

Purification of a lysophosphatidic acid-hydrolysing lysophospholipase from rat brain

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A lysophosphatidic acid (LPA)-hydrolysing lysophospholipase was purified from rat brain and characterized. This membrane-bound lysophospholipase was solubilized by using n-octyl glucoside and purified by sequential cation, hydrophobic and gel-filtration chromatography. The purified protein has a mass of 80 kDa as assayed by SDS/PAGE. This lysophospholipase catalysed the hydrolysis of a variety of lysophosphatidic acids, but with different rates, depending on the length and degree of saturation of the *sn*-1 acyl group (1-oleoyl-LPA \approx 1-stearoyl-

LPA > 1-palmitoyl-LPA > 1-myristoyl-LPA). This enzyme had no measurable catalytic activity when other lysophospholipids, monoacylglycerol or phosphatidic acid were used as substrates. On the basis of its chromatographic properties, substrate specificity and cellular localization, we conclude that this lysophospholipase differs from those previously purified and speculate that it has an important function in terminating biological responses to LPA.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycero-3-phosphate) is a naturally occurring lipid which induces a variety of biological effects, including platelet aggregation [1,2], alterations in neuronal cell morphology [3], induction of smooth-muscle contraction [4,5], mediation of a chemotactic response in *Dictyostelium* [6], and DNA synthesis and cell division in a number of different cell types [7,8]. In addition, LPA can regulate second-messenger production [7–14], possibly by interacting with a specific G-protein-coupled receptor that we have recently characterized [15].

The multiplicity of biological effects of LPA necessitates that the level of this lipid be tightly controlled in order to maintain cellular homeostasis. Lysophospholipases play an important role in metabolizing lysophospholipids. Several lysophosphatidylcholine-preferring lysophospholipases have been purified from a wide range of cells and tissues [16–22]. However, none has been shown to hydrolyse LPA preferentially. In this study, we describe the purification and characterization of this novel LPA-selective lysophospholipase.

MATERIALS AND METHODS

Materials

[³H]Stearoyl-LPA (1-[³H]stearoyl-*sn*-glycero-3-phosphate; sp. radioactivity 180 Ci/mmol) was generously given by Dr. David Ahern (DuPont–New England Nuclear, Boston, MA, U.S.A.). Non-radioactive stearoyl-LPA (Avanti Polar Lipids, Alabaster, AL, U.S.A.) was used to lower the specific radioactivity of [³H]LPA to 360 μ Ci/mmol. 1-Stearoyl-monoacylglycerol was purchased from Serdary Research Laboratories (London, Ontario, Canada). Other lipids, including 1,2-dipalmitoyl phosphatidic acid, 1-stearoyl-*sn*-glycero-3-phosphocholine (stearoyl-lysophosphatidylcholine), 1-stearoyl-*sn*-glycero-3-phosphoethanolamine (stearoyl-lysophosphatidylcholine), 1-oleoyl-*sn*-glycero-3-phosphate (oleoyl-LPA), 1-myristoyl-*sn*-glycero-3-phosphate (myristoyl-LPA), 1-palmitoyl-*sn*-glycero-3-

phosphate (palmitoyl-LPA) and phosphatidylcholine were purchased from Avanti Polar Lipids and were reported by the manufacturer to be \sim 98 % pure. n-Octyl glucoside and phenylmethanesulphonyl fluoride were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Dithiothreitol, bacitracin, soybean trypsin inhibitor and benzamide were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Acrylodated intestinal fatty-acid-binding protein (ADIFAB) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Bolton–Hunter reagent was obtained from and used according to directions from DuPont–New England Nuclear. All other reagents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

LPA-lysophospholipase purification

For this, 50 frozen rat brains (Pel-Freeze, Rogers, AR, U.S.A.) were defrosted at room temperature in 20 mM Hepes (pH 8.0)/1 mM EDTA (1 brain/4 ml) containing 100 μ g/ml bacitracin, 10 μ g/ml soybean trypsin inhibitor, 1 mM benzamide and 1 mM phenylmethanesulphonyl fluoride. The tissue was disrupted by using a Polytron homogenizer, and the resulting homogenate was centrifuged at 10000 g for 30 min at 4 °C. All LPA-hydrolysing lysophospholipase activity was associated with the pellet. Lysophospholipase activity in the pellet was solubilized by homogenizing in n-octyl glucoside (1 %, w/v). The solubilized material was clarified by centrifugation at 100000 g for 45 min at 4 °C.

