J Amer Oil Chem Soc (2007) 84:645–651
DOI 10.1007/s11746-007-1081-1

ORIGINAL PAPER

Conversion of Phosphatidylcholine to Phosphatidylglycerol with Phospholipase D and Glycerol

George J. Piazza · William N. Marmer

Received: 25 January 2007 / Revised: 26 March 2007 / Accepted: 26 April 2007 / Published online: 26 June 2007

Abstract When phosphatidylcholine (PtdCho) is acted upon by the enzyme phospholipase D (PLD) in the presence of glycerol and water, at least two products can arise: phosphatidic acid (PtdOH) from hydrolysis and PtdGly from transphosphatidylation. Commercial PLD preparations from *Streptomyces chromofuscus*, *Streptomyces* sp., and cabbage were examined for their ability to selectively promote PtdGly formation in a two phase aqueous-organic solvent system. Factors examined were enzyme amount, pH, glycerol concentration, and the type of organic solvent. The reaction of PtdCho to give products such as PtdGly was followed by HPLC using a stationary phase consisting of a polymerized poly (vinyl alcohol) on silica gel with ELSD. The identities of all products were confirmed by retention times and HPLC-MS analyses. Under all tested conditions PLD from *S. chromofuscus* gave at most a 15% yield of PtdGly. Higher amounts of added glycerol inhibited this PLD. Nearly quantitative conversion to PtdGly was obtained with cabbage PLD when the mol ratio of glycerol to PtdCho was at least 64 (mol water/glycerol = 105). With PLD from *Streptomyces* sp. a nearly quantitative yield of PtdGly was obtained when the mol ratio of glycerol to PtdCho was at least 5.3 (mol water/glycerol = 1,266), demonstrating that this PLD had a higher selectivity for glycerol than cabbage PLD. When the glycerol concentration was very low, the level of PtdOH increased, and cardiolipin (diphosphatidylglycerol) was generated. The highest mol ratio PtdGly to PtdOH was observed when the solvents isopropyl ether or dichloromethane were used.

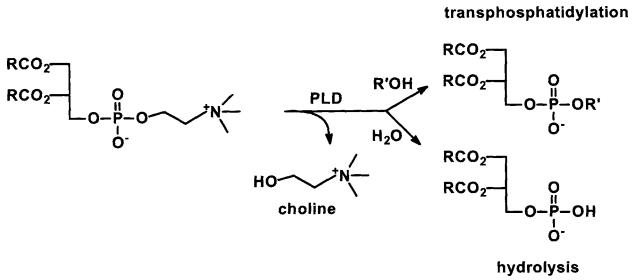
Keywords Atmospheric pressure chemical ionization · Cardiolipin · Electrospray ionization · High performance liquid chromatography · Phosphatidic acid · Phosphatidylcholine · Phosphatidylglycerol · Phospholipase D

Introduction

Phospholipase D (PLD) catalyzes phosphate ester hydrolysis of phosphatidylcholine (PtdCho) to give choline and phosphatidic acid (PtdOH). Generation of PtdOH has received much attention, as it is known to stimulate a number of physiological events [1]. However, PLD can also catalyze a transphosphatidylation reaction with primary alcohols to generate new phospholipids (PL); ethanol and 1-butanol are preferentially used over water as the nucleophile by 1,000-fold or more [2]. A diagram of the hydrolysis and transphosphatidylation reactions is shown in Scheme 1 where PtdCho (RCO_2, fatty ester) is converted to PtdOH (lower pathway) with water and to a new phospholipid with a primary alcohol (R′OH) (upper pathway). Glycerol has been used in the transphosphatidylation reaction to convert the readily available neutral PtdCho to acidic phosphatidylglycerol (PtdGly) [3, 4]. As glycerol has recently become available in large amounts due to increasing biodiesel production, research on the synthesis of PtdGly is warranted as a means of finding an additional use for excess glycerol [5]. Although PtdGly is a naturally

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

G. J. Piazza (✉) · W. N. Marmer
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA
e-mail: george.piazza@ars.usda.gov



Scheme 1 Pathway of the action of phospholipase D (PLD) on phosphatidylcholine (PtdCho) with displacement of choline by alcohol (R′OH, transphosphatidylation) or water (hydrolysis)

occurring PL, it is normally found in only small amounts, and yet it is known to have unique surfactant/lubricant properties, particularly when mixed with appropriate proteins. For example PtdGly is found in relatively large amounts in mammalian lung compared to other mammalian membranes [6]. The development and production of surfactant preparations for therapeutic practices for relief of a variety of diseases such as neonatal respiratory distress syndrome is currently an area of intense research, and access to an additional source of PtdGly would be helpful.

