 10.0) of the wild-type lipase, however the pH stability range of the wild-type enzyme (pH 7.5–11.0) was broader than that of the recombinant enzyme (pH 8.0–10.5). Little difference in pH optimum and stability might be due to the different refolding processes of both lipase forms. The findings (high pH optimum and alkaline stability) are in agreement with those for alkaline lipases reported for strains *Acinetobacter* [18,19] and *Bacillus* [17]. The lipase from *Acinetobacter* sp. RAG-1 was found to be stable at pH 5.8–9.0, with optimal activity at 9.0 [18]. *Actinetobacter* sp. SY-01 lipase had an optimal lipase activity at pH 10.0 and was stable at pH 9.0–11.0 [19]. *B. thermocatenulatus* lipase BTL2 showed an optimal activity at pH 7.5–9.0 and stability in a pH range from 7.0 to 11.0 [17]. Having an optimal activity and stability at an akaline pH range is of importance of lipases that are used as detergent additives and catalysts.

Effect of organic solvents on lipase activity

Another important characteristic of protein catalysts used in organic synthesis reactions is that the enzymes are stable and active in organic solvents. The effect of 10–30% (v/v) of organic solvents (Table 1) on lipase activity was investigated by incubating the lipase LipA for 30 min at 30 °C in 0.1 M phosphate buffer (pH 8.0). Acetonitrile and 1-butanol showed an obvious inhibitory effect on the lipase LipA activity. The residual lipase activity towards olive oil in the presence of 10–30% (v/v) 1-butanol or acetonitrile was 40–86%. The addition of 10% (v/v) of other organic solvents stimulated the lipase LipA and increased the activity by 9–67% (Table 1). Increasing the solvent concentration from 10 to 30%, these organic solvents showed different effects on the lipase activity. The addition of 30% (v/v) of DMFA, acetone, ethanol, and isopropanol decreased the lipase activity by 44–57% in comparison to the control sample. The addition of 30% (v/v) of DMSO and methanol showed no significant effect on the lipase activity, the residual lipase activity remained (100–104%). The addition of 30% (v/v) of *tert*-butanol, hexane, dichloromethane, ethyl acetate and especially ethanolamine induced the lipase activation and increased the activity strongly by 41–234% in comparison to the control sample.

Table 1
Effect of organic solvents on lipase activity

Solvent	Remaining activity (%) at concentration (%) of	
	10	30
Methanol	110	104
Ethanol	143	56
Isopropanol	139	56
1-Butanol	53	71
tert-Butanol	118	141
Dichloromethane	167	155
Ethyl acetate	109	198
Acetonitrile	86	40
Acetone	116	55
Ethanolamine	137	334
Hexane	152	153
DMSO	164	100
DMFA	128	43

One microgram of the refolded lipase was incubated in 0.1 M phosphate buffer, pH 8.0 at 30 °C for 30 min with 10 or 30% (v/v) of different solvents. The lipase activity was measured by pH-stat assay with 1% olive oil as a substrate at pH 9.0 and 55 °C.

In general, most organic solvents stimulated the activation of the lipase LipA as well as the wild-type enzyme from *Ralstonia* sp. M1,[2] whereas a strong inhibitory effect of organic solvents on activity was found for the lipase from *Acinetobacer* sp. SY-01 [19] and no significant effect for the lipase from *Acinetobacter* sp. RAG-1 [18]. The residual activity of the lipase LipA and the wild-type lipase increased to 1.5–3.3 times and up to 2.7 times in the presence of 10–30% (v/v) of many organic solvents, respectively. In contrast to the lipase LipA, the lipase from *Acinetobacter* sp. SY-01 showed a residual activity between 9 and 58%, except for methanol (100%) and the lipase from *Acinetobacter* sp. RAG-1 had a remaining activity between 72 and 100%, except for pyridine (0–25%). High stability and activity of the lipase LipA in organic solvents are potentials for applications of this lipase in the organic synthesis reactions.

