

# Identification, Purification, and Characterization of a Thermally Stable Lipase from Rice Bran. A New Member of the (Phospho) Lipase Family<sup>1</sup>

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A thermally stable lipase (EC 3.1.1.3.) was first identified in rice (*Oryza sativa*) bran, and the enzyme was purified to homogeneity using octyl-Sepharose chromatography. The enzyme was purified to 7.6-fold with the final specific activity of  $0.38 \mu\text{mol min}^{-1} \text{mg}^{-1}$  at  $80^\circ\text{C}$  using  $[9,10\text{-}^3\text{H}]$ triolein as a substrate. The purified enzyme was found to be a glycoprotein of 9.4 kD. Enzyme showed a maximum activity at  $80^\circ\text{C}$  and at pH 11.0. The protein was biologically active and retained most of its secondary structure even at  $90^\circ\text{C}$  as judged by the enzymatic assays and far-ultraviolet circular dichroism spectroscopy, respectively. Differential scanning calorimetric studies indicated that the transition temperature was  $76^\circ\text{C}$  and enthalpy  $1.3 \times 10^5 \text{ Calorie mol}^{-1}$  at this temperature. The purified lipase also exhibited phospholipase A<sub>2</sub> activity. Colocalization of both the hydrolytic activities in reverse-phase high-performance liquid chromatography and isoelectric focusing showed that the dual activity was associated with a single protein. Further, a direct interaction between both the substrates and the purified protein was demonstrated by photoaffinity labeling, using chemically synthesized analogs of triolein and phosphatidylcholine (PC). Apparent  $K_m$  for triolein (6.71 mM) was higher than that for PC (1.02 mM). The enzyme preferentially hydrolyzed the *sn*-2 position of PC, whereas it apparently exhibited no positional specificity toward triacylglycerol. Diisopropyl fluorophosphate inhibited both lipase and phospholipase activities of the purified enzyme. This enzyme is a new member from plants in the family of lipases capable of hydrolyzing phospholipids.

Lipases (EC 3.1.1.3.) are versatile enzymes that catalyze the hydrolysis of ester linkages, primarily in neutral lipids such as triglycerides. They hydrolyze the acyl chains either at primary (Ransac et al., 1990; Rogalska et al., 1993) or secondary positions (*Candida antarctica* lipase, *Geotrichum candidum* lipase B, lipase from *Vernonia anthelmintica*, etc.). However, a few lipases do not show any positional specificity (Rollof et al., 1987; Hiromasa et al., 1998). Plant lipases hydrolyze triacylglycerols at much lower rates (usually  $<0.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ) as compared with animal or microbial lipases. All the members of the lipase gene family have a conserved Ser, which is the nucleophile essential for catalysis. The active site triad of lipases consisting of Ser-His-Asp/Glu is reminiscent of the Ser proteases (Blow, 1990). In addition to triglycerides, lipases are also known to degrade Tween and water-soluble and insoluble esters. There are reports of a few animal and microbial lipases that hydrolyze phospholipids (Durand et al., 1978; Fauvel et al., 1981; Jensen et al., 1982; van Oort et al., 1989; Thirstrup et al., 1994). Enzymatic activity of many lipases has been shown to be modulated by calcium (Rosenstein and Gotz, 2000), ricinoleic acid (Ory et al., 1962), and bile salts (Miled et al., 2000).

In plants, the regulation, in some cases the location, and the exact physiological roles of lipases are not very clear. Oilseed (*Brassica napus*) lipases that are the best described have been shown to be localized in oil bodies (Ory et al., 1968; Lin and Huang, 1983) or glyoxysomes (Muto and Beevers, 1974; Rosnitschek and Theimer, 1980). They are known to play an essential role in the mobilization of seed-storage lipids to support germination and post-germinative embryonal growth. Lipases are generally considered to be absent in most dry seeds and are probably synthesized de novo after the germination; however, a triacylglycerol lipase has been purified from ungerminated dry seeds of *Vernonia galamensis* (Ncube et al., 1995).

Rice (*Oryza sativa*) bran oil is typically an oleic-linoleic-type fatty acid, and its physical-chemical properties qualify it for a good quality edible oil (Salunkhe et al., 1992). However, complete utilization of bran oil suffers from the fact that there is a large accumulation of free fatty acids (FFA), which has been attributed to the presence of lipase activity (Funatsu et al., 1971). Identifying and characterizing the lipases from bran is essential to devise efficient methods to overcome the problem of rice bran oil instability. Two soluble lipases have been purified from rice bran. Lipase I has a molecular mass of 40 kD and a pH optimum of about 7.5. It is activated by calcium and cleaves preferentially fatty acids from the *sn*-1 and *sn*-3 positions of triacylglycerols (Funatsu et al., 1971). Lipase II has a molecular mass of 32 kD, a pI of 9.1, and pH optimum of about 7.5 (Aizono et al.,

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1976; Fujiki et al., 1978). Here, we report the identification, purification, and biophysical and biochemical characterization of a new lipase from rice bran. The striking feature of this lipase is its unusual thermal stability. In addition, this enzyme is a new member in the family of lipases capable of hydrolyzing phospholipids as well.

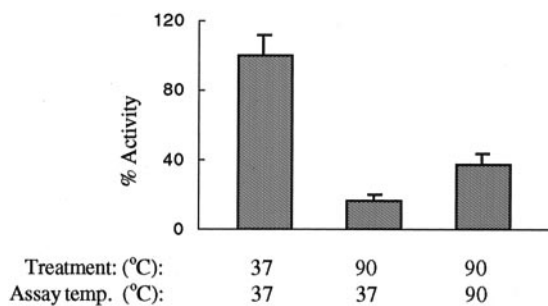
## RESULTS AND DISCUSSION

### Identification of a Thermostable Lipase from Rice Bran

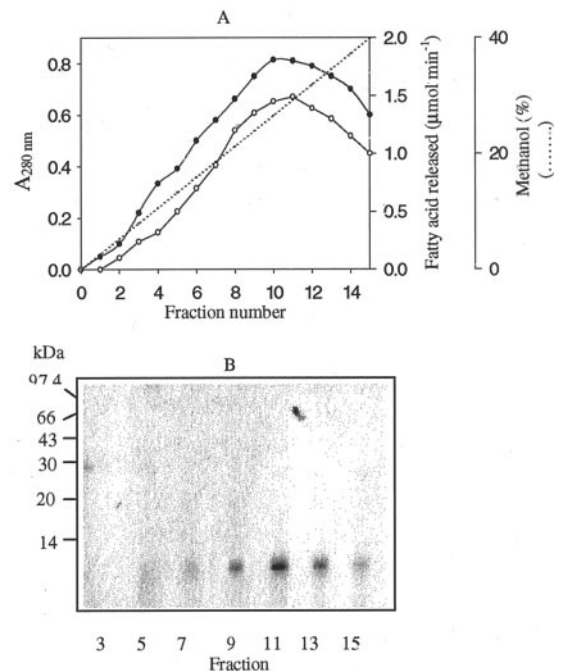
Rice bran oil has been shown to be unstable due to the presence of lipolytic activity. One of the methods to increase the shelf life up to 3 months involves dry heating at 105°C for 3 h (Houston, 1972). To test thermal stability, we pretreated the bran extracts at either 37°C or 90°C for 15 min. Lipolytic activity was measured using <sup>3</sup>H-triolein as substrate. The 90°C pretreated extract showed 16% and 34% activity at 37°C and 90°C, respectively (Fig. 1). The fact that the activity in the extract pretreated at 90°C was higher when measured at 90°C as compared with 37°C suggested the presence of a thermally stable lipase in rice bran.