As noted above PLD has been used to make synthetic PtdGly. However, the commercially available PLD enzymes are expensive and relatively unstable. New rapid assays for PtdGly formation that simultaneously measure the formation of the hydrolysis product PtdOH and other unwanted byproducts would be very helpful for the discovery of new PLD enzymes or for testing modifications of existing PLD enzymes. Here we describe the development of an HPLC/ELSD and HPLC/MS assay that can be used to conveniently assay PLD. We have demonstrated the utility of the assay using three commercially available PLD enzymes.

Materials and Methods

Materials

PLD (E.C. 3.1.4.4) preparations from *Streptomyces chromofuscus* (lyophilized powder, ≥2,000 units/mg solid), *Streptomyces* sp. (lyophilized powder ≥1,300 units/ mg solid) and cabbage (lyophilized powder ≥100 units/mg solid) were purchased from Sigma (St Louis, MO). One unit will liberate 1.0 μmol of choline from L-α-phosphatidyl choline per hr at pH 5.6 at 30°C. Anhydrous glycerol (99.5%) was purchased from Fluka Biochemika (Buchs, Switzerland). Phosphatidylcholine, phosphatidic acid, and other phospholipids were 1,2 dioleoyl derivatives and were purchased from Sigma. All other reagents were of the highest purity available.

Activity Assays

Activity assays were conducted as follows except as noted in the text. To each vial (1.8 mL) was added PtdCho (1.7 mg) containing two acyl oleates (PtdCho-diO) (2.16 μmol) in 860 μl CH_2Cl_2, 10 μl glycerol (0.137 mmol), and various amounts of PLD in 260 μl 0.2 M sodium acetate buffer, pH 5.5, containing 0.05 M $CaCl_2$. Samples were mixed at 20 °C on a laboratory rotator (36 cm diameter) at 20 rev/min. After 24 h samples were partitioned between 8 mL $CHCl_3$/CH_3OH (2:1, v/v) and 8 mL aqueous 5% Na_2SO_4. The aqueous fraction was extracted with 5 mL $CHCl_3$/CH_3OH. The combined $CHCl_3$/CH_3OH extracts were taken to dryness under a stream of nitrogen. The solid was dissolved in 5 mL $CHCl_3$/CH_3OH. The solution was filtered through a 0.45 μm PVDF membrane and taken to dryness under a nitrogen stream. The samples were dissolved in 0.5 mL $CHCl_3$/CH_3OH and analyzed by HPLC.

Separation and Quantitation of Products

Products of PLD action (PdtOH and other phospholipids) were separated from each other and unreacted PtdCho at 22 °C on a YMC PVA-Sil S-5 120-Å column (250 H 2.0 mm) (Waters, Milford, MA). Products and unreacted PdtCho were detected with a Varex MK III evaporative light-scattering detector (ELSD) (Alltech, Deerfield, IL) operated at 60 °C, with N_2 as the nebulizing gas at a flow rate of 1.5 L/min. Mobile phase composition was eluent A: hexane/*t*-methylbutyl ether (98:2 v/v) containing 5 mM acetic acid and 5 mM triethylamine; eluent B: isopropanol/acetonitrile/chloroform/acetic acid (84:8:8:0.025 v/v); eluent C: isopropanol/water (1:1 v/v) containing 1 mM acetic acid and 1 mM triethylamine. The solvent gradient system was 0–30 min A/B (80/20 v/v) to A/B/C (42/52/6 v/v); 30–44 min A/B/C (42/52/6 v/v) to A/B/C (32/52/16 v/v); 44–50 min A/B/C (32/52/16 v/v); 50–56 min A/B/C (32/52/16 v/v) to A/B (80/20 v/v) at a flow rate of 0.5 mL/min. Detector response was converted to mass using calibration curves prepared from PtdCho, PtdOH, and PtdGly. The ranges of concentrations used were 5–50 μg, 4–40 μg, and 4–40 μg per10-μL injection, respectively. Response curves were computer fitted with second order quadratic equations.

Product Characterization

Starting phospholipid (PtdCho), PtdOH, PtdGly, and other phospholipids were characterized by HPLC-MS. Products were detected with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (Micromass ZMD, Waters, Milford, MA) using the above HPLC conditions. The APCI detector was set to scan over the mass range of *m/z* 500–2,000 at 400 amu per s. The corona,

cone, and extractor voltages were 2,500, 32, and 4 V, respectively. The source and APCI heater temperatures were 110 and 500°C, respectively. The ESI detector was set to scan over the mass range of *m/z* 400–2,500 at 300 amu per s. The capillary, cone, and extractor voltages were 2,480, 42, and 1 V, respectively. The source and desolvation heater temperatures were 145 and 154 °C, respectively.