Effect of ions and inhibitors on lipase activity

The effect of 0.1, 1, and 10 mM of various metal ions was determined by incubating the lipase LipA for 30 min in 0.1 M Tris–HCl buffer, pH 8.0. In general, all assayed metal ions showed no significant effect on the lipase LipA activity, except for Zn^{2+} and Fe^{3+} (Table 2). The addition of 0.1, 1, and 10 mM of Zn^{2+} inhibited the lipase significantly, the residual lipase activity was 65, 34, and 30% in comparison to the control sample, respectively. Also Fe^{3+} showed a moderately inhibitory effect on lipase activity. The addition of 0.1, 1, and 10 mM of Fe^{3+} decreased the lipase activity by 14–23%. In contrast to Zn^{2+} and Fe^{3+} ions, incubating the lipase with 0.1, 1, and

Table 2
Effect of metal ions and inhibitors on lipase activity

Compound	Remaining activity (%) at concentration (mM) of		
	0.1	1	10
$CaCl_2$	114	99	83
$CuCl_2$	119	121	101
$FeCl_3$	82	77	86
$MgCl_2$	98	110	85
$MnCl_2$	106	93	122
$SnCl_2$	112	101	103
$ZnCl_2$	65	34	30
DEPC	105	93	85
EDTA	72	73	71
PMSF	99	85	57
2-Mercaptoethanol	82	68	95

One microgram of the refolded lipase was incubated in 0.1 M Tris–HCl buffer, pH 8.0 at 30 °C for 30 min with 0.1–10 mM of different metal ions and inhibitors. The lipase activity was measured by pH-stat assay with 1% olive oil as a substrate at 55 °C and pH 9.0.

10 mM of Cu^{2+} and Sn^{2+} ions increased the lipase activity slightly by 1–22%. Other metal ions including Ca^{2+}, Mg^{2+}, and Mn^{2+} in different concentrations of 0.1, 1, and 10 mM showed an converse effect, inhibitory (up to 17%) and inducible (up to 22%).

In contrast to the lipase LipA, the lipase from *Acinetobacter* sp. RAG-1 showed an increase in activity by 10 and 27–33% by incubating with 1 mM of Zn^{2+} and 1–10 mM of Fe^{3+}, respectively, but a decrease in activity by 63 and 84% by incubating with 1 and 10 mM of Cu^{2+}, respectively [18]. Both recombinant lipases showed more or less similar effect of Ca^{2+}, Mg^{2+}, and Mn^{2+} on the activity. In contrast to the recombinant lipase LipA, most metal ions had a moderately—strongly inhibitory effect on the activity of the wild-type lipase. The residual activity of the wild-type lipase after treatment with 0.1–10 mM of metal ions was between 92 and 16%, except for 1 mM Sn^{2+}.[2]

All inhibitors including DEPC, EDTA, PMSF, and 2-mercaptoethanol showed a slightly—moderately inhibitory effect on lipase activity by incubating the lipase with 0.1, 1, and 10 mM for 30 min in 0.1 M Tris–HCl buffer, pH 8.0, except for 0.1 mM DEPC (Table 2). The addition of 0.1, 1, and 10 mM of the chelating agent EDTA decreased the lipase activity by 27–29%, indicating that EDTA accesses the putative Ca^{2+} binding site and removes ions. The wild-type lipase was slightly inhibited (up to 11%) by adding of EDTA with lower concentrations (0.1–1 mM), but slightly stimulated (16%) by incubating the enzyme with 10 mM of EDTA.[2] However, EDTA strongly inhibited the enzyme activity of the lipase from *Acinetobacter* RAG-1 with 90% activity loss. The addition of 0.1 mM of PMSF showed no effect on lipase activity, however the addition of 1 and 10 mM decreased the lipase activity by 15 and 43%, respectively. The addition of 1–10 mM of PMSF to the wild-type lipase showed a slight increase and decrease in activity by 18 and 6%, respectively.[2] PMSF is expected to inhibit lipase as it contains a serine as part of the catalytic triad. Incubating with 0.1 mM of DEPC increased the lipase activity slightly of the recombinant by 5%, but decreased the wild-type lipase activity by 19%. Higher concentration (10 mM) of DEPC indicated an opposite effect, decreased the activity of the recombinant one (by 15%) and increased that of the wild type (by 31%). The inhibition by histidine residue modifier DEPC suggests the possibility of histidine residues being involved in catalysis as well. The addition of 0.1, 1, and 10 mM of 2-mercaptoethanol decreased the lipase activity by 18, 32, and 5%, respectively, indicating a putative disulfide bridge is required for activity. 2-Mercaptoethanol showed a similar effect on enzyme activity (slight decrease) for both lipases from *Ralstonia* sp. M1 and *Acinetobacter* RAG-1, but increased the activity of the wild-type lipase from *Ralstonia* sp. M1 by up to 12%.

