Purification and properties of lysophospholipase isoenzymes from pig gastric mucosa

Hiroaki SUNAGA,*† Hiroyuki SUGIMOTO,* Yukio NAGAMACHI† and Satoshi YAMASHITA*‡

*Department of Biochemistry and †First Department of Surgery, Gunma University School of Medicine, Maebashi 371, Japan

Two lysophospholipases, named gastric lysophospholipases I and II (enzymes I and II), were purified 3730- and 2680-fold from pig gastric mucosa. The preparations showed 22 and 23 kDa single protein bands on SDS/PAGE respectively. Both enzymes lacked transacylase activity and appeared to exist as monomers. Their activities were not affected by Ca^{2+} , Mg^{2+} or EDTA. Enzyme I was most active at pH 8.5 and hydrolysed a variety of lysophospholipids including acidic lysophospholipids and the acyl analogue of platelet-activating factor, whereas enzyme II was most active at pH 8 and its activity was confined to lysophosphatidylcholine and lysophosphatidylethanolamine. When 1-palmitoylglycerophosphocholine was used as substrate, enzymes I and II showed half-maximal activities at 11 and 12 μM respectively. The enzymes exhibited no phospholipase B, lipase

or general esterase activity. Enzyme II was significantly inhibited by lysophosphatidic acid whereas enzyme I was only moderately inhibited. Peptide mapping with V8 protease and papain revealed structural dissimilarity between the two enzymes. Antiserum raised against enzyme I did not recognize enzyme II, but did recognize the small-sized lysophospholipase purified from rat liver. Anti-(enzyme II) consistently did not cross-react with enzyme I or the liver enzyme. These antisera specifically recognized neither the 60 kDa lysophospholipase transacylase purified from liver nor any peritoneal macrophage protein. Thus gastric mucosa contains two different small-sized lysophospholipases: one is closely related to the small-sized lysophospholipase of liver, but the other appears to be a novel isoform.

INTRODUCTION

Lysophosphatidylcholine (lysoPC) is produced from phosphatidylcholine (PC) by the action of phospholipase A_2 which is activated in response to a variety of hormones, mitogens and inflammatory stimuli. Although the other product, fatty acid especially arachidonic acid, has attracted special attention because of its conversion into eicosanoids, less attention has been paid to lysoPC. This phospholipid has long been known to be cytolytic [1], but more recently it was shown that it elicits pleiotropic effects, such as inhibition of membrane fusion [2], stimulation of monocyte chemotaxis [3], T-cell activation [4,5], stimulation of mast-cell secretion [6], modulation of vasodilatation [7,8], inhibition of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ pump [9] and electrophysiological alterations in Purkinje fibres [10–12]. LysoPC is known to modulate the activities of protein kinase C [13], guanylate cyclase and adenylate cyclase [14]. Some of the above physiological and pathological effects of lysoPC may be attributable to the modulation of these signal-transducing enzymes.

Lysophospholipase catalyses the hydrolysis of lysoPC to fatty acid and *sn*-glycero-3-phosphocholine (GPC). Effectors modulating its activities, such as acylcarnitine, fatty acid [15–17] and phosphatidic acid [18], have been identified. It is very likely that this enzyme plays an important role in the regulation of the intracellular level of lysoPC. Mammalian lysophospholipases may be classified into two groups according to their molecular mass [19]. Large-sized enzymes (50–63 kDa) were obtained from the microsomal fraction of liver [20–22] and the cytosolic fractions of lung [23], heart [16] and liver [18]. All of the cytosolic

large-sized enzymes exhibited not only hydrolase, but also transacylase, activities, suggesting the formation of acyl-enzyme intermediates. However, the microsomal large-sized enzyme obtained from rat liver did not have such activity [23]. Small-sized enzymes (17.4–28 kDa) were purified from the cytosolic fraction of liver [20], heart [15] and blood cells [17,24–26]. No small-sized enzymes were reported to possess transacylase activity. The relationship between the reported lysophospholipases has not been well studied.