Results and Discussion

HPLC Analysis of Phospholipids

In prior research, the conversion of PtdCho to PtdGly was frequently followed by TLC using either TLC/FID [7] or radiolabel [8, 9] for detection. While HPLC has also been used to separate products of PLD action, it has not been used for following PtdGly synthesis [10]. HPLC-based separations on a silica gel stationary phase generally lacked sufficient resolving power to separate PtdGly and PtdOH, and the need to add water to the silica gel requires a long reconditioning time. Also irreversible adsorption of solutes can give poor separation performance with repeated use [11]. More recently, polar-bonded phases have been developed for PL separations. A report by Christie and Urwin demonstrated lipid class analysis on a commercially available column containing a stationary phase consisting of a polymerized poly (vinyl alcohol) on silica gel (PVA-Sil®) [12]. The phospholipids from potato tubers were generally well resolved. Subsequent research in several laboratories has documented the utility of this stationary phase [11, 13–16]. Peak shapes are nearly symmetrical, and this stationary phase is compatible with solvents ranging from hexane to water. As noted most of the HPLC methods developed to date do not separate PtdOH and PtdGly [17], and this issue has not been addressed in the PVA-Sil work [11–16].

Different gradient systems were examined for their ability to separate the PLs discussed above on PVA-Sil. A ternary gradient system for following PtdGly production was developed for the PVA-Sil HPLC column and is described in ''Materials and Methods''. This system is compatible with detection by ELSD and by APCI- and ESI-MS. Using the developed gradient system, several commonly encountered glycerol-based phospholipid standards were injected onto the PVA-Sil column. Each of the standard phospholipids contained one or two oleates as acyl groups. These are indicated by the designation ''monoO'' and ''diO'' only when necessary to clarify the discussion. The phospholipids were detected using ELSD or MS. The chromatographic and MS characteristics of these standards are summarized in Table 1. The phospholipids that are of major interest here contain choline (PtdCho) or glycerol (PtdGly) as head groups or have no head group (PtdOH) as a result of hydrolysis. It can be seen that these phospholipids have substantially different retention times and are well separated from each other during the chromatographic procedure. For comparison Table 1 also shows the properties of the phospholipids containing the ethanolamine (PtdEtn) and serine (PtdSer) head groups. The retention time of PtdEtn is substantially different than that of PtdCho. However PtdSer elutes closely to PtdCho.

The chromatographic characteristics of the lyso derivative of phosphatidyl choline (PtdCho-monoO) are shown in the last entry of Table 1. These would be of potential interest if an impure source of PLD were being assayed because the lyso derivative is a product of the action of lipase or phospholipase A on PtdCho-diO. As noted in the table, PtdCho-monoO elutes about 12 min after PtdCho-diO, and therefore the detection of potential fatty acyl hydrolysis is readily made by the HPLC method.

When using MS as the detector, PLD activity can be followed by observing the change in the base peaks of phospholipids even if only poor separation of the phospholipids is obtained. Base peaks obtained with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are shown in Table 1 in the three columns on the right. Positive and negative ions were observed with APCI, and negative ions with ESI. The base peaks obtained are of two types. One type is *m/z* plus or minus a proton depending on whether positive or negative ion detection is used. The other type is *m/z* plus or minus a charged adduct such as $NH(CH_2CH_3)_3^+$ (M + 101) or CH_3COO^- (M + 59) also depending on whether positive or negative ions are being detected. The base peak for the PL derivatives obtained as $m/z = [M + 1]^+$ or as $m/z = [M - 1]^-$ are shown in bold print. Previous research using ESI to detect PL found similar patterns in base peak detection, although the most prominent (nonproton) adduct obtained depends upon the composition of the eluent [18].

PLD from *Streptomyces chromofuscus*

Reactions of PLD from *Streptomyces chromofuscus* were conducted with PtdCho-diO in the presence of glycerol as described above. The amount of PLD added to each reaction was varied between 0.1 and 995.2 units, and the amount of added glycerol from 0.137 mmol to 2.29 mmol. Reactions were analyzed by HPLC with detection by ELSD as described above. When one unit or more PLD was added, complete reaction of PtdCho was noted, and the major reaction product was PtdOH. PtdGly was at most only 15% of the product. When the amount of glycerol was increased in an attempt to increase the PtdGly, the activity

Table 1 Retention times on HPLC and base peaks observed with several MS detection methods