Cation-exchange chromatography of a LPA-hydrolysing lysophospholipase

The solubilized enzyme was applied on to a 21.5 mm \times 15 cm CM-3SW h.p.l.c. column (Toso Haas, Philadelphia, PA, U.S.A.) which had been pre-equilibrated in 20 mM Hepes (pH 8.0)/1 mM EDTA containing 0.1 % n-octyl glucoside and 10 % ethylene glycol (Buffer A) at a flow rate of 10 ml/min. Protein was eluted from the column by linearly increasing the NaCl concentration in buffer A from 0 to 1 M over a time period of 25 min. A flow rate of 10 ml/min was used throughout, and fractions were

Table 1 Summary of lysophospholipase purification

The data shown are representative of five independent experiments.

	Total protein (mg)	Specific activity (nmol/h per mg)	Total activity (nmol/h)	Enrichment (fold)	Total enrichment (fold)	Yield (%)
Starting material	13257	1.64	21848			
n-Octyl glucoside	4869	4.57	22234	2.8	2.8	100
CM column	102	10.89	1111	2.4	6.7	5.1
Ether column	0.160	925	148	84.9	570.5	0.67
Gel filtration	0.0035	38000	133	41.1	23391	0.60

collected at 1 min intervals and assayed for lysophospholipase activity. The enzyme was unstable at this stage of the purification procedure, and it was essential for the next step to be carried out as rapidly as possible.

Hydrophobic chromatography of a LPA-lysophospholipase

The fractions eluted from the cation column which contained lysophospholipase activity were pooled and further purified by hydrophobic interactive chromatography. $(\text{NH}_4)_2\text{SO}_4$ was added to the sample to a final concentration of 1 M. The sample was then applied to a 7.5 mm \times 7.5 cm ether-5PW h.p.l.c. column (Toso Haas) which had been pre-equilibrated in Buffer A containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The protein was eluted by linearly decreasing the concentration of $(\text{NH}_4)_2\text{SO}_4$ from 1 M to 0 over a period of 25 min, at a flow rate of 1 ml/min throughout. Fractions were collected at 1 min intervals and assayed for lysophospholipase activity. After hydrophobic chromatography, the lysophospholipase was more stable and could be stored on ice overnight without significant loss of activity.

Gel-filtration chromatography of a LPA-lysophospholipase

The lysophospholipase activity obtained from the ether-column fractions were applied to a GF-450 h.p.l.c. column (Zorbax 450XL; DuPont, Wilmington, DE, U.S.A.). Lysophospholipase activity was eluted with buffer A containing 0.5 M NaCl at a flow rate of 2.5 ml/min. Fractions were collected at 1 ml intervals and assayed for lysophospholipase activity.

Radiometric assay of lysophospholipase activity

Lysophospholipase activity was measured in a buffer containing 20 mM Hepes, pH 8.0, and 1 mM EDTA in final assay volume of 0.5 ml. Reactions were initiated by addition of substrate (^3H]LPA, final concn. 250 μM) and were incubated for 1 h at 37 $^\circ\text{C}$. Reactions were terminated by extraction of non-esterified ^3H]fatty acid by the Dole method as previously described [19–21]. Under the conditions used, all reactions were linear with respect to protein concentration and time.

Fluorometric assay of lysophospholipase activity

Hydrolysis of different lysophospholipids by purified lysophospholipase was determined by using the fluorescent fatty-acid-binding protein ADIFAB, and non-radiolabelled substrate as described previously [23]. Briefly, the standard reaction mixture contained 4 $\mu\text{g/ml}$ ADIFAB, purified lysophospholipase and substrate (final concn. 5 μM) in 20 mM Hepes (pH 8.0)/1 mM