Stomach mucosa contains high levels of phospholipase A_2 activity and mRNA [27,28]. The product, lysophospholipid, is known to exert ulcerogenic effects on gastric mucosa [29,30] whereas prostaglandins act in a protective manner [31]. Thus lysophospholipases as well as phospholipase A_2 are involved in the regulation of ulceration and cytoprotection of gastric mucosa. Although purification of gastric phospholipase A_2 has been reported [32], gastric lysophospholipase has been only partially characterized [33,34]. The present paper describes the purification of two small-sized lysophospholipases from the cytosolic fraction of pig gastric mucosa, both of which lacked transacylase activity. On the basis of immunological and other lines of evidence, the two enzymes were concluded to be distinct isoenzymes. The 22 kDa enzyme, named gastric lysophospholipase I or enzyme I, showed preference for a variety of lysophospholipids, whereas the 23 kDa enzyme, named gastric lysophospholipase II or enzyme II, showed significant preference for lysoPC and lysophosphatidylethanolamine. Immunological studies indicated that enzyme I was closely related to liver lysophospholipase I described by De Jong et al. [20], but enzyme II appears to be a novel lysophospholipase isoform.

Abbreviations used: lysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; GPC, *sn*-glycero-3-phosphocholine; GP, *sn*-glycero-3-phosphate; GPE, *sn*-glycero-3-phosphoethanolamine; GPS, *sn*-glycero-3-phosphoserine; GPI, *sn*-glycero-3-phospho-*myo*-inositol.

‡ To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

1-[¹⁴C]Palmitoyl-GPC (55 mCi/mmol) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*methy*-³H]choline (1,2-dipalmitoyl-[³H]-GPC; 62 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). 1-Palmitoyl-[³H]GPC was prepared from 1,2-dipalmitoyl-[³H]GPC by digestion with snake venom phospholipase A₂, followed by TLC. 1-Palmitoyl-*sn*-glycero-3-phosphate (1-palmitoyl-GP) and 1-palmitoyl-GPC were from Serdary Research Laboratories (London, U.K.) and Bachem Feinchemikalien AG (Bubendorf, Switzerland) respectively. 1-Palmitoyl-*sn*-glycero-3-phosphoethanolamine (1-palmitoyl-GPE), 1-oleoyl-GPE, 1-acyl-*sn*-glycero-3-phosphoserine (1-acyl-GPS) and 1-acyl-*sn*-glycero-3-phospho-*myo*-inositol (1-acyl-GPI) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). 1-Stearoyl-2-arachidonoyl-GP and 1-oleoyl-2-acetyl-GPC were obtained from Sigma (St. Louis, MO, U.S.A.). 1,2-Dipalmitoyl-GPC and *p*-nitrophenylacetate were from Wako (Osaka, Japan). DEAE-Cellulofine A-500 and butyl-Toyopearl were from Seikagaku Kogyo (Tokyo, Japan) and Tosoh (Tokyo, Japan) respectively. Sephacryl S-300 and Blue Sepharose CL-6B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Protein Pak G-ether column used for HPLC was obtained from Millipore (Milford, MA, U.S.A.).

Enzyme assays

Unless otherwise indicated, activity was determined isotopically. The assay mixture contained 20 mM Tris/HCl, pH 8.5, 0.3 mM 1-[¹⁴C]palmitoyl-GPC (750 d.p.m./nmol) and enzyme in a total volume of 0.1 ml. The assay mixture without enzyme was sonicated for 5 min in a Bransonic B-220 sonifier (Shelton, CT, U.S.A.). The reaction was started by adding the enzyme and allowed to proceed for 15 min at 37 °C. The liberated palmitic acid was extracted and counted by a modified Dole's method as described previously [17,18,35]. When substrate specificity was studied, enzyme activity was assayed colorimetrically as described previously [18] using the same reaction mixture as above except that various unlabelled lysophospholipids were used instead of 1-[¹⁴C]palmitoyl-GPC.