### Purification of Lipase

To purify the thermally stable lipase, extract from delipidated rice bran was passed through an octyl-Sepharose column and the bound proteins were eluted with a linear gradient of 0% to 40% (v/v) methanol. Each fraction was evaluated for protein and lipase activity (Fig. 2A). Analysis of alternate fractions on a 15% (w/v) SDS-PAGE is shown in Figure 2B. A single band was observed in the later fractions upon silver staining but there was no pro-



**Figure 1.** Thermally stable lipolytic activity in rice bran. Aliquots of protein extract from delipidated rice bran were preincubated either at 37 °C or 90 °C for 15 min and then assayed for lipolytic activity at indicated temperatures. Assays were performed using <sup>3</sup>H-triolein as substrate. After 10 min of the reaction period, lipids were extracted and separated on a silica-TLC. The lipid standards were run in adjacent lanes and identified by iodine staining. Radioactive spots corresponding to the unreacted substrate and the product (FFA) were then scraped off from the TLC plates and quantified by liquid scintillation counter. Activity obtained at 37°C in the extracts pre-treated at 37°C was considered as 100%, which was 62 nmol min<sup>-1</sup> mg<sup>-1</sup>. Values are means (± SE) for six independent determinations.

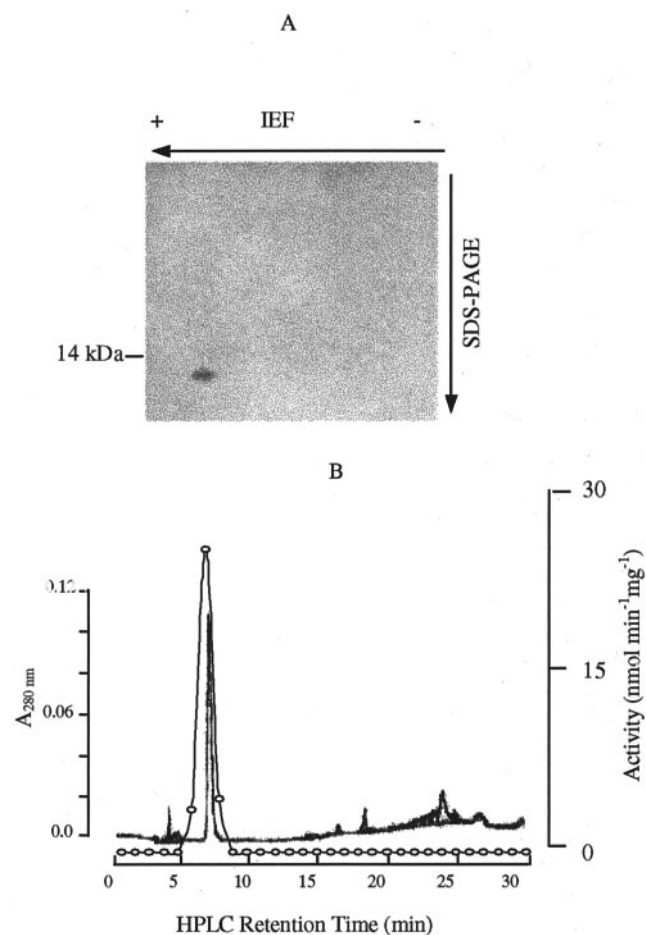


**Figure 2.** Chromatographic elution profile and SDS-PAGE analysis of lipase purification. A, Elution profile of an octyl-Sepharose column is shown. Bran extract was loaded onto the column that was pre-equilibrated with 10 mM Tris-HCl (pH 7.5). Elution was carried out with a linear gradient of 0%–40% of methanol (.....), and 10-mL fractions were collected. All the fractions were monitored for protein concentration (●) and assayed for lipase activity (○) using [<sup>3</sup>H]triolein as the substrate. B, SDS-PAGE of the octyl-Sepharose fractions. Equal volume aliquots were resolved on a 15% SDS-PAGE and visualized by silver staining.

tein detected in the initial fractions. In the absence of salt, there could be a specific hydrophobic interaction with the lipase and the matrix. Under these conditions, no other protein was found to bind to the matrix. Purity of the preparation was confirmed by two-dimensional gel electrophoresis (Fig. 3A) and a single band was observed in the acidic region (pH 4.1) upon silver staining. A single protein peak was obtained when the purified lipase was chromatographed in an analytical reverse-phase HPLC column and the activity was localized with the protein peak. (Fig. 3B). Purification is summarized in Table I. The overall purification was 7.6-fold, with an activity yield of 23%. Lipase purification was achieved to an apparent homogeneity in a single step.

### Physical Characterization

When the purified protein was passed through a gel filtration Superdex-75 FPLC column, activity was found to be associated with both the exclusion volume and the included low-*M<sub>r</sub>* peaks (data not shown). Both the peaks were analyzed on SDS-PAGE and were found to migrate at the same position (Fig. 4A), suggesting that the protein was forming large aggregates. The aggregation was concentration de-



**Figure 3.** Confirmation of the purity of lipase. A, Two-dimensional IEF/SDS-PAGE analysis of the purified protein. The first dimension of this gel is an isoelectric focusing using ampholytes with a pH range of 3.0 to 10.0. The gel was run in the acidic direction for 3 h. The second dimension is a 15% SDS-PAGE. Protein was visualized by silver staining the gel. B, HPLC profile. Purified protein (100  $\mu$ g) was applied to a C18-reverse phase HPLC column and 1-ml fractions were collected. Protein elution was monitored by absorbance at 280 nm (solid line). Lipase activity was measured after dialyzing the fractions, using radiolabeled triolein as substrate (open circle).

pendent as assessed by scattering at 600 nm (data not shown). The purified enzyme was resolved on HPLC and the eluted sample was analyzed for molecular mass by MALDI (Fig. 4B) and found to be 9.4 kDa.