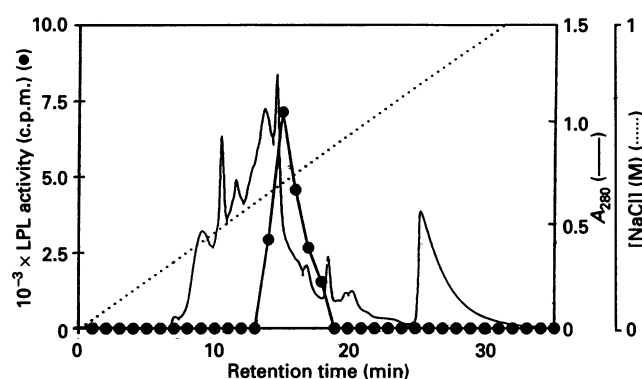


Figure 1 Cation-exchange chromatography of rat brain lysophospholipase

n-Octyl glucoside-solubilized lysophospholipase activity (LPL) from rat brain was applied to a CM-silica column. The material was eluted by increasing the NaCl concentration (.....). A_{280} was monitored (—) and fractions were collected and assayed for lysophospholipase activity (●) with ^3H]LPA as a substrate.

EDTA. Fluorescence was measured at 432 nm with a Perkin-Elmer LS-50B fluorimeter with an excitation wavelength of 390 nm.

Protein measurements

The protein concentration in samples was determined by the method of Bradford [24], with reagents obtained from Bio-Rad (Richmond, CA, U.S.A.). Samples with a low concentration of protein were quantified by using Quanti-Gold (Diversified Biotech, Newton Center, MA, U.S.A.) as directed by the manufacturer.

RESULTS

Lysophospholipase purification

^3H]LPA was readily hydrolysed by rat brain homogenates. All of this enzyme activity was associated with the particulate fraction. The lysophospholipase was quantitatively solubilized by homogenizing the pellet in 1% n-octyl glucoside, indicating that this enzyme was membrane-associated (Table 1). The solubilized material was applied to a cation-exchange h.p.l.c. column. All of the lysophospholipase activity bound to the cation-exchange column. LPA-hydrolysing lysophospholipase activity was eluted as a single peak (Figure 1). The peak of enzyme activity from the cation-exchange column was further

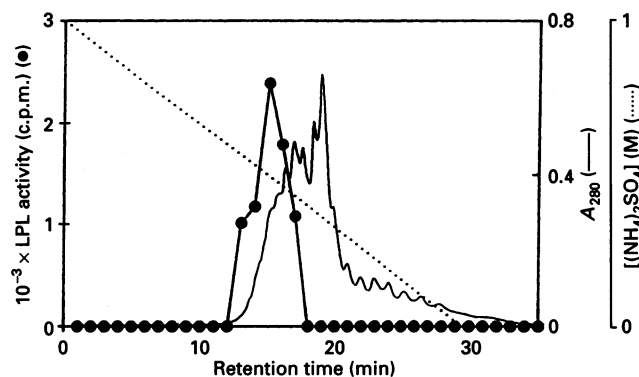


Figure 2 Hydrophobic chromatography of rat brain lysophospholipase

Fractions (14–17 from the CM column) were pooled, and an equal volume of 2 M $(\text{NH}_4)_2\text{SO}_4$ was added before the mixture was applied to an ether hydrophobic-interactive column. The material was eluted by decreasing the $(\text{NH}_4)_2\text{SO}_4$ concentration (.....). A_{280} was monitored (—) and fractions were collected and assayed for lysophospholipase activity (●) with $[^3\text{H}]$ LPA as a substrate.

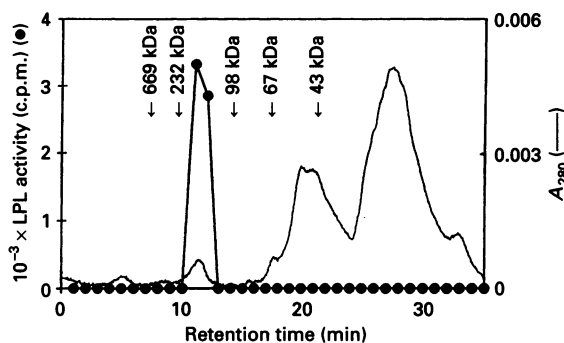


Figure 3 Gel filtration of rat brain lysophospholipase

Fractions 13–17 from the hydrophobic-interactive column were pooled, concentrated to a final volume of 1 ml by ultrafiltration, applied to the gel-filtration column and eluted. A_{280} was monitored (—) and fractions were collected and assayed for lysophospholipase activity (●). The material that was eluted in fractions 11–12 was pooled and used in all subsequent experiments. The molecular-mass standards used were: thyroglobulin, 669 kDa; catalase, 232 kDa; lysozyme, 98 kDa; BSA, 67 kDa; ovalbumin, 43 kDa.