Table 3
Effect of detergents on lipase activity

Detergent	Remaining activity (%) at concentration (mM) of		
	0.2	1	5
Tween 80	241	330	244
Tween 60	222	294	289
Tween 40	238	306	273
Triton X-100	119	139	11
Triton X-45	142	145	69
SDS	99	152	17

One microgram of the refolded lipase was incubated in 0.1 M phosphate buffer, pH 8.0 at 30 °C for 30 min with 0.2, 1, and 5% of different detergents. The lipase activity was measured by pH-stat assay with 1% olive oil as a substrate at 55 °C and pH 9.0.

Effect of detergents on lipase activity

The effect of 0.2, 1, and 5% (w/v) of detergents including Tween 80, Tween 60, Tween 40, Triton X-100, Triton X-45, and SDS was measured by incubating the lipase LipA for 30 min at 30 °C in 0.1 M phosphate buffer, pH 8.0. The addition of 0.2–5% (w/v) of Tween 80, Tween 60, and Tween 40 induced the activation of the recombinant (Table 3) as well as the wild-type lipase.[2] The residual lipase activity increased to 2–3 times and up to 1.7 times in comparison to the control sample, respectively. The addition of 0.2–1% (w/v) of Triton X-100 and X-45 increased the lipase LipA activity by 19–45%, whereas increasing the concentration of Triton X-100 and Triton X-45 to 5% (w/v) showed an opposite effect, decreased the lipase activity dramatically to 11 and 69%, respectively. No effect on lipase activity was observed when the lipase was incubated with only 0.2% (w/v) of SDS for 30 min. However, the addition of 1% (w/v) of SDS increased the lipase LipA activity strongly, the residual activity increased to 152% in comparison to the control sample. Increasing the concentration of SDS to 5% (w/v) reduced the lipase activity dramatically to 17%.

The addition of 0.1–5% (w/v) of Triton X-100, Triton X-45, and SDS decreased the activity of the wild-type lipase by 23–59%, except for 1 mM of Triton X-100 (increase by 14%) and 5% (w/v) of SDS decreased the activity dramatically to 7%. The lipase BTL2 from *B. thermocatenulatus* was slightly activated by the addition of 1% (w/v) of Triton X-100 and Tween 80, the lipase activity increased by around 30 and 6%, respectively [17]. The addition of 1% (w/v) of Tween 20 or 1% (w/v) of cholate decreased the lipase activity slightly by 7 and 20%, respectively. However, SDS inhibited the lipase completely, the residual lipase activity was only 5% of that of the control sample.

Substrate specificity

Among triglycerides, the lipase LipA showed the highest activity towards tributyrin (C4 acyl group) measured at pH 9.0 and 55 °C. The highest activity towards tributyrin was fixed as 100% (Fig. 5). The typical profile of chain length specificity of this lipase is showed in Fig. 5. In general, the lipase LipA activity decreased in the line of increasing the number of the carbon chain length, except for two substrates: tricaprylin, and trilaurin (Fig. 5). The activity towards tricaproin (C6 acyl group) relative to tributyrin was around 69%. The activity of the lipase LipA towards tricaprin (C10 acyl group), tricaprylin (C8 acyl group), trimyristin (C14 acyl group), and tripalmitin (C16 acyl group) remained around 26–37% of that towards tributyrin. The relative activity of triolein (C18:1 acyl group), and trilaurin (C12 acyl group) in comparison to tributyrin was around 15–18%. Tristearin (C18 acyl group) was the poorest substrate (8%). A similar preference for triacylglycerols of short length chain fatty acids has been observed for the wild-type lipase from *Ralstonia* sp. M1[2] and the recombinant lipase from *B. thermocatenulatus* [17] (see Table 4).