Compound name	Formula	Formula weight	Isotopic dist.	Retention time (min)[a]	Base peak (*m/z*)[b]		
					APCI+	APCI-	ESI-
PtdCho-diO (1,2-dioleoyl-*sn*-glycero-3-phosphocholine)	$C_{44}H_{84}NO_8P$	786.11	785 (59.3%) 786 (30.2%) 787 (8.5%)	30.5	**786**[c]	820[d]	845[e]
PtdEta-diO (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine)	$C_{41}H_{78}NO_8P$	744.03	743 (61.4%) 744 (29.1%) 745 (7.7%)	18.6	845[f]	**742**[g]	**742**[g]
PtdGly-diO (1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol))	$C_{42}H_{79}O_{10}P$	775.06	774 (60.6%) 775 (29.3%) 776 (8.1%)	15.3	876[f]	**773**[g]	**773**[g]
PtdSer-diO (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine)	$C_{42}H_{78}NO_{10}P$	788.06	787 (60.4%) 788 (29.4%) 789 (8.2%)	30.1	888[f]	**786**[g]	**786**[g]
PtdOH-diO (1,2-dioleoyl-*sn*-glycerol-3-phosphate	$C_{39}H_{73}O_8P$	700.97	700 (63.0%) 701 (28.2%) 702 (7.2%)	18.0	802[f]	**699**[g]	**699**[g]
PtdCho-monoO (1-oleoy-*sn*-glycero-3-phosphocholine)	$C_{26}H_{52}NO_7P$	521.67	521 (72.9%) 522 (22.1%) 523 (4.3%)	42.2	**522**[c]	580[e]	506[h]

[a] PVA-Sil S-5 column used according to the procedure in ''Materials and Methods''

[b] *m/z* rounded to whole number. The lowest *m/z* of the isotopic group is reported. *m/z* values in bold font are either +1 or –1 from isotopic formula weight

[c] $[M + H]^+$

[d] $[M + Cl]^-$

[e] $[M + CH_3COO]^-$

[f] $[M + NH(CH_2CH_3)_3]^+$

[g] $[M–H]^-$

[h] $[M\text{-}CH_3]^-$

of PLD was inhibited. Thus it is concluded that it is not possible to produce high levels of PtdGly using PLD from *S. chromofuscus*.

Cabbage PLD

Tests of cabbage PLD showed conversion of PtdCho to PtdGly. Assays were conducted as described above with 0.137 mmol glycerol added. At various times reactions were extracted and subjected to HPLC analysis with detection by ELSD. The results from one time course experiment are shown in Fig. 1. Here it is seen that conversion to PtdGly is achieved with little by-product formation. Reactions conducted with higher levels of PLD or over longer time spans gave nearly quantitative formation of PtdGly. When the amount of glycerol was reduced, the amount of PtdGly was decreased, and PtdOH was increased. For example, when the glycerol was decreased by a factor of four (34.2 μmol) about one-half of the product was PtdOH. Therefore these results show that to achieve high formation of PtdGly with minimal by-product contamination, it is necessary to conduct reactions with a relatively high amount of glycerol. Procedures to recover the glycerol would be required for efficient PtdGly production.

PLD from *Streptomyces* sp.

Tests of another PLD from *Streptomyces* sp. were made using identical reaction conditions, but with varying amounts of glycerol. It was found that much lower levels of glycerol were required by this PLD. This is shown graphically in Fig. 2. Here HPLC traces with detection with ELSD are shown at four levels of added glycerol. The top trace shows the results of an assay containing only 11.4 μmol glycerol, and it can be seen that most of the product is PtdGly with only a small amount of PtdOH and an unidentified by-product at retention time (RT): 20.5 min. The amount of PtdOH tended to increase as the amount of glycerol was