### Thermal Stability of the Lipase

To determine the thermal stability of the lipase activity, aliquots were incubated at 20°C, 0°C, 40°C,

60°C, 80°C, and 100°C for 1 h and then the enzyme activities were measured using <sup>3</sup>H-triolein as the substrate. Results showed that the enzyme was active up to 40°C and the activity declined sharply to 65% at 60°C and then gradually decreased thereafter (Fig. 5A). To monitor the structural stability of this enzyme with respect to temperature, CD spectra were recorded at various temperatures in the region of far and near UV. Spectra revealed a profile of a sharp negative ellipticity at 232 nm. These results showed that the enzyme retained more than 90% of its secondary structure even at 90°C (Fig. 5B). Little denaturation was observed and was found to be reversible upon rescans of the sample after cooling to 20°C (Fig. 5C). The protein was subjected to DSC and Figure 5D shows the excess heat capacity versus temperature profiles as determined by DSC. The DSC transition corresponding to the thermal denaturation was reversible. The denaturation enthalpy ( $\Delta H$ ) was  $1.3 \times 10^5$  Calorie mol<sup>-1</sup> at  $T_m$  76°C.

The pancreatic lipases and the reported plant triacylglycerol lipases lose activity at high temperatures, but some microbial lipases have been reported to be resistant to temperature up to 50°C (Aisaka and Terada, 1980). Rice bran lipase was thermally stable as judged by the CD and differential scanning spectroscopic studies. In addition, the enzyme retained 65% activity after 1 h of incubation at 60°C. The CD spectrum was characterized by a distinct negative peak at 232 nm. Aromatic residues and disulfides could contribute significantly in this spectral range. A similar peak was observed in the spectra of two Ser proteinases, namely human tryptase (Schechter et al., 1995) and bovine chymotrypsin, that was attributed to the conserved Trp residue in its environment (Yang et al., 1986).

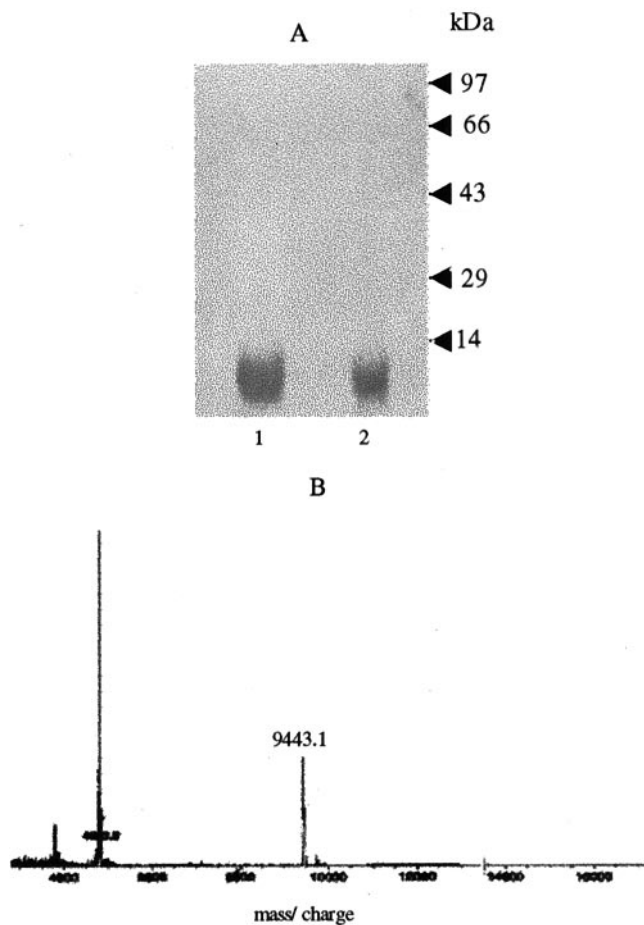
### Enzymatic Characterization of the Purified Lipase

Lipase activity was further characterized using triolein in the form of sonicated vesicles. Lipase showed maximum activity at 80°C and pH 11.0 (Fig. 6A and B, respectively). CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid} and digitonin had an inhibitory effect even at 0.5 mM concentration. SDS inhibited 50% of the activity at 2 mM. NP-40 and Triton X-100 decreased the activity marginally at lower concentrations and then the activity was restored at higher concentration (4 mM) of the detergent (Fig. 7A). Lipase activity was not altered significantly by Ca<sup>2+</sup>; however, other divalent cat-

**Table 1.** Purification of lipase from rice bran

Rice bran (200 g) was delipidated and then used for protein extraction. Activities were determined using 10 mM <sup>3</sup>H-triolein in 10 mM Tris-HCl, pH 7.5, at 80°C.

Fraction	Protein mg	Total Activity $\mu\text{mol min}^{-1}$	Specific Activity $\mu\text{mol min}^{-1} \text{mg}^{-1}$	Yield %	Purification -fold
Crude extract	250	12.5	0.05	100	1
Octyl-Sepharose	7.5	2.85	0.38	22.8	7.6



**Figure 4.** Lipase forms large aggregates. A, Purified protein was loaded on an analytical column (10 × 310 mm) that was equilibrated with 10 mM Tris (pH 7.5) containing 100 mM NaCl. Elution volumes for markers were as follows: blue dextran 2000 (2,000 kD), 7.8 mL; bovine serum albumin (67 kD), 8.3 mL; ovalbumin (43 kD), 9.3 mL; carbonic anhydrase (29 kD), 10.58 mL; lysozyme (14 kD), 11.75 mL. Silver stained SDS-PAGE of the void volume active peak I (lane 1) and the included active peak II (lane 2). B, The purified enzyme was resolved on HPLC and the eluted sample was analyzed by MALDI-TOF.

ions like  $Mg^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ , and  $Cd^{2+}$  at 2 mM inhibited activity about 70% (Fig. 7B). Potassium acetate enhanced the activity around 4- to 5-fold in the range of 20 to 200 mM, whereas sodium acetate showed a 2-fold increase at 20 mM and no effect at higher concentrations at pH 7.5. Sodium chloride up to 50 mM showed a marginal increase and potassium chloride showed a marginal decrease in the activity (Fig. 7C). The isolated lipase hydrolyzed diacylglycerol and phosphatidylethanolamine (data not shown); however, the hydrolytic activities with other lipid substrates were not performed.