Table 4
Comparison of the properties of the wild-type lipase from *Ralstonia* sp. M1[2] and the recombinant lipase LipA expressed in *E. coli*

Properties	The wild-type lipase	The recombinant lipase LipA
Molecular mass	34 kDa (estimated)	32.5 kDa (deduced)
pH_{opt}	10.0	10.75
pH_{stab}	8.0–10.5	7.5–11.0
T_{opt}	55–60 °C	50–55 °C
T_{stab}	25–75 °C	25–45 °C
Organic solvent stability	Highly resistant	Highly resistant
Detergent stability	Inducible	Inducible
Substrate specificity		
Triacylglycerols	C4 > C8 > C10	C4 > C8 > C10
p-NP esters	C14	C8
Natural oils and fats	Similar profile	Similar profile

pH_{opt}, optimal pH; pH_{stab}, pH stability; T_{opt}, optimal temperature; T_{stab}, thermostabiligy; *p*-NP, *p*-nitrophenyl; C4, tributyrin; C8, tricaprylin; C10, tricaprin; *p*-NP C14, *p*-nitrophenyl myristate; and *p*-NP C8, *p*-nitrophenyl caprylate.

To determine the substrate specificity, also the lipase LipA activity towards *p*-NP esters of different carbon chain length was measured spectrometrically at 405 nm (for C2–C8 acyl group) and 420 nm (for C14–C16 acyl group), pH 8.0 and 55 °C. The *p*-NP esters showed a different substrate specificity profile in comparison to triglycerides. Among *p*-NP esters, the lipase LipA showed the highest activity towards *p*-NP caprylate (C8 acyl group) and fixed as 100%. The typical profile of chain length specificity of this lipase towards *p*-NP esters is showed in Fig. 6. The activity towards *p*-NP myristate (C14 acyl group) relative to *p*-NP caprylate was around 67%. The activity of the lipase LipA towards *p*-NP palmitate (C16 acyl group), caproate (C6 acyl group), and propionate (C3 acyl group), was 39, 25, and 12% of that towards *p*-NP caprylate, respectively. *p*-NP acetate (C2 acyl group) was the poorest substrate (1%) in comparison to *p*-NP caprylate. The wild-type lipase from

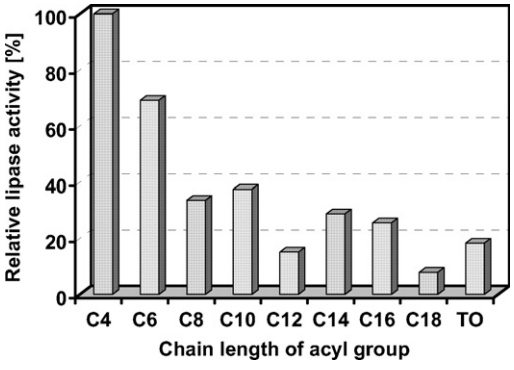

Fig. 5. Substrate specificity of the lipase LipA. Acyl-chain length specificity of purified and refolded lipase LipA was determined from its activity towards various triglycerides (C4–C18 acyl group). The lipase activity was measured by pH-stat assay with 10 mM of different triacylglycerols (C4–C18 acyl group) at pH 9.0 and 55 °C. Percentages shown are relative to maximum activity (C4 acyl group). TO, triolein.

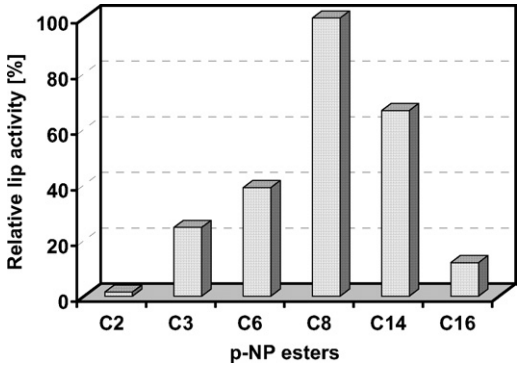

Fig. 6. Substrate specificity of the lipase LipA. Acyl-chain length specificity of purified and refolded lipase LipA was determined form its activity towards various *p*-NP (C2–C16 acyl group). The lipase activity was measured by spectrophotometric assay at 405 nm (C2–C8 acyl group) and 420 nm (C14–C16 acyl group): 0.1 mM pNP ester, pH 8.0, 55 °C, 3 min. Percentages shown are relative to maximum activity (C8 acyl group).