Enzyme purification

All procedures were carried out at 4 °C. Unless otherwise stated, solutions used for homogenization and chromatography were supplemented with protease inhibitors (2 µg/ml leupeptin, 0.25 µg/ml pepstatin A, 4 µg/ml antipain, 0.25 µg/ml chymostatin and 20 µg/ml benzamide), 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol. A fresh pig stomach was obtained from a local slaughterhouse. Gastric mucus was removed by scraping the mucosal surface with a glass slide, followed by rinsing with 2 litres of 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 0.25 M sucrose and 1 µg/ml pepstatin A. The mucosa (225 g) was removed from the muscle layer, minced and homogenized for 2 min with a Polytron blender (Kinematica, Littau, Switzerland) in 2.5 vol. of 20 mM histidine/HCl, pH 6.5, containing 0.25 M sucrose. The homogenate was centrifuged at 5000 *g* for 20 min, and the supernatant was centrifuged at 100000 *g* for 90 min. The resulting supernatant (460 ml) was applied to a DEAE-Cellulofine A-500 column (7 cm × 5 cm) equilibrated with 20 mM histidine/HCl, pH 6.5. Almost all the lysophospholipase activity passed through the column. The eluted fraction was adjusted to pH 8.5 with Tris and adsorbed to a DEAE-Cellulofine A-500 column (3.5 cm × 20 cm) equilibrated with 20 mM Tris/HCl, pH 8.5. The enzyme was

eluted with a 1.2-litre linear gradient of NaCl (0–0.75 M) in 20 mM Tris/HCl, pH 8.5. Active fractions were combined, adjusted to 0.5 M NaCl and 0.01% (w/v) Triton X-100, and loaded on to a butyl-Toyopearl 650M column (2 cm × 12 cm) equilibrated with 20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl and 0.01% (w/v) Triton X-100. The column was washed with 3 vol. of the same buffer and eluted with a linear gradient made from 100 ml of 20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl and 0.01% (w/v) Triton X-100 and the same volume of 20 mM Tris/HCl, pH 7.5, containing 20% (v/v) ethylene glycol and 1% (w/v) Triton X-100, followed by elution with the latter solution. To the pooled eluate was added solid (NH₄)₂SO₄ to 60% saturation. The mixture was centrifuged at 100000 *g* for 60 min. The precipitate was dissolved in 5 ml of 20 mM Tris/acetic acid, pH 6.0, containing 0.01% (w/v) Triton X-100 and passed through a Sephacryl S-300 column (3 cm × 50 cm). The active fractions were pooled and applied to a Blue-Sepharose CL-6B column (0.7 cm × 5 cm) equilibrated with 20 mM Tris/acetic acid, pH 6.0, containing 0.01% (w/v) Triton X-100. After being washed with 3 vol. of the same buffer, the column was eluted with a pH gradient made from 15 ml each of 20 mM Tris/acetic acid, pH 6.0, containing 0.01% (w/v) Triton X-100, and 20 mM Tris/HCl, pH 8.5, containing 0.01% (w/v) Triton X-100. Activity was eluted in two peaks. The second peak was used as enzyme II. The first peak (enzyme I) was filtered through a 0.45 µm Versapor filter (Gelman Sciences Japan, Tokyo, Japan), made 0.5 M with (NH₄)₂SO₄, applied to a Protein Pak G-ether column (0.82 cm × 7.5 cm) equilibrated with 20 mM Tris/HCl, pH 7.5, containing 0.5 M (NH₄)₂SO₄ and 0.01% (w/v) Triton X-100, and eluted by decreasing (NH₄)₂SO₄ concentration (0.5–0 M) in the absence of protease inhibitors at a flow rate of 30 ml/h using a Waters model 650 HPLC system.