#### Dual Enzyme Activity, Lipase, and Phospholipase A<sub>2</sub>

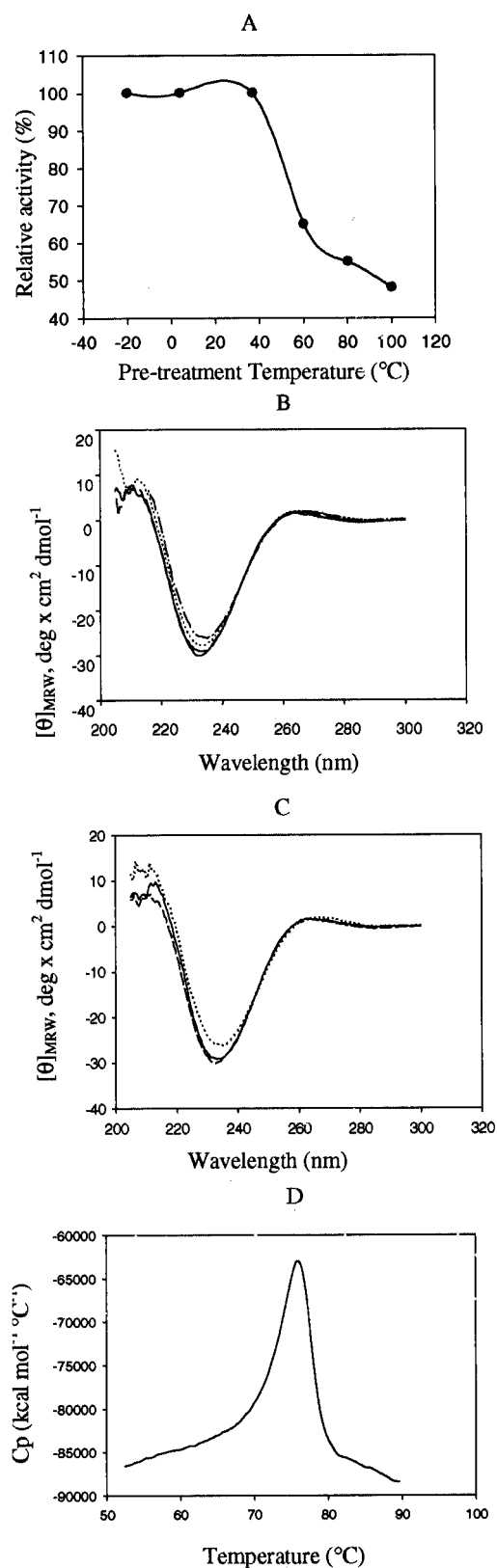
During the course of investigating the substrate specificity, it was noted that the lipase also displayed

phospholipase activity ( $121 \text{ nmol min}^{-1} \text{ mg}^{-1}$ ) using [2-palmitoyl-9,10-<sup>3</sup>H]phosphatidylcholine as a substrate. It could be possible that a single polypeptide was responsible for both the activities or there could be a contaminating activity present in the purified rice bran lipase preparation. To investigate these possibilities, phospholipase was purified using the lipase purification procedure and found a constant ratio of specific activities for triolein and PC. The purified lipase and phospholipase preparations were monitored in isoelectric-focusing gel and reverse-phase HPLC column. In-gel assay and the analysis of fractions revealed that both the activities were colocalized, suggesting a single polypeptide was responsible for both the hydrolytic activities. To further confirm, radiolabeled photoactive analogs of PC and triolein were synthesized. Both the analogs were used as substrates by the purified enzyme in the dark and as irreversible inhibitors upon cross-linking under UV light (data not shown). Figure 8 shows the photoaffinity labeling of the protein with both the analogs. Labeling of a single polypeptide with both the analogs unequivocally demonstrated that the purified protein specifically interacted with triolein as well as PC analogs.

To gain insight if both the substrates have independent catalytic or binding sites, <sup>32</sup>P-PC hydrolysis was monitored in the presence of unlabeled triolein. Triolein competed the radiolabeled substrate although not as efficiently as the unlabeled PC (Fig. 9A). Further, lipase and phospholipase activities were monitored in the presence of Ser modifier (Fig. 9B) and both the activities were compromised. These experiments indicated that both triolein and PC hydrolysis required Ser in the catalytic site. In the immunoinhibition assays, with polyclonal antisera raised against the purified protein, lipase activity was selectively inhibited (Fig. 9C) suggesting that the binding sites for triolein and PC could be different. These results suggested that the purified lipase has a common catalytic site but independent binding sites for substrates such as triolein and PC.

#### Phospholipase Activity of the Purified Lipase

Phospholipase A<sub>2</sub> activity was linear with respect to time and protein concentrations, and temperature optimum was found to be approximately 80°C. (data not shown). We investigated the phospholipase activity toward <sup>32</sup>P-PC <sup>3</sup>H-lysophosphatidic and <sup>14</sup>C-phosphatidic acid that were dispersed in the form of sonicated vesicles. Upon enzymatic hydrolysis, there was a release of lysophosphatidylcholine (LPC) from PC (Fig. 10A) but the enzyme did not hydrolyze phosphatidic acid (Fig. 10B) or lysophosphatidic acid (LPA) (Fig. 10C). In general, non-specific acyl hydrolases are able to hydrolyze various mono-, and diacyl lipids. There are a few non-specific acyl-ester hydrolases reported in potato (*Solanum tuberosum*) tuber,



**Figure 5.** Thermal stability of the purified lipase. A, Enzymatic activity. Enzyme was preincubated for 1 h at different temperatures and the residual activity measured at 37°C under the standard assay conditions. Activity of the enzyme pretreated at 37°C was considered

rice bran, barley (*Hordeum vulgare*) endosperm (for review, see Ref Huang, 1987) and sunflower seed (Teissere et al., 1995). The lipase reported in this study is a true lipase. It was shown earlier that a few lipases/phospholipases required  $\text{Ca}^{2+}$  for their activity (Channon and Leslie, 1990; Scott et al., 1990); however, rice bran lipase did not require calcium (Fig. 10D).

#### Positional Specificity of the Enzyme toward Triolein and PC

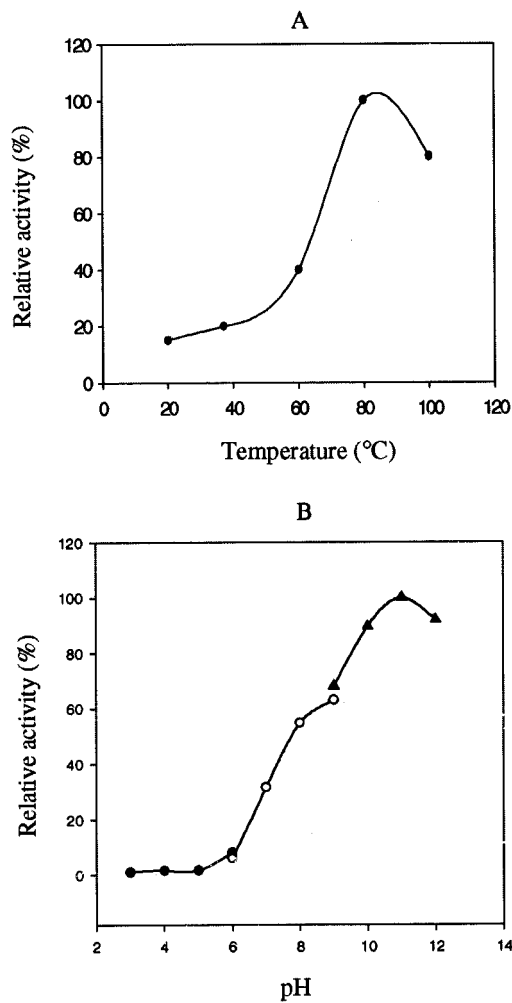
To determine the positional specificity toward triacylglycerols, a time course for the release of possible intermediates from  $^3\text{H}$ -triolein labeled at all the fatty acids was performed. Analyses of reaction products revealed the accumulation of fatty acids. Diacylglycerol (DAG) formation was observed at the early time points (Fig. 11A) but its level came down at later time points. These results indicated that the enzyme hydrolyzed fatty acids at all *sn*-positions. At present, it remains to be elucidated if the enzyme could directly act at all the positions or if isomerization of fatty acid was involved as suggested for some lipases (Ory, 1969).