Ralstonia sp. M1 hydrolyzed most preferentially the substrate myristate (C14 acyl group). The lipase *Acinetobacter* sp. RAG-1 had a similar preference towards medium fatty acid esters of *p*-NP. However, the lipase *Acinetobacter* sp. SY-01, related to the lipase from *Acinetobacter* sp. RAG-1, showed the maximum activity toward *p*-NP acetate and the activity decreased gradually with increase of chain length [19].

Natural substrate specificity

Among natural oils and fats, the activity towards olive oil was fixed as 100% measured at pH 9.0 and 55 °C. The specificities of this lipase towards natural oils and fats are shown in Fig. 7. In general, the lipase LipA showed activity towards all assayed natural oils and fats more than activity toward olive oil (Fig. 7). The lipase LipA showed the highest activity towards cottonseed oil (CTS) with 317%. The activity towards corn oil (CRN), palm oil (PLM), wheatgerm oil (WGM), and soybean oil (SBN) relative to olive oil was 227, 222, 210, and 195%, respectively. The activity of the lipase LipA towards beef tallow (BTL), rapeseed (RSD), and coconut oil (CCN) was as high as 170–176% of that towards olive oil. The relative activity of linseed oil (LSD), and peanut oil (PNT) in comparison to olive oil was around 118–126%. The profiles of substrate specificity of both the recombinant LipA and the wild-type enzymes towards natural fats and oils were very similar. Both enzymes hydrolyzed preferentially cottonseed oil, corn oil, palm oil, and wheatgerm oil among natural fats and oils.

Conclusion

The recombinant LipA from *Ralstonia* sp. M1 is an alkaline, thermophilic, highly organic-solvent-resistant, and detergent-inducible lipase. This lipase showed similar effects of temperature, pH, detergents, organic solvents, metal ions, and inhibitors on the activity and stability, as well as similar profiles of the substrate specificity as the wild-type lipase.[2] Furthermore, the physicochemical behaviors of the lipase LipA are more or less similar to those of the lipase from *Acinetobacter* sp. RAG-1 than SY-01, both lipases belong to the subfamily I.2 too. These physicochemical characteristics of the lipase LipA from *Ralstonia* sp. M1 make a potential of applications of this lipase in the detergent industry and organic synthesis.

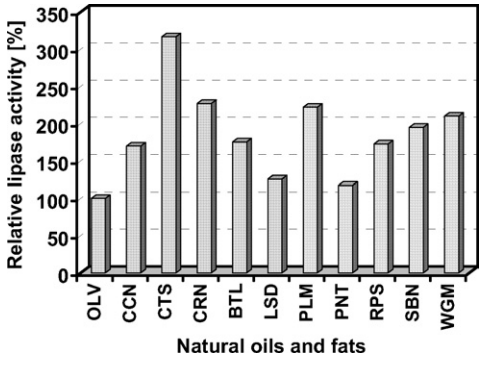

Fig. 7. Hydrolysis of different natural oils and fats by the lipase LipA. The lipase activity was measured by pH-stat assay with 1% different natural oils and fats at pH 9.0 and 55 °C. Percentages shown are relative to maximum activity (olive oil). OLV, olive oil; CCN, coconut oil; CTS, cottonseed oil; CRN, corn oil; BTL, beef tallow; LSD, linseed oil; PLM, palm oil; PNT, peanut oil; RPS, rapeseed oil; SBN, soybean oil; and WGM, wheatgerm oil.

Acknowledgments

This work has been supported by the Foundation of Fundamental Sciences (Vietnamese Ministry of Science and Technology) for the fiscal year 2001–2005. Dinh Thi Quyen gratefully acknowledges a grant for equipment from the International Foundation of Science (Sweden, 2003) and a scholarship from the Korea Science and Engineering Foundation (KOSEF, 2003).