enriched by sequential hydrophobic interactive chromatography (Figure 2) followed by gel-filtration chromatography (Figure 3). The lysophospholipase activity chromatographed with an apparent molecular mass of approx. 150 kDa. This purification procedure resulted in an enrichment of approx. 23000-fold, with a final yield of approx. 0.6% (Table 1). The purity of the isolated lysophospholipase was assessed by SDS/PAGE (Figure 4). A single band with a molecular mass of 80 kDa was observed.

Lysophospholipase substrate specificity

The ability of the purified lysophospholipase to hydrolyse a variety of LPAs with different fatty acids in the *sn*-1 position and different lysophospholipids was determined by a fluorometric assay [23]. Figure 5 illustrates the results obtained by using this assay with 1-stearoyl-LPA as a substrate. Results obtained by this method (610 nmol of fatty acid released/min per mg of protein)

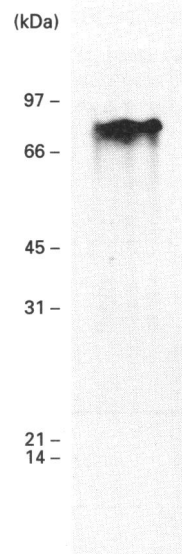


Figure 4 SDS/PAGE of purified rat brain lysophospholipase

The purified lysophospholipase from the gel-filtration column was iodinated by using Bolton–Hunter reagent and analysed by SDS/PAGE. The molecular-mass standards (kDa) used were (from top to bottom) phosphorylase *b*, BSA, aldose, triose phosphate isomerase, trypsin inhibitor and lysozyme.

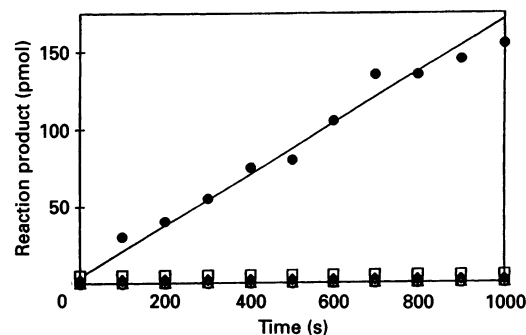


Figure 5 Substrate specificity of purified rat brain lysophospholipase

The purified lysophospholipase (10 ng/ml) was assayed with the indicated substrates (●, 1-stearoyl-LPA; □, 1-stearoyl-lysophosphatidylcholine; △, 1-stearoyl-lysophosphatidylethanolamine; ○, 1-stearoyl-lysophosphatidylinositol; ▲, 1-stearoyl-lysophosphatidylserine) by a fluorometric method [23]. This assay utilized ADIFAB (4 $\mu\text{g}/\text{ml}$) and substrate (5 μM) in a buffer consisting of 20 mM HEPES, pH 8.0, and 1 mM EDTA. The data shown are from a single experiment, four of which were performed.

are in agreement with those determined by using 1- $[^3\text{H}]$ stearoyl-LPA in radiometric assays (Table 1). LPAs with different *sn*-1 substitutions were used as substrates, and the rates of hydrolysis of this enzyme were calculated (Table 2). 1-Oleoyl-LPA and 1-stearoyl-LPA were most readily hydrolysed, followed by 1-palmitoyl-LPA and 1-myristoyl-LPA. Other lysophospholipids, such as 1-stearoyl-lysophosphatidylcholine, 1-stearoyl-lysophosphatidylethanolamine, 1-stearoyl-lysophosphatidylserine and 1-stearoyl-lysophosphatidylinositol, were not hydrolysed by this lysophospholipase (Figure 5). The purified enzyme was unable to hydrolyse other lipids, including phosphatidic acid, phosphatidylcholine, monoacylglycerol and arachidonoyl-CoA when this procedure was used (results not shown).

Table 2 Substrate specificity of purified lysophospholipase

Purified lysophospholipase (10 ng/ml) was incubated with the indicated substrates, and the rates of the hydrolysis were quantified by a fluorimetric assay [23]. The data shown above are means \pm S.D. from three separate experiments using two different preparations of enzyme.