Release of lysophosphatidylcholine (LPC) from  $^{32}\text{P}$ -PC indicated that the enzyme had phospholipase  $A_2$  activity. We confirmed the *sn*-2 specificity by using PC that was radiolabeled at *sn*-2 position. Quantitation of the products showed that there was only a release of FFA (Fig. 11B). These results demonstrated that the purified rice bran lipase was specific for the hydrolysis at *sn*-2 position of PC. LPC, DAG, or PA were not formed during the reaction, indicating that the purified phospho(lipase) did not have phospholipase  $A_1$ , phospholipase C and D type of activities. It was surprising that the enzyme preferred hydrolysis at *sn*-2 position in PC but did not show such positional specificity for triolein.

#### Substrate Dependence of the Enzyme toward Triolein and PC

The dependence of the enzyme toward the vesicles of triolein and PC under physiological pH and temperature was studied. Based on the Lineweaver-Burk plots, apparent  $K_m$  for triolein and PC were 6.71 mM and 1.02 mM, respectively (data not shown). Earlier

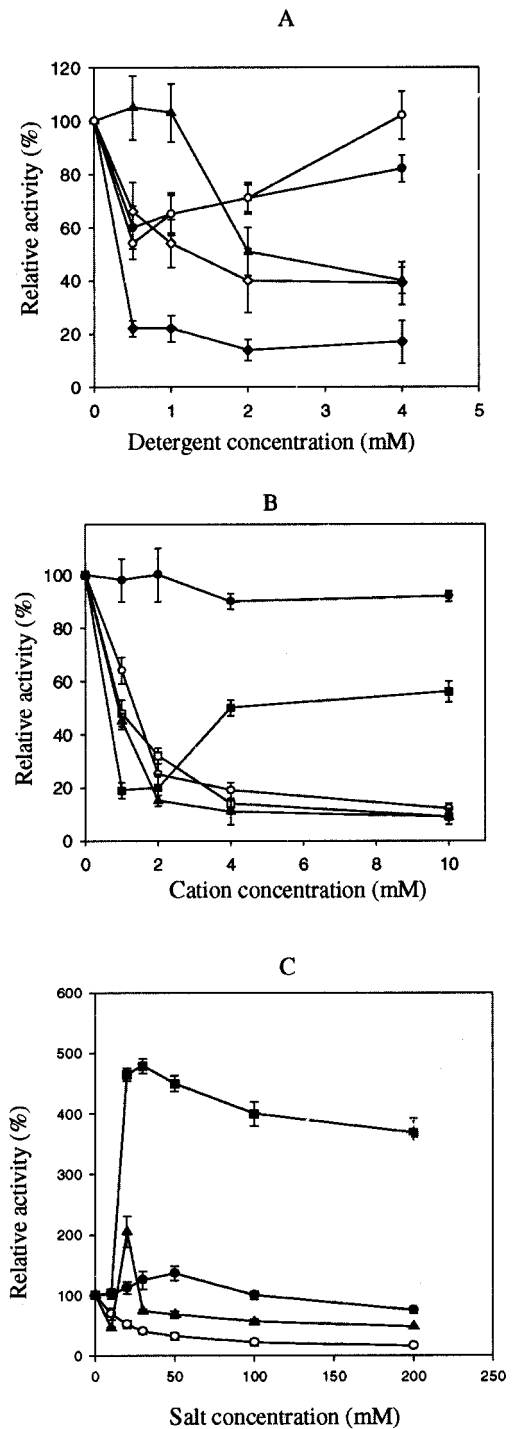
as 100% ( $83 \text{ nmol min}^{-1} \text{ mg}^{-1}$ ). B, Near- and far-UV circular dichroism (CD) spectra of purified protein at 20°C (solid), 40°C (long dash), 60°C (dotted) and 90°C (dash-dot). C, Near- and far-UV CD spectra of native (long dash), unfolded (dotted) and refolded (solid) protein. Sample was unfolded at 90°C and then allowed to fold for 10 min at 20°C. Protein used was  $40 \mu\text{g mL}^{-1}$ . Spectra shown are averages of four scans. D, The differential scanning calorimetry (DSC) data. The sample was scanned from 20°C to 90°C, cooled to 20°C, and rescanned from 20°C to 90°C. The scan rate was  $90^\circ\text{C h}^{-1}$ . Protein concentration was  $70 \mu\text{g mL}^{-1}$ .



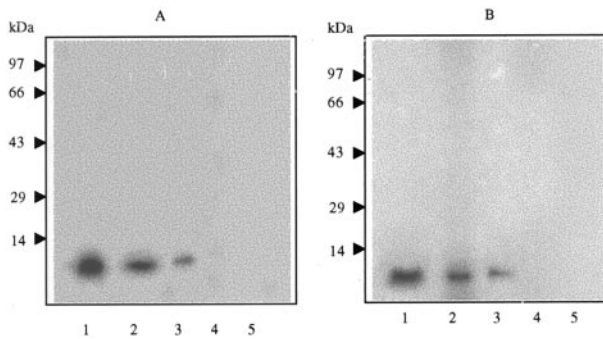
**Figure 6.** Optimum temperature (A) and pH (B) profiles of the purified lipase. A, Enzyme activity was measured using <sup>3</sup>H-triolein as a substrate at different temperatures and pH 7.5. Activity at 80°C (353 nmol min<sup>-1</sup> mg<sup>-1</sup>) corresponds to 100%. B, Activity was measured at different pH and 37°C. Activity at pH 11.0 (416 nmol min<sup>-1</sup> mg<sup>-1</sup>) corresponds to 100%. Buffers used for activity of lipase as a function of pH: citrate buffer (closed circle); Tris-HCl (open circle); phosphate buffer (triangle).

reports indicated that the  $K_m$  for 44 kD rice bran lipase was 7.4 mM (Shastry and Raghavendra, 1971). Kinetic experiments showed that the enzyme preferentially used PC as compared with triolein and the overall catalytic efficiency ( $V_{max}/K_m$ ) for PC was 20-times higher than that for triolein. Although the catalytic rates of the rice bran lipase are comparable to the reported plant lipases, reasons for its lower catalytic efficiency under physiological conditions of pH and temperature as compared with most other known animal or microbial lipases are not known.