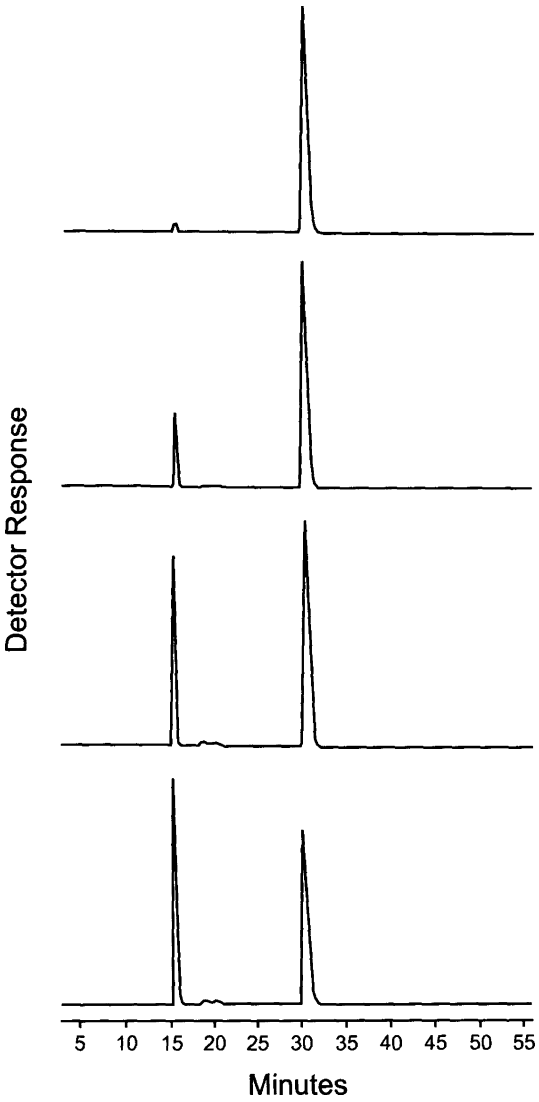


Fig. 1 Time course of cabbage PLD action on PtdCho at a relatively high glycerol concentration. An HPLC trace with detection by ELSD is shown for each assay conducted for different lengths of time. Each assay was conducted as described in ''Materials and Methods'' with 3.0 units PLD in a mixture of aqueous buffer and CH_2Cl_2 with 0.137 mmol glycerol and 2.16 μmol PtdCho. HPLC traces are shown from top to bottom for reaction times of 1, 2.5, 4.5 and 6.5 h, respectively. Traces show that PtdCho, retention time (RT): 30.5 min, is converted progressively and almost exclusively to phosphatidylglycerol (PtdGly; RT: 15.3 min)

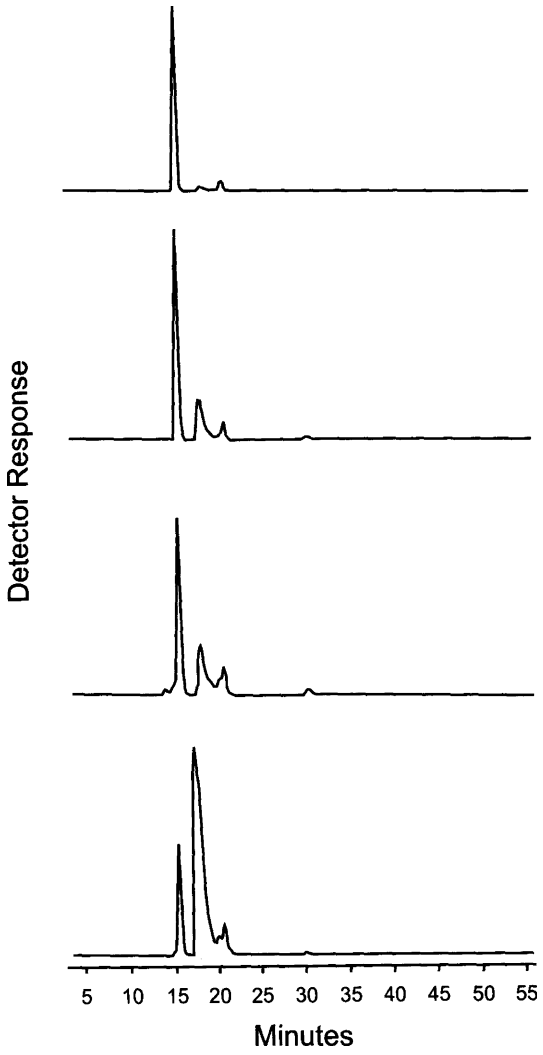


Fig. 2 Influence of the amount of glycerol on *Streptomyces* sp. PLD action on PtdCho. An HPLC trace with detection by ELSD is shown for each assay containing variable amounts of glycerol. Each assay was conducted as described in '' Materials and Methods'' for 24 h using 1.0 unit PLD in a mixture of aqueous buffer and CH_2Cl_2 with 2.16 μmol PtdCho. HPLC traces are shown from top to bottom for 11.4, 5.72, 3.81 and 1.91 μmol glycerol, respectively. Traces show complete or nearly complete reaction of PtdCho (RT: 30.5 min) and conversion to primarily PtdGly (RT: 15.3) at glycerol 11.4 μmol (top trace), and as the amount of glycerol is reduced progressively more PtdOH (RT: 18 min) is formed (bottom trace). A small amount of an unidentified by-product at RT: 20.5 min is also formed

decrease further (lower traces). Nevertheless the amount of glycerol needed for high PtdGly formation was about 12-fold lower with PLD from *Streptomyces* sp. than with PLD from cabbage, a significant improvement. However when PtdGly formation is high compared to PtdOH, the amount of glycerol required (11.4 μmol) is still approximately fivefold higher than the amount of PtdCho (2.16 μmol).