Substrate	Activity (nmol/min per mg of protein)
1-Oleoyl-LPA	640 \pm 48
1-Stearoyl-LPA	610 \pm 24
1-Palmitoyl-LPA	360 \pm 46
1-Myristoyl-LPA	100 \pm 20
1-Stearoyl-glycerol	0

DISCUSSION

LPA induces a spectrum of profound biological effects, which indicates that this compound may play a pivotal role in both physiological and pathophysiological processes [1–5,7–14]. As a consequence, attenuation of the LPA signal is crucial for the maintenance of homeostasis. LPA could potentially be catabolized by a variety of pathways, involving acyltransferase, transacylase, phosphatase or lysophospholipase action. We have previously characterized a LPA receptor, using 1-[³H]stearoyl-LPA, and observed that this ligand was rapidly converted into non-esterified fatty acid [15]. These data suggested that the principal mechanism of LPA degradation was by a lysophospholipase. In the present study, we have described the purification and characterization of this lysophospholipase and demonstrate that it selectively hydrolyses LPA.

Several different lysophospholipases have been purified from a number of cells and tissues [16–22,25]. To date, all are anionic enzymes that are located in the cytosol. Furthermore, all appear to hydrolyse lysophosphatidylcholine preferentially [23]. The lysophospholipase described here differs from these other enzymes in several respects. Firstly, this enzyme is membrane-bound and required detergent for solubilization. Secondly, this enzyme also had distinct chromatographic properties. At pH 8.0 it bound to a cation-exchange column and chromatographed as a 150 kDa protein upon gel filtration. In contrast, SDS/PAGE analysis indicated a molecular mass of approx. 80 kDa, suggesting that this protein chromatographed as a dimer on the gel-filtration column. Thus this lysophospholipase has a different mass from any previously described lysophospholipase (for review see [26]). Finally this lysophospholipase selectively hydrolysed LPA and had no measurable enzyme activity with other lysophospholipids.

Some of the other lysophospholipases purified to date have had other enzymic activities, such as acyltransferase, transacylase and phospholipase A₂ activity (for review see [26]). The enzyme described here was assayed for these activities; however, no such activity was found. It was also conceivable that the enzyme may have had both a phosphohydrolase and a monoacylglycerol lipase activity. However, since the enzyme had no measurable activity towards monoacylglycerol, this rather unexpected possibility can be eliminated. Thus we conclude that this enzyme lacks other enzymic activities.

The high degree of substrate specificity of this lysophospholipase has not been observed for any other lysophospholipase studied [23]. In addition to displaying a specificity for the polar head group, the acyl group in the *sn*-1 position determined the rate of LPA catalysis. 1-Oleoyl-LPA and 1-stearoyl-LPA were

the preferred substrates, followed by 1-palmitoyl-LPA and 1-myristoyl-LPA, which were more slowly hydrolysed. Interestingly, a similar rank order of potencies for these LPAs was observed in both mitogenesis assays [7,27] and in LPA-receptor affinity assays [15]. Thus this enzyme appears to be ideally suited for terminating LPA's biological effects. Indeed, there is precedence in the literature that showed this to be the case. LPA is known to induce platelet aggregation [1,28,29]. Furthermore, 1-*O*-hexadecyl-*sn*-glycero-3-phosphate is 30 times more potent than the acyl analogue. One explanation for the observed difference in potencies may be that the acyl derivative is more readily hydrolysed by the enzyme described here, thus decreasing its effective concentration.

Significant quantities of LPA are produced after cell activation [1,30,31] and, when released into the extracellular milieu, interact with specific membrane-associated receptors on target cells and tissue [15]. The novel LPA-selective lysophospholipase described here is also membrane-bound, and thus may be in close proximity to the LPA receptor. This potent lipid mediates biological responses that are often associated with inflammatory and proliferative disease (for review see [32]). It is therefore essential that the LPA signal is terminated to restore balance to the biological system, and the lysophospholipase described here may be crucial in this process.

We gratefully acknowledge Dr. David Ahern of New England Nuclear for generously giving us the [³H]stearoyl-LPA.

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Received 3 December 1993/19 January 1994; accepted 31 January 1994