Identification of the reaction products

The enzymes were incubated with 1-[¹⁴C]palmitoyl-GPC (750 d.p.m./nmol) or 1-palmitoyl-[¹⁴C]GPC (2500 d.p.m./nmol) under the standard assay conditions. The reaction was stopped by adding 3 ml of chloroform/methanol (2:1, v/v). The mixture was then shaken with 0.6 ml of 0.09% (w/v) NaCl and centrifuged at 2000 *g* for 5 min. The lower phase from the 1-[¹⁴C]palmitoyl-GPC incubation was separated on a silica gel H 60 plate (Merck, Darmstadt, Germany) with *n*-hexane/diethyl ether/acetic acid (80:30:1, by vol. or 50:50:1, by vol.), and the entire chromatogram was divided into equal segments. Each segment was counted in 5 ml of a toluene/Triton X-100 scintillator to identify the lipid product. The upper layer from the 1-palmitoyl-[³H]GPC incubation was developed on a silica-gel H 60 plate with methanol/0.6% (w/v) NaCl/30% (w/v) NH₄OH (20:20:1, by vol.). The chromatogram was scraped off and counted to identify the water-soluble reaction product.

SDS/PAGE

This was carried out by the method of Laemmli [36] with 18% (w/v) gels. The gel was stained with the Wako silver stain kit.

Peptide mapping

A modification of the method of Cleveland et al. [37] was used. The purified enzyme was dissolved in 0.125 M Tris/HCl, pH 6.8, containing 10% (v/v) glycerol, 0.5% (w/v) SDS and 0.001% (w/v) Bromophenol Blue, treated in a boiling-water bath for 2 min, and digested with the indicated amount of *Staphylococcus aureus* V8 protease or papain (Wako) for 60 min at 37 °C. After the addition of 2-mercaptoethanol and SDS to final concen-

trations of 10 and 2 % (w/v) respectively, the sample was treated in a boiling-water bath for 2 min, and separated by SDS/PAGE.

Preparation of antisera

Rabbits were anaesthetized and injected in popliteal lymph nodes with 100 µg of purified enzyme I or II emulsified in complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.) with 5 mg of killed *Mycobacterium butyrium* (Difco) and then intramuscularly with 2×10^{10} killed *Bordetella pertussis*. After 4 and 6 weeks, booster injections were given subcutaneously into the backs with 100 µg of enzyme emulsified in complete Freund's adjuvant with 5 mg of killed *M. butyrium*. Antisera were obtained 2 weeks after the final booster injection.

Immunoblotting

The purified enzymes and tissue extracts were separated by SDS/PAGE. The proteins were transferred to a nitrocellulose membrane (Hybond-C) with a semi-dry electroblotter (Sartorius, Goettingen, Germany). The membrane was pretreated with 5 % (w/v) dried skimmed milk in 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl at 4 °C overnight, washed and then treated with anti-lysophospholipase sera diluted 1:250 or 1:500 for 2 h. This was followed by extensive washing with 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl. Immunoreactive proteins were located by treatment with Protein A-peroxidase complex (Zymed Laboratories, San Francisco, CA, U.S.A.) diluted 1:2500 for 30 min and then with the peroxidase immunostaining kit (Wako).

Preparation of rat peritoneal macrophages

Male Wistar rats weighing about 300 g were anaesthetized and injected intraperitoneally with 1 ml of 2 % (w/v) glycogen. After 3 days, the rats were killed and peritoneal macrophages were collected by rinsing the peritoneal cavity with 20 ml of cold PBS, followed by centrifugation at 600 *g* for 5 min and washing. Macrophages obtained from each animal (2×10^7) were separately suspended in 0.5 ml of 20 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose and protease inhibitors (see above), sonicated in an Ohtake 5202 sonicator (4×15 s; output of 50 W) on ice, and then centrifuged at 1500 *g* for 15 min. The resulting supernatant was used for immunoblot analysis.

Protein determination

Protein concentration was determined by the method of Bradford [38] using a Bio-Rad protein assay kit (Richmond, CA, U.S.A.). For samples containing Triton X-100, the Bio-Rad DC protein assay kit was used. 2-Mercaptoethanol was removed in an Amicon microconcentrator (Danvers, MA, U.S.A.). BSA was used as the standard.