Since PLD from *Streptomyces* sp. was the best enzyme tested regarding relatively limited glycerol use, two more experiments designed to optimize reaction conditions were performed. In the first experiment reactions were performed with aqueous buffer at different pH values using CH_2Cl_2 as the organic phase. A high amount of glycerol (0.137 mmol) was used to suppress formation of PtdOH. The results of these experiments are shown in Fig. 3. Here it is seen that optimal conversion of PtdCho to PtdGly was achieved in the pH range of 5.2–6.2.

Experiments were performed at low levels of glycerol in order to determine the partitioning of PtdCho into PtdGly and PtdOH. When the level of glycerol was lowered, the

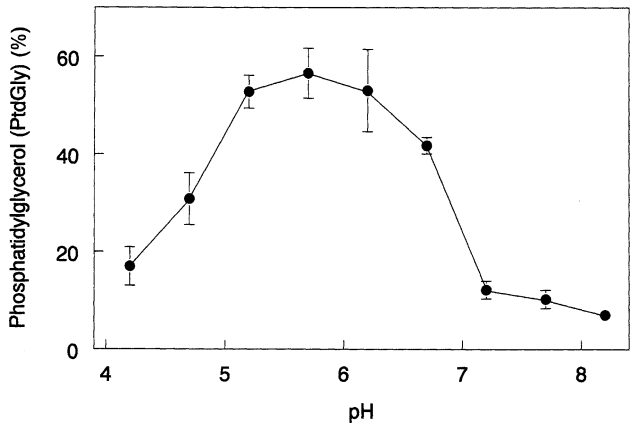


Fig. 3 Influence of the buffer pH on the synthesis of PtdGly from PtdCho using *Streptomyces* sp. PLD. The percent yield of PtdGly was determined by HPLC using ELSD. Calibration curves were prepared with standard PtdCho and PtdGly. These were used to convert the ELSD response to mass. The buffer contained 100 mM Hepes buffer (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), 100 mM Mes buffer (2-[*N*-morpholino]ethanesulfonic acid), 100 mM acetic acid, and 50 mM $CaCl_2A2H_2O$. Each assay was conducted as described in ''Materials and Methods'' for 24 h using 0.05 unit of PLD in a mixture of aqueous buffer and CH_2Cl_2 with 0.137 mmol glycerol and 2.16 μmol PtdCho. The data are the mean ± SE for three repetitions

unidentified product at RT 20.2 increased, particularly when solvents ethyl acetate and ethyl ether were used. An example is shown in Fig. 4 where an ELSD trace of reaction products is displayed. This reaction was conducted in ethyl ether with 1.91 μmol glycerol added.

The unknown material was analyzed more extensively to determine its chemical structure. Analysis of the material by HPLC-MS conclusively showed that the material was formed from double phosphatidylation of phosphodiglyceride on glycerol to give cardiolipin (diphosphatidylglycerol, Ptd_2Gly). As shown in Fig. 5, the

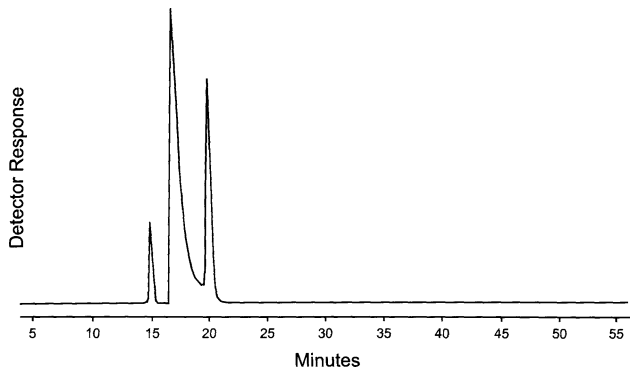


Fig. 4 An HPLC trace with detection by ELSD of the reaction products from PtdCho in ethyl ether. Each assay was conducted as described in ''Materials and Methods'' for 24 h using 0.75 unit of PLD in a mixture of aqueous buffer and ethyl ether with 1.91 μmol glycerol and 2.16 μmol PtdCho. The products and their HPLC retention times are cardiolipin (diphosphatidylglycerol, Ptd_2Gly), 20.2 min; PtdOH, 17.0 min; and PtdGly, 15.3 min