Rice bran (phospho) lipase being a small protein and hence amicable for manipulations, provides a good model enzyme to study the interaction with substrate. Unique properties like stability and broad substrate specificity of lipases are of particular inter-



**Figure 7.** Influence of detergents on activity of the purified lipase using <sup>3</sup>H-triolein as a substrate (A). Enzyme activity was measured using <sup>3</sup>H-triolein as a substrate in the presence of different concentrations of NP-40 (filled circle); Triton X-100 (open circle); SDS (triangle); CHAPS (filled diamond); and digitonin (open diamond). Effect of different concentrations of divalent cations (B) CaCl (closed circle); Zn sulfate (open circle); CuCl (closed square); CdCl (open square); MgCl (closed triangle). Influence of salts on the enzyme activity (C) NaCl (closed circle); KCl (open circle); K acetate (closed square); Na acetate (closed triangle). Values are means ( $\pm$  SE) for three independent determinations.



**Figure 8.** Photoaffinity labeling of the purified lipase. Purified lipase (10  $\mu\text{g}$ ) was pre-incubated with the synthesized photoanalogs, [ $^{125}\text{I}$ ]N $_3$ PC (A) or [ $^{125}\text{I}$ ]N $_3$ TAG (B) and increasing concentrations, 0  $\mu\text{M}$  (lane 1), 250  $\mu\text{M}$  (lane 2), and 500  $\mu\text{M}$  (lane 3) of the natural substrate phosphatidylcholine (PC; A) or triolein (B) for 15 min at 4°C. These samples were then crosslinked by exposing to short-UV for 5 min at 4°C. Protein was TCA precipitated. The autoradiograms were obtained following SDS-15% PAGE of the cross-linked samples. Lane 4, The photoprobes irradiated prior to the addition of the enzyme. Lane 5, Photolyzed analogs without the enzyme.

est not only for fundamental understanding of the mechanism of action but also for their implications in various industrial applications.

## MATERIALS AND METHODS

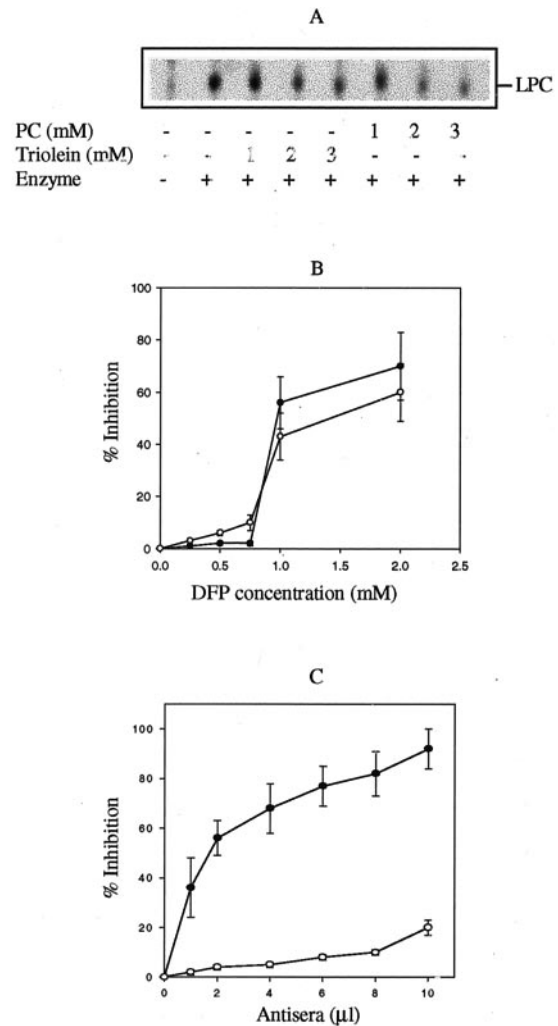
### Materials

[9,10- $^3\text{H}$ (N)]Triolein (10 Ci  $\text{mmol}^{-1}$ ), [2-palmitoyl-9,10- $^3\text{H}$ ]PC (92.3 Ci  $\text{mmol}^{-1}$ ), [1-oleoyl-9,10- $^3\text{H}$ ]lysophosphatidic acid (50 Ci  $\text{mmol}^{-1}$ ), [glycerol- $^{14}\text{C}$ ]phosphatidic acid (100 mCi  $\text{mmol}^{-1}$ ) and  $\text{Na}^{125}\text{I}$  (17 Ci  $\text{mg}^{-1}$  0.1  $\text{mL}^{-1}$ ) were obtained from Perkin Elmer Applied Biosystems (Foster City, CA).  $^{32}\text{P}$ -PC was synthesized from mung bean by in vivo labeling with  $^{32}\text{P}$ -orthophosphate (Herman and Chrispeels, 1980). Phospholipids were quantified by digesting the organic phosphate with perchloric acid and the digested phosphate was determined colorimetrically (Bartlett, 1959). Superdex 75, octyl-Sepharose 4 Fast Flow matrix and ampholytes were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Thin layer chromatography plates and trifluoroacetic acid were from Merck (Rahway, NJ). Ammonium persulfate and reagents for electrophoresis were from Bio-Rad Laboratories (Hercules, CA). Protein assay reagents were obtained from Pierce Chemical (Rockford, IL). All other reagents were obtained from Sigma-Aldrich (St. Louis). Rice (*Oryza sativa*) bran was obtained from local mills.