RESULTS AND DISCUSSION

Purification of lysophospholipase from pig gastric mucosa

A combination of protease inhibitors was used to prevent proteolytic modification of the enzyme. As much gastric mucus as possible was removed by scraping and rinsing the mucosal surface of the stomach. The remaining mucus was removed by passing the crude extracts through a DEAE-Cellulofine column at pH 6.5. At this pH, over 70 % of the lysophospholipase activity was recovered in the flow-through fraction whereas gastric mucus was largely adsorbed on the column. The pH was adjusted to 8.5, and the eluted fraction was again applied to a DEAE-Cellulofine column. At this pH, virtually all the enzyme activity was adsorbed on the column, and was eluted as a sharp

Figure 1 Separation of enzymes I and II by Blue-Sepharose CL-6B chromatography

Activity (●) and protein (○) were separated into two peaks. The unadsorbed fraction (enzyme I) was further purified by HPLC on Protein-Pak G-ether, and the adsorbed fraction (enzyme II) was eluted from the column in a homogeneous state by a pH gradient (—) constructed using 20 mM Tris/acetic acid, pH 6.0, and Tris/HCl, pH 8.5.

Table 1 Enzyme purification

Fraction	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (-fold)	Yield (%)
Crude extract	3750	2330	0.62	1	100
DEAE-Cellulofine I	1380	1660	1.2	1.9	71
DEAE-Cellulofine II	64.3	476	7.4	12	20
Butyl-Toyopearl 650M	2.65	480	181	292	21
Sephacryl S-300	0.35	546	1560	2520	23
Blue-Sepharose					
Enzyme I	0.23	495	2150	3470	21
Enzyme II	0.01	16.6	1660	2680	0.7
Protein Pak G-ether					
Enzyme I	0.20	462	2310	3730	20

peak with 0.03 M NaCl, resulting in a 6-fold purification. The NaCl concentration was then adjusted to 0.5 M, and the eluate was applied to a butyl-Toyopearl column; enzyme was eluted by decreasing the concentration of NaCl while increasing the concentrations of ethylene glycol and Triton X-100. This step was very effective, resulting in a 24-fold purification. As the eluted enzyme was unstable, it was immediately precipitated with 60 % satd. $(\text{NH}_4)_2\text{SO}_4$ and passed through Sephacryl S-300. A low concentration of Triton X-100 (0.01 %, w/v) was an absolute requirement for efficient recovery of the enzyme from the column. On the Blue-Sepharose column, the enzyme activity was separated into two peaks (Figure 1). The unadsorbed major peak of activity (enzyme I) was further purified by G-ether HPLC, and the enzyme adsorbed on Blue-Sepharose (enzyme II) was eluted with a pH gradient from 6.0 to 8.5. The final preparations gave single protein bands on SDS/PAGE with molecular masses of 22 and 23 kDa (see below). Table 1 shows a typical enzyme purification. The overall purifications of enzymes I and II were

Figure 2 Identification of the reaction products

Enzyme I (a, b; 150 ng) or enzyme II (c, d; 300 ng) was incubated with 1-[^{14}C]palmitoyl-GPC (a, c) or 1-palmitoyl-[^3H]GPC (b, d), and the products soluble in chloroform/methanol (2:1, v/v) (a, c) and water (b, d) were analysed by silica-gel TLC. Arrows indicate the locations of the authentic standards: lysoPC (LPC), palmitic acid (Pal), choline (Cho), phosphocholine (PCho) and GPC.

3730- and 2680-fold with yields of 20 and 0.7 % respectively. The enzymes could be kept at -80°C for at least 3 months without any appreciable loss of activity.