References

[1] M. Martinelle, M. Holmquist, K. Hult, On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase, Biochim. Biophys. Acta 1258 (1995) 272–276.
[2] K.-E. Jaeger, T. Eggert, Lipases for biotechnology, Curr. Opin. Biotechnol. 13 (2002) 390–397.
[3] M. Chihara-Siomi, K. Yoshikawa, N. Oshima-Hirayama, K. Yamamoto, Y. Sogabe, T. Nakatani, T. Nishioka, J. Oda, Purification, molecular cloning, and expression of lipase from *Pseudomonas aeruginosa*, Arch. Biochem. Biophys. 296 (1992) 505–513.
[4] S. Wohlfarth, C. Hoesche, C. Strunk, U.K. Winkler, Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1, J. Gen. Microbiol. 138 (1992) 1325–1335.
[5] E.R. Sullivan, J.G. Leahy, R.R. Colwell, Cloning and sequence analysis of the lipase and lipase chaperone-encoding genes from *Acinetobacter calcoaceticus* RAG-1, and redefinition of a proteobacterial lipase family and an analogous lipase chaperone family, Gene 230 (1999) 277–286.
[6] R.G. Kok, J.J.v. Thor, I.M. Nugteren-Roodzant, B. Vosman, K.J. Hellingwerf, 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 (1995) 3295–3307.
[7] S. Aoyama, N. Yoshida, S. Inouye, Cloning, sequencing and expression of the lipase gene from *Pseudomonas fragi* IFO-12049 in *E. coli*, FEBS Lett. 242 (1988) 36–40.
[8] L.G. Frenken, M.R. Egmond, A.M. Batenburg, J.W. Bos, C. Visser, C.T. Verrips, Cloning of the *Pseudomonas glumae* lipase gene and determination of the active site residues, Appl. Environ. Microbiol. 58 (1992) 3787–3791.
[9] S. Jorgensen, K.W. Skov, B. Diderichsen, Cloning, sequence and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes, J. Bacteriol. 173 (1991) 559–567.

[10] M.A. Taipa, K. Liebeton, J.V. Costa, J.M. Cabral, K.E. Jaeger, Lipase from *Chromobacterium viscosum* : biochemical characterization indicating homology to the lipase from *Pseudomonas glumae*, Biochim. Biophys. Acta 1256 (1995) 396–402.
[11] D. Litthauer, A. Ginster, E. Skein, *Pseudomonas luteola* lipase: a new member of the 320-residue *Pseudomonas* lipase family, J. Enzyme Microb. Technol. 30 (2002) 209–215.
[12] J.L. Arpigny, K.E. Jaeger, Bacterial lipolytic enzymes: classification and properties, Biochem. J. 343 (1999) 177–183.
[13] L.G. Frenken, J.W. Bos, C. Visser, W. Muller, J. Tommassen, C.T. Verrips, An accessory gene, lipB, required for the production of active *Pseudomonas glumae* lipase, Mol. Microbiol. 9 (1993) 579–589.
[14] D.T. Quyen, C. Schmidt-Dannert, R.D. Schmid, High-level formation of active *Pseudomonas cepacia* lipase after heterologous expression of the encoding gene and its modified chaperone in *Escherichia coli* and rapid in vitro refolding, Appl. Environ. Microbiol. 65 (1999) 787–794.
[15] E.K. Kim, W.H. Jang, J.H. Ko, J.S. Kang, M.J. Noh, O.J. Yoo, Lipase and its modulator from *Pseudomonas* sp. strain KFCC 10818: proline-to-glutamine substitution at position 112 induces formatin of enzymatically active lipase in the absence of the modulator, J. Bacteriol. 183 (2001) 5937–5941.
[16] U.K. Laemmli, Cleavage of structure proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[17] D.T. Quyen, C. Schmidt-Dannert, R.D. Schmid, High-level expression of a lipase from *Bacillus thermocatenulatus* BTL2 in *Pichia pastoris* and some properties of the recombinant lipase, Protein Expr. Purif. 28 (2003) 102–110.
[18] E.A. Snellman, E.R. Sullivan, R.R. Colwell, Purification and properties of the extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1, Eur. J. Biochem. 269 (2002) 5771–5779.
[19] S.J. Han, J.H. Back, M.Y. Yoon, P.K. Shin, C.S. Cheong, M.H. Sung, S.P. Hong, I.Y. Chung, Y.S. Han, Expression and characterization of a novel enantioselective lipase from *Acinetobacter* species SY-01, Biochimie 85 (2003) 501–510.