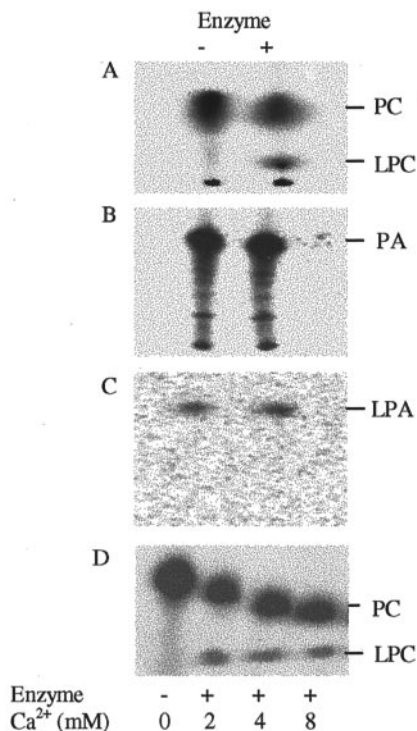
### Purification of Lipase

Rice bran was delipidated with (10 g 100  $\text{mL}^{-1}$ ) diethyl ether and stirred for 12 h at 4°C in 10 mM Tris-HCl, pH 7.5 and 1.0 mM EDTA. The extract was passed through two layers of cheesecloth and centrifuged at 3,000g for 30 min. The clear bran extract was loaded onto an octyl-Sepharose that had been pre-equilibrated with 0.01 M Tris-HCl, pH 7.5 at a flow rate of 2  $\text{mL min}^{-1}$ . The column was washed with

the same buffer until the effluent showed a negligible  $A_{280}$ . The enzyme was eluted with a linear gradient of 0% to 40% (v/v) methanol and fractions of 10 mL were collected. Protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as the standard. Samples were electrophoresed using Laemmli discontinuous buffer system (Laemmli, 1970) on 15% SDS-PAGE gels (10  $\times$  10 cm) at 100 V and stained with silver (Nesterenko, 1994). Purified lipase was used as im-



**Figure 9.** Binding and catalytic sites for triolein and PC in the purified rice bran lipase. A, An autoradiogram showing the competition with unlabeled triolein and PC.  $^{32}\text{P}$ -PC was used as the substrate. B, The enzyme was preincubated with different concentrations of diisopropyl fluorophosphate (DFP) and then measured the activity using PC (closed circle) or triolein (open circle) as substrates. Percent inhibition is calculated with respect to the control where DFP was not added. C, Immunoinhibition of the lipase and phospholipase activities of the purified enzyme. The enzyme was preincubated at various dilutions of the antisera, raised against the purified protein, or preimmune sera at 37°C for 30 min and then the activity was measured on triolein (closed circle) or PC (open circle). Percent inhibition is calculated with respect to the preimmune sera controls. Values are means ( $\pm$  SE) for three independent experiments.



**Figure 10.** Purified rice bran lipase shows phospholipase activity. Autoradiograms showing the TLC profile of the reaction products obtained when  $^{32}\text{P}$ -PC (A),  $^{14}\text{C}$ -phosphatidic acid (B), or  $^3\text{H}$ -lysophosphatidic acid (C) were supplied as substrates. TLC autoradiograms where  $^{32}\text{P}$ -PC was used as substrate and the assay was performed in the presence of increasing concentrations of calcium (D). Reaction products were resolved on TLC using chloroform:methanol:water (65:25:4, v/v).

munogen in rabbits to raise polyclonal antibodies. (Harlow and Lane, 1988).

**Two-Dimensional Gel Electrophoresis**

Protein was analyzed on two-dimensional gel using a Bio-Rad mini-protean II two-dimensional gel apparatus. The first dimension of the gel was run in the acidic direction using ampholytes with pH range of 3.0–10.0. The run was performed at 500 V for 3 h. The second dimension was 15% acrylamide SDS-gel and the protein was visualized by silver staining.

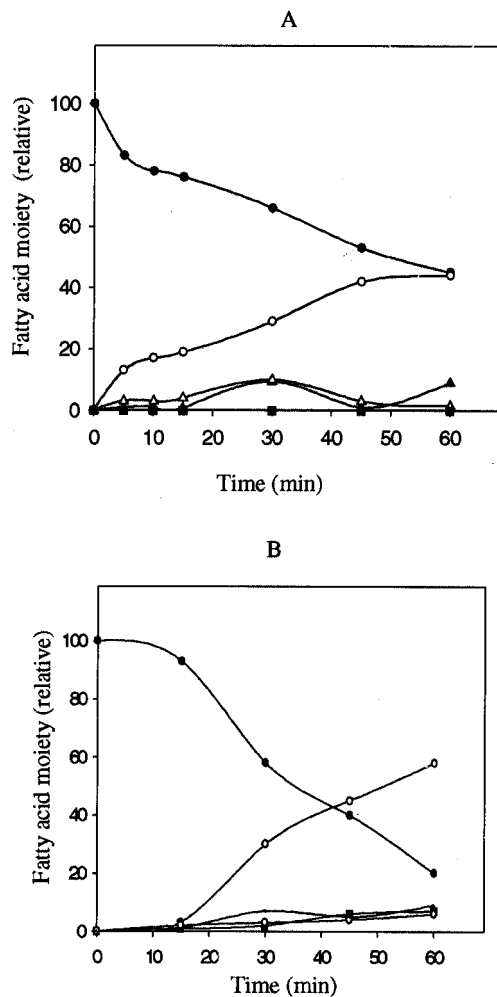
**Reversed-Phase HPLC**

Purified lipase was resuspended in 100  $\mu\text{L}$  of water plus 0.1% trifluoroacetic acid (HPLC grade) and loaded onto a C18 reverse phase column (Vydac reversed phase C18 column, 10- $\mu\text{m}$  particle size, 22-mm i.d., 25-cm length). Prior to loading, the column was pre-equilibrated with water plus 0.1% (v/v) trifluoroacetic acid. Protein was eluted from the column using a linear gradient of 0% to 70% (v/v) acetonitrile (HPLC grade) plus 0.1% (v/v) trifluoroacetic acid and a flow rate of 1.0  $\text{mL min}^{-1}$ . The elution profile was monitored by  $A_{280}$ . Fractions were collected in 1 min

intervals and each fraction was evaluated for the presence of enzyme activity after dialyzing the samples extensively.

**Size-Exclusion FPLC**

Purified lipase was concentrated using a Centricon (5-kD cut-off) and applied onto an analytical Superdex 75 FPLC column fitted with Bio-Rad Biologic low-pressure chromatography system with a buffer consisting of 0.01 M Tris-HCl, pH 7.5 containing 100 mM sodium chloride. Elution was carried out with the same buffer at a flow rate of 1.0  $\text{mL min}^{-1}$ . Fractions were collected in 1-min intervals.



**Figure 11.** A, Formation of intermediates during the hydrolysis of triolein.  $[9,10\text{-}^3\text{H}]$ Triolein was used as a substrate in the time course reaction. Reaction products, FFA (open circle); 1,3-DAG (filled triangle); 1,2-DAG (open triangle); MAG (filled square) and the unreacted substrate (filled circle) were resolved on silica-TLC using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) as the solvent system and quantified by liquid scintillation counter. B, Positional specificity of the purified enzyme toward PC.  $[2\text{-palmitoyl-}9,10\text{-}^3\text{H}]$ PC was used as substrate in a time course reaction. The reaction products FFA (open circle), DAG (closed square), LPC (closed triangle), and unreacted substrate PC (closed circle) were resolved on silica-TLC and quantitated.