Reaction products

To confirm the identity of the purified enzymes, they were incubated with labelled 1-palmitoyl-GPC and the radioactive products were identified by TLC. To analyse the lipid product of the enzymes, we used 1-[^{14}C]palmitoyl-GPC as substrate. After incubation, the lipid produced was extracted with chloroform/methanol (2:1, v/v) and chromatographed on a silica-gel plate using *n*-hexane/diethyl ether/acetic acid (80:30:1, by vol. or 50:50:1, by vol.). As shown in Figure 2, the lipid produced by enzymes I and II co-migrated with authentic palmitic acid on TLC. When 1-palmitoyl-[^3H]GPC was used as substrate and the water-soluble product was analysed by TLC, radioactivity co-migrated with authentic GPC. These results indicate that both enzymes I and II hydrolysed 1-palmitoyl-GPC to palmitic acid and GPC, confirming that the purified enzymes were indeed lysophospholipases. We next examined whether or not the purified enzymes possessed transacylase activity. Lipid produced from 1-[^{14}C]palmitoyl-GPC was developed with chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:0.25, by vol.), but no PC formation could be demonstrated (results not shown), indicating the absence of transacylase activity in enzymes I and II.

Figure 3 Effect of various substrate concentrations

(a) Enzyme I (20 ng); (b) enzyme II (40 ng). Activity was assayed using various concentrations of 1-[^{14}C]palmitoyl-GPC as substrate.

Properties of the enzymes

From elution profiles on a Sephacryl S-200 column, the molecular masses of native enzymes I and II were estimated to be 21 and 23 kDa respectively (results not shown). These values were very close to those determined on SDS/PAGE (22 and 23 kDa respectively), indicating that both lysophospholipases exist as monomers. The activities of the enzymes were not affected by Ca^{2+} , Mg^{2+} or EDTA. Thus no metal ions were included in the standard assay mixture. Both enzymes were active over wide pH ranges. Some differences in the activity were noted with the buffers used. Activity was somewhat higher in phosphate buffer than acetate and Tris buffers. In Tris buffer, enzyme I was the most active at pH 8.5 and enzyme II at pH 8, but enzyme II was almost fully active at pH 8.5 as well. We routinely used Tris/HCl buffer, pH 8.5, for both enzyme assays. Figure 3 shows the effect of various concentrations of 1-palmitoyl-GPC on the activities of enzymes I and II. As in the case of amnionic and macrophage lysophospholipases [17,39] and liver lysophospholipase transacylase [18], the enzymes did not follow normal saturation kinetics. The substrate-dependence curves were sigmoidal and the double-reciprocal plots were upward concave. Half-maximal activities were obtained at 11 and 12 μM 1-palmitoyl-GPC with enzymes I and II respectively. Based on a detailed kinetic study of macrophage lysophospholipase, Stafford et al. [40] recently showed that the atypical kinetics of lysophospholipase were not related to the aggregation state of the substrate as previously thought [17,39], but were characteristic of the enzyme itself.

Substrate specificity

We next examined the substrate specificities of enzymes I and II. Because of limited availability of labelled lysophospholipids, we used the colorimetric method for enzyme assay. The fatty acid liberated from lysophospholipids was converted into enoyl-CoA using acyl-CoA synthetase and acyl-CoA oxidase, and the H_2O_2 formed by the latter reaction was colorimetrically estimated [41]. As shown in Table 2, enzyme I utilized various lysophospholipids with the following order of effectiveness: 1-acyl-GPS > 1-oleoyl-GPC = 1-palmitoyl-GPC > 1-oleoyl-GPE > 1-acyl-GPI > 1-stearoyl-GPC > 1-palmitoyl-GPE. Enzyme II predominantly

Table 2 Substrate specificity

The activities of enzymes I (100 ng) and II (72 ng) were assayed colorimetrically with different substrates at 0.8 mM (*p*-nitrophenylacetate was used at 0.4 mM). ND, Not detectable.

Substrate	Activity ($\mu\text{mol}/\text{min per mg}$)	
	Enzyme I	Enzyme II
1-Palmitoyl-GPC	2.1	1.8
1-Stearoyl-GPC	1.1	1.5
1-Oleoyl-GPC	2.1	3.4
1-Palmitoyl-GPE	0.5	0.7
1-Oleoyl-GPE	1.7	2.1
1-Acyl-GPI	1.6	0.2
1-Acyl-GPS	2.3	0.5
1-Oleoyl-2-acetyl-GPC	4.2	0.2
1,2-Dipalmitoyl-GPC	ND	ND
Triolein	ND	ND
<i>p</i> -Nitrophenylacetate	ND	ND