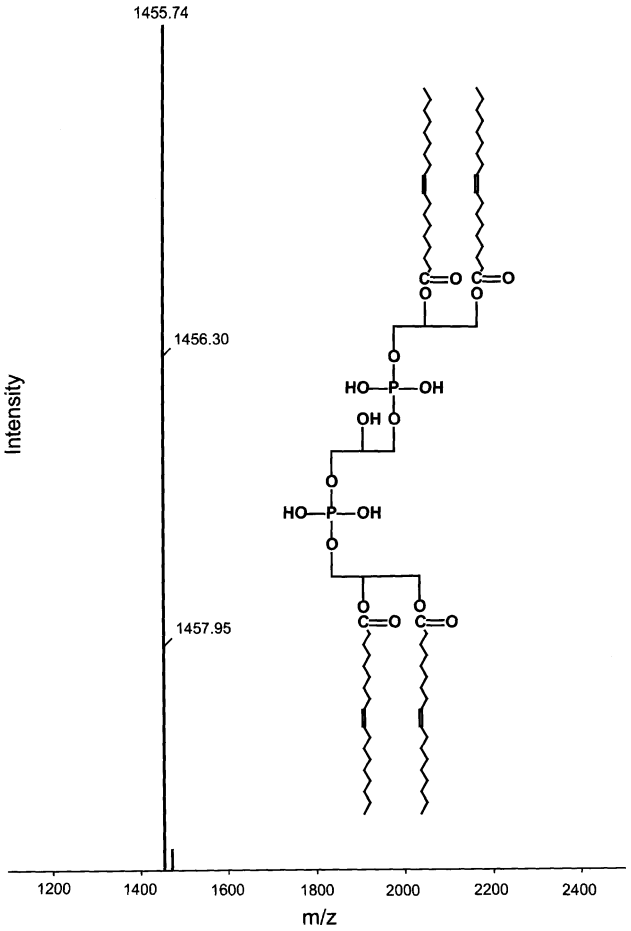


Fig. 5 HPLC/MS/ESI with negative ion detection of cardiolipin (diphosphatidylglycerol, Ptd_2Gly) eluting at approximately 20 min showing base peak at *m/z* 1,455 corresponding to M – 1. The chemical structure of Ptd_2Gly is shown in the figure

material gave a strong $[M–H]^{-1}$ fragment (*m/z* = 1,455) using ESI with negative ion detection. An identical fragmentation pattern was observed using negative ion APCI. In positive ion APCI a base peak from the addition of triethylamine was observed: $[M + NH(CH_2CH_3)_3]^+$ (*m/z* = 1,558).

The transphosphatidylation reaction was conducted in a variety of solvents with a low amount of glycerol (1.91 μmol) to determine which solvents promoted the highest PLD activity and to determine if a particular solvent was better able to direct PtdCho into PtdGly rather than PtdOH. The results of these experiments are displayed in Table 2. The solvents listed at the top of the table supported PLD reaction on PtdCho while solvents listed at the bottom did not. The solvents *t*-butylmethyl ether, ethyl acetate, and ethyl ether supported the greatest conversion of PtdCho to products during the 24 h reaction period. However, the highest ratio of PtdGly to PtdOH was given by the solvents that gave slower reaction, dichloromethane and isopropyl ether. The situation was complicated how-

Table 2 Influence of solvent on PLD action on PtdCho

Solvent	Remaining PtdCho[a] (%)	PtdGly/ PtdOH	PtdGly + Ptd_2Gly[b])/PtdOH
Dichloromethane	46.3 ± 7.6[c]	0.97 ± 0.14	1.56 ± 0.22
t-butylmethyl ether	8.8 ± 0.7	0.23 ± 0.03	0.46 ± 0.05
Isopropyl ether	75.0 ± 4.3	0.95 ± 0.05	1.92 ± 0.09
Ethyl acetate	10.9 ± 1.2	0.21 ± 0.02	0.63 ± 0.05
Ethyl ether	12.4 ± 3.0	0.45 ± 0.13	0.66 ± 0.08
Hexane	100		
Tetrahydrofuran	100		
Trimethyl pentane	100		
Dimethylsulfoxide	100		

[a] Each assay was conducted as described in ''Materials and Methods'' for 24 h using 0.75 unit of PLD in a mixture of aqueous buffer and solvent with 1.91 μmol glycerol and 2.16 μmol PtdCho

[b] Cardiolipin (diphosphatidylglycerol); see Fig. 5 for chemical structure

[c] Standard error of the mean for seven repetitions

ever by the production of the dimer Ptd_2Gly. Nevertheless the highest ratio of PtdGly and its dimer relative to PtdOH was still given by dichloromethane and isopropyl ether.