## Mass Spectrometry

Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) was recorded using KOMPACT SEQ (Kratos Analytical Inc) MALDI mass spectrometer in positive ion mode.  $\alpha$ -Cyano-4-hydroxy cinnamic acid and sinapinic acid dissolved in a mixture containing 60% (v/v) acetonitrile, 40% (v/v) water, and 0.1% (v/v) trifluoroacetic acid were used as matrices. The purified enzyme was resolved on HPLC and the eluted sample (100 pmol) was spotted along with matrix solution on MALDI target plate and evaporated. Data was acquired on a linear time-of-flight detector (1.7 m) using a pulsed N<sub>2</sub> laser (337 nm). Ions were extracted into the linear TOF using an extraction potential of 20 kV in high-mass detection mode.

## DSC

DSC measurements were performed on a VP-DSC microcalorimeter (Microcal Inc., Northampton, MA). Sample solutions for DSC measurements were prepared by dialyzing the purified protein against 0.01 M phosphate buffer at pH 7.5 exhaustively. The protein concentration was 0.07 mg mL<sup>-1</sup>. Samples and reference buffers were degassed by stirring gently under vacuum prior to measurements. Protein unfolding events were recorded between 20°C and 90°C with a scan rate of 90°C h<sup>-1</sup>. To check the reversibility of the observed transitions, rescans were performed after slowly cooling to 20°C. The scans were analyzed after subtraction of an instrument base line recorded with water in both cells using the software ORIGIN from Microcal.

## CD Spectroscopy

CD spectra were recorded on a Jasco J-720 spectrophotometer (Jasco Research Ltd. Victoria, British Columbia) equipped with a thermostatted cell holder. Spectra were recorded at 0.05 mg mL<sup>-1</sup> in a 1-cm path length quartz cuvette sealed with a teflon stopper. A resolution of 0.1 nm and scanning speed of 20 nm min<sup>-1</sup> with a 2-s response time were employed. Spectra presented are an average of three consecutive spectra. For monitoring thermal stability, spectra were recorded at 20°C, 40°C, 60°C, and 90°C. Equilibration time of 5 min was included at each temperature interval. To check the reversibility, the sample was cooled to 20°C and rescanned.

## Enzyme Assays

In radiometric assays, activity was measured using either [9,10-<sup>3</sup>H]triolein or [2-palmitoyl-9,10-<sup>3</sup>H]PC as a substrate (2.5 mM) in a total volume of 100  $\mu$ L of assay buffer (0.01 M Tris-HCl, pH 7.5, unless otherwise noted). The mixture was incubated at 37°C for 1 h, unless otherwise mentioned, before addition of 400  $\mu$ L of chloroform:methanol (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer (1959). The organic phase was dried (speed vacuum centrifugation), lipids were resuspended in chloroform and resolved on thin-layer silica gel plates (silica Gel 60 F-254) using one of the following solvent

systems: chloroform:methanol:water (65:25:4, v/v), chloroform:methanol:ammonia (65:30:4, v/v), petroleum ether:diethyl ether:acetic acid, (70:30:1, v/v). The lipids were visualized either by autoradiography or with iodine vapor and spots corresponding to the unreacted substrate and the products were scraped off and quantitated by liquid scintillation counting. For triolein hydrolysis, the substrate (2.5 mM) was emulsified with 1% gum Arabic (unless mentioned). For PC hydrolysis, sonicated vesicles of labeled PC were used (250,000 cpm per assay) as substrate. For analyzing the hydrolysis of other phospholipids, the PC was replaced with the phospholipid to be investigated. Control incubations were carried out for zero time and in the absence of enzyme. The control value was subtracted from the actual assay value and the specific activity was calculated after the correction. In a colorimetric assay, *para*-nitrophenyl laurate (2.5 mM) was used as the substrate and the hydrolytic product *para*-nitrophenol was monitored at 410 nm. The absorbance was measured against the reference cell to which water had been added instead of enzyme solution. Because of the simple detection of the released colored product, this assay was used for the initial screening of enzyme activity during the purification procedures. Hydrolysis of non-radiolabeled lipid substrates (diacylglycerol and phosphatidylethanolamine) was detected qualitatively by silica-TLC. Incubations and lipid extractions were carried out essentially as outlined above except that the reaction volume was 0.5 mL. Quantitative estimation of enzyme activity was not done for the non-radiolabeled substrates.

## Synthesis of 1,2-Dipalmitoyl 3-[12-((3-Iodo (<sup>125</sup>I) 4-Azidosalicyl) Amino) Dodecanoyl]-sn-Glycerol (<sup>125</sup>I<sub>3</sub>TAG)

12-[(4-Azidosalicyl) amino]dodecanoic acid (ASD) was synthesized from the *N*-hydroxysuccinimide ester of *p*-azidosalicylic acid (Rajasekharan et al., 1993). 1,2-Dipalmitoyl glycerol (1 mmol) was then acylated with the synthesized ASD-anhydride (4 mmol) by stirring the mixture for 30 h at room temperature in dry chloroform. *N,N*-Dimethyl-4-aminopyridine (0.5 mmol) was used as catalyst. The reaction flask was flushed with nitrogen and sealed. After the reaction, the solvent was evaporated under reduced pressure and the residue was treated with 15 mL of chloroform. The insoluble suspension was removed by filtration and the clear solution was loaded onto silicic acid (20 g) column that had been pre-equilibrated with chloroform. The column was washed with chloroform and then eluted with mixtures of chloroform:methanol (1:1, v/v). The purity was checked by TLC using chloroform:methanol:water (98:2:0.5, v/v). The yield was approximately 46%. The purified 1,2-dipalmitoyl-3-[12-(4-azidosalicyl) amino dodecanoyl]-sn-glycerol was iodinated using Na<sup>125</sup>I and chloramine-T (Ji et al., 1985). The iodinated product was purified using reverse-phase column chromatography. The efficiency of iodination was 59–63%. All operations involving azide were carried out under dim safe light.

### Synthesis of 1,2-di[12-(4-Azidosalicyl)Amino]Dodecanoyl]Glycerophosphocholine (azido-PC)

The synthesis of azido-PC was achieved by direct acylation of CdCl<sub>2</sub> complex of glycerophosphocholine with ASD-anhydride (Gupta et al., 1977). The purified product was iodinated as described earlier. The yield was around 52%.

### Photoaffinity Labeling

The photolabeling experiments were carried out in a final volume of 50  $\mu$ L containing 10  $\mu$ g of lipase in 0.01 M Tris-HCl, pH 7.5, 0.1 mM 2-mercaptoethanol and photoprobe (0.5  $\mu$ Ci, 0.5  $\mu$ M) as described earlier (Tumaney and Rajasekharan, 1999). Mixture was preincubated on ice in the dark for 5 min, in a microfuge tube cap and irradiated for 3 min with a hand-held UV-lamp with the filter removed (5000  $\mu$ W/cm<sup>2</sup>, model UVG-54, UV products) at a distance of 8 cm.

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