Figure 4 Inhibition of enzymes I (a) and II (b) by phosphatidic acid, lysophosphatidic acid and non-esterified fatty acid

To test the inhibitory effects of 1-oleoyl-GP (●), 1-palmitoyl-GP (○), palmitic acid (□) and 1-stearoyl-2-arachidonoyl-GP (■), the lipids were added to assay tubes as chloroform solutions and dried under a stream of N_2 . The reaction mixture was then added and sonicated to achieve thorough mixing. The reaction was started by the addition of 120 or 73 ng of enzyme I or II respectively.

utilized 1-acyl-GPC and 1-acyl-GPE. The order was 1-oleoyl-GPC > 1-oleoyl-GPE > 1-palmitoyl-GPC > 1-stearoyl-GPC > 1-palmitoyl-GPE. 1-Acyl-GPS and 1-acyl-GPI were poor substrates of enzyme II. Thus enzyme I exhibited a rather broad specificity toward lysophospholipids including acidic lyso-

Figure 5 Peptide mapping

Purified enzymes I (lane 1) and II (lane 2) were separated by SDS/PAGE and silver-stained. Some 200 ng of enzymes I (lanes 3 and 5) and II (lanes 4 and 6) were digested for 60 min at 37 °C with 2.5 μg of *Staphylococcus aureus* V8 protease (lanes 3 and 4) or 0.25 μg of papain (lanes 5 and 6) in the presence of SDS and then electrophoresed. The arrows on the left indicate the locations of the size markers: egg albumin, 45 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa (from top to bottom).

phospholipids. In addition, enzyme I efficiently deacylated 1-oleoyl-2-acetyl-GPC, an acyl analogue of platelet-activating factor and hence resembled 25 kDa bovine liver lysophospholipase I [42]. In contrast with this rather broad substrate specificity of enzyme I, enzyme II was specific for 1-acyl-GPC and 1-acyl-GPE and exhibited little activity toward 1-acyl-2-acetyl-GPC. Neither enzyme hydrolysed diacylphospholipids, triolein and *p*-nitrophenylacetate at measurable rates. 1-Stearoyl-2-[1- ^{14}C]arachidonoyl-*sn*-glycerol (Amersham) was not hydrolysed (results not shown). These results indicate that the lysophospholipase activities observed here are not due to phospholipase B, lipase or general esterase. The marked difference in substrate specificity indicates that enzymes I and II are distinct isoenzymes.

Inhibition by phosphatidic acid, lysophosphatidic acid and fatty acid

Inhibition of lysophospholipase by low concentrations of acyl-carnitine and fatty acid was demonstrated with enzymes purified from heart [15,16] and macrophages [17,25]. More recently, rat liver supernatant was found to contain endogenous inhibitors of lysophospholipase transacylase, which were identified as phosphatidic acid and non-esterified fatty acid [18]. In addition, lysophosphatidic acid was also shown to be inhibitory to the enzyme [18]. In view of these observations, we examined whether or not enzymes I and II showed different sensitivities to these inhibitor lipids. Enzyme II was more sensitive to lysophosphatidic acid than enzyme I (Figure 4), supporting the above idea. Phosphatidic acid and fatty acid had little effect.

Peptide mapping

The molecular masses of enzymes I and II were estimated to be 22 and 23 kDa respectively on SDS/PAGE (Figure 5, lanes 1 and 2). We examined qualitative differences between enzymes I and II by peptide-mapping analysis with *Staphylococcus aureus* V8 protease and papain by the method of Cleveland et al. [37]. After protease digestion in the presence of SDS, the cleaved enzymes were separated by SDS/PAGE (Figure 5, lanes 3–6). V8 protease and papain produced strikingly different patterns of digestion. When V8 protease was used, enzyme I produced several small peptides and enzyme II larger fragments. Papain treatment gave rise to a large fragment from enzyme I, but several smaller fragments