Thus, it was shown that products generated by PLD enzymes can be efficiently identified and quantified by HPLC/ELSD. Under the aqueous/organic reaction conditions used in this study the commercial preparation of PLD from *Streptomyces* sp. had the highest selectivity for glycerol, and nearly quantitative yields of PtdGly were obtained even when the mol ratio of water to glycerol was in excess of 1,000. However, because *Streptomyces* sp. has a high affinity for alcohol compared to water, lowering the glycerol concentration too much resulted in cardiolipin formation as well as PtdOH formation. The developed HPLC method makes product measurements efficiently compared to older assay methods that rely on TLC, and using MS as the detection method allows for rapid product identification in those cases where identification by retention time is insufficient. Using MS as the detector would allow PLD assays to be made using phospholipids with much fatty acyl heterogeneity and will help in the preparation of ''industrial grades'' of PtdGly.

Acknowledgments C. Frank Fox provided technical assistance.

References

1. Litcovitch M, Czarny M, Fiucci G, Tang X (2000) Phospholipase D: molecular and cell biology of a novel gene family. Biochem J 345:401–415
2. Jenkins GM, Frohman MA (2005) Phospholipase D: a lipid centric review. Cell Mol Life Sci 62:2305–2316
3. Sato R, Itabashi Y, Suzuki A, Hatanaka T, Kuksis A (2004) Effect of temperature on the stereoselectivity of phospholipase D toward glycerol in the transphosphatidylation of phosphatidylcholine to phosphatidylglycerol. Lipids 39:1019–1023
4. Barenholz Y, Amselem S (2005) Enzymatic transesterification or hydrolysis of phospholipids in aqueous media. U.S. Patent 6,924,130
5. Dasari MA, Kiatsimkul PP, Sutterlin WR, Suppes GJ (2005) Low-pressure hydrogenolysis of glycerol to propylene glycol. Appl Catal A: Gen 281:225–231
6. Serrano AG, Pérez-Gil J (2006) Protein-lipid interactions and surface activity in the pulmonary surfactant system. Chem Phys Lipids 141:105–118
7. Iwasaki Y, Mizumoto Y, Okada T, Yamamoto T, Tsutsumi K, Yamane T (2003) An aqueous suspension system for phospholipase D-mediated synthesis of PS without toxic organic solvent. J Am Oil Chem Soc 80:653–657
8. Morris AJ, Frohman MA, Engebrecht J (1997) Measurement of phospholipase D activity. Anal Biochem 252:1–9
9. Aurich I, Hirche F, Ulbrich-Hofmann R (1999) The determination of phospholipase D activity in emulsion systems. Anal Biochem 268:337–342
10. Filomena F, Gasperi V, Mazzei C, Maccarrone M (2005) Radiochromatographic assay of N-acyl-phosphatidylethanolamine-specific phospholipase D activity. Anal Biochem 339:113–120
11. Deschamps FS, Chaminade P, Ferrier D, Baillet A (2001) Assessment of the retention properties of poly(vinyl alcohol) stationary phase for lipid class profiling in liquid chromatography. J Chromatogr A 928:127–137
12. Christie WW, Urwin RA (1995) Separation of lipid classes from plant tissues by high performance liquid chromatography on chemically bonded stationary phases. J High Resolut Chromatogr 18:97–100
13. Beermann C, Green A, Möbius M, Schmitt JJ, Boehm G (2003) Lipid class separation by HPLC combined with GC FA analysis: comparison of seed lipid compositions from different *Brassica napus* L. varieties. J Am Oil Chem Soc 80:747-753
14. Fagan P, Wijesundera C (2004) Liquid chromatographic analysis of milk phospholipids with on-line pre-concentration. J Chromatogr A 1054:241–249
15. Rabinovich-Guilatt L, Dubernet C, Gaudin K, Lambert G, Couvreur P, Chaminade P (2005) Phospholipid hydrolysis in a pharmaceutical emulsion assessed by physicochemical parameters and a New Analytical Method. Eur J Pharm Biopharm 61:69-76
16. Sommer U, Herscovitz H, Welty FK, Costello CE (2006) LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures. J Lipid Res 47:804-814
17. Barroso B, Bischoff R (2005) LC-MS analysis of phospholipids and lysophospholipids in human bronchoalveolar lavage fluid. J Chromatogr B 814:21–28
18. Malavolta M, Bocci F, Boselli E, Frega NG (2004) Normal phase liquid chromatography-electrospray ionization tandem mass spectrometry analysis of phospholipid molecular species in blood mononuclear cells: application to cystic fibrosis. J Chromatogr B 810:173–186