Figure 6 Immunoblot analysis

(a) Enzyme I (200 ng, lanes 1 and 5), enzyme II (250 ng, lanes 2 and 6), cytosol of pig gastric mucosa (120 μ g, lanes 3 and 7) and liver large-sized lysophospholipase (100 ng, lanes 4 and 8) were subjected to immunoblot analysis using anti-(enzyme I) serum (lanes 1–4) or anti-(enzyme II) serum (lanes 5–8). (b) Enzyme I (200 ng, lanes 1 and 4), enzyme II (250 ng, lanes 2 and 5) and liver small-sized lysophospholipase (150 ng, lanes 3 and 6) were probed with anti-(enzyme I) serum (lanes 1–3) and anti-(enzyme II) serum (lanes 4–6). (c) Enzyme I (200 ng, lanes 1 and 5), enzyme II (250 ng, lanes 3 and 6) and 1500 μ g supernatant of rat peritoneal macrophages (75 μ g, lanes 2, 4 and 7) were subjected to immunoblot analysis using anti-(enzyme I) serum (lanes 1 and 2), anti-(enzyme II) serum (lanes 3 and 4) and preimmune serum (lanes 5–7).

together with a large one from enzyme II. No peptide common to both enzymes was obtained. These results indicate considerable structural differences between enzymes I and II.

Immunological characterization of enzymes I and II

To confirm the above conclusion, we raised polyclonal antibodies against individual enzymes in rabbits by repeated injection of the purified enzymes, and used them for immunoblot analysis. Antiserum raised against enzyme I hardly recognized enzyme II (Figure 6a, lane 2), and antiserum against enzyme II did not react with enzyme I (lane 5). When the cytosol of pig gastric mucosa was probed with anti-(enzyme I), a protein band with the same mobility as enzyme I could be detected (lane 3). When anti-(enzyme II) was used, different bands were stained with the major band being slightly retarded on SDS/PAGE when compared with enzyme II. The reason for this was not known. Anti-(enzyme I) and anti-(enzyme II) antibodies could also react with 22 and 23 kDa proteins in the cytosol of rat gastric mucosa respectively (results not shown). Neither antiserum recognized the purified rat liver 60 kDa lysophospholipase transacylase (lanes 4 and 8). These results clearly indicate that enzymes I and

II are structurally distinct from each other and also from the large-sized lysophospholipase.

We next examined the relationship of enzymes I and II to small-sized lysophospholipases in other tissues. De Jong et al. [20] showed that bovine liver contains lysophospholipase with a molecular mass of 25 kDa [22]. The corresponding enzyme with a molecular mass of 24 kDa was purified from rat liver supernatant to homogeneity (H. Sugimoto, unpublished work) and tested for cross-reactivity with the antibodies. Figure 6(b) shows that anti-(enzyme I) serum cross-reacted with the rat liver enzyme, but anti-(enzyme II) did not. This indicates that enzyme I is closely related to the hepatic enzyme, but enzyme II is not. Macrophages have been reported to contain two small-sized lysophospholipases, and the enzymes were purified from two cell lines, P388D₁ and WEHI265.1 [17,25]. They were shown to have considerably higher molecular masses (27 and 28 kDa), and hence thought to be different from the present enzymes. We performed experiments to examine whether the enzymes in the two tissues were immunologically related. Rats were intra-peritoneally injected with glycogen, and macrophages were collected from the peritoneum, disrupted by sonication, centrifuged and subjected to immunoblot analysis. As shown in Figure 6(c), there was no obvious protein band in the macrophage supernatant capable of specifically reacting with anti-(enzyme I) and anti-(enzyme II) antibodies. The results shown here are representative of three experiments. These findings are consistent with the view that macrophage lysophospholipases are not closely related to enzymes I and II and therefore to liver lysophospholipase either. Considering the marked developmental and functional differences between stomach and macrophages, these findings are not surprising.

In summary, the cytosol of gastric mucosa contains two immunologically distinguishable small-sized lysophospholipases, enzymes I and II, which exhibit markedly different substrate specificities. Enzyme I is closely related to the small-sized lysophospholipase of liver. Enzyme II seems to be a novel small-sized isoenzyme.

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