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Multiple Activities of Oleate-Activated Phospholipase D Exist in Rat Brain Microsomes Solubilized by Decanoyl *N*-Methylglucamide

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Phospholipase D (PLD) has emerged as an enzyme involved in signal transduction, vesicle trafficking, and membrane remodeling.[1] The enzyme was discovered in plants 50 years ago, but distribution of PLD has been found to be extremely widespread in the most of biological sources.[1,2] The PLD activity in mammalian tissue was initially detected in 1973 by Saito and Kanfer[3] and activation of PLD by oleate was reported in 1982 by the same group.[4] Since then the PLD assay system containing oleate has been tested and the oleate-activated PLD was found in a variety of mammalian tissues.[5-8] Further evidence obtained in a variety of biochemical studies implicate the existence of multiple isoforms of PLDs.[9] Two isoforms of PLD identified in mammalian sources indicate that PLD1 localized in the perinuclear region is dependent on ADP-ribosylation factor (ARF) and phosphatidylinositol 4,5-bisphosphate (PIP_2), whereas PLD2 in plasma membrane is regulated by PIP_2 but not by ARF.[10] In addition to membrane-associated PLDs, a cytosolic PLD activity was identified in human neutrophils and bovine lung.[11] Therefore resolution of multiple isoforms and search for their physiological roles have been major focusing effort in signal-activated PLDs. Recent advances in the PLD study have brought new insights into the molecular and cellular roles of PLD. PLDs have been cloned from caster bean, rice, maize, Arabidopsis, yeast, human, and mouse.[12] Analysis of the cDNA sequence of PLD has led to the identification of probable catalytic and regulatory domains.[13]

In spite of many attempts to solubilize membrane-associated PLD, no animal PLDs have been fully purified and characterized.[14-19] In this work, the oleate-activated PLD of microsomal membrane from rat brain was solubilized using decanoyl N-methylglucamide (MEGA-10). The stability of solubilized PLD improved substantially from previous study[19] and the source was further fractionated into 4 fractions by chromatography on Sephacryl S-300. Here we report a partial characterization of multiple PLD activities in brain microsomes, which are activated by oleate.

Experimental

Radioactive 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine (specific activity 111 mCi/mmole) was purchased from Amersham (Aylesbury, England). Phosphatidic acid and phosphatidylethanol were prepared from phosphatidylcholine using cabbage PLD according to the procedure described previously.[20] Sodium oleate, decanoyl N-methylglucamide, and Sephacryl S-300 were obtained from Sigma (St. Louis, USA). Precoated silica gel 60 TLC plate was purchased from Merck (Darmstadt, Germany). Brain microsomal fraction was obtained from female Wistar rats (four weeks old). The brains were homogenized in ten volume of 10% sucrose solution and centrifuged at 10,000×g for 10 minutes at 4°C. The supernatant was recentrifuged at 100,000×g for 1 hour to obtain microsomal pellet. Initially the microsomal pellet was

washed to eliminate undesirable membrane proteins by sonicating the pellet with 50 mM HEPES buffer (pH 7.0) containing 0.2% MEGA-10 and 1.5 M sodium chloride (3 mL per rat). The sonicated microsomal fraction was centrifuged at 100,000×g for 1 hour and the washing supernatant was discarded. The resulting pellet was solubilized with 50 mM HEPES buffer (pH 7.0) containing 0.4% MEGA-10 and 1.5 M sodium chloride. The high concentration-detergent solubilized microsomal fraction was recentrifuged at 100,000×g for 1 hour and the second supernatant was used as a solubilized PLD enzyme source. The solubilized enzyme was applied on Sephacryl S-300 column (φ2.5×60 cm). The column was preequilibrated with 50 mM HEPES buffer (pH 7.0) containing 0.4% MEGA-10 and 1.5 M sodium chloride and eluted with the same buffer at 23 °C. The column work was carried out at 23 °C since 0.4% MEGA-10 buffer solution tended to gel at lower temperature.

PLD activity was determined essentially according to Taki and Kanfer[14] except using different detergent MEGA-10 and adding PA as an activator. The oleate-activated PLD was measured by the radioactivity of hydrolysis and transphosphatidylation products of 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine in the presence of ethanol. The standard reaction mixture contained 0.5 mM of PC with 0.1 μCi 1,2-di[1-^{14}C]palmitoyl phosphatidylcholine, 2 mM oleate, 1.5 mM PA, 50 mM of HEPES buffer (pH 7.0), 5 mM $CaCl_2$, 10 mM KF, 0.1% (w/v) MEGA-10, 1.5% ethanol, and indicated amounts of protein from each enzyme source in a total volume of 100 μL. The reaction was initiated by addition of enzyme solution and incubated for 2 hr at 30 °C. The products were separated using TLC as described previously.[21]

Results and Discussion

The solubilized PLD contained a rather high concentration of MEGA-10 and sodium chloride. Therefore any conventional purification methods such as ion exchange chromatography, affinity chromatography and electrophoresis were not applicable except gel filtration. Figure 1A shows an elution profile of PLD activity measured immediately after the gel filtration using Sephacryl S-300. Four peaks of the PLD activity eluted on Sephacryl S-300 column. PLD I, II, III, IV were designated according to the elution order from the column. When the eluted fractions were stored at 4 °C, peak I and II fractions geled and phase separation occurred within a few weeks. PLD activities were detected only in upper phases and increased markedly compared with those of freshly eluted sources (Figure 1B). Thus we used the upper phase to characterize PLD I and II activities. However peak III and IV did not gel upon cooling.

The PLD I activity had optimum pH of 6.5. The highest activity of PLD I was observed at 2 mM oleate and at 0.3 mM PA. The total activity of PLD II recovered was more than those of the other PLDs. The optimum pH of PLD II was 7.0 and the maximum activity was obtained at 2 mM oleate and at 0.6 mM PA. The PLD activities in both peak I and II were not affected by Ca^{2+} ions. The total activity of PLD III was small compared with those of PLD I and II. However the enzyme was stable at 4 °C for more than 8 weeks. The optimum pH of PLD III was 7.0 and maximum activity was observed at 2 mM oleate and at 1.0 mM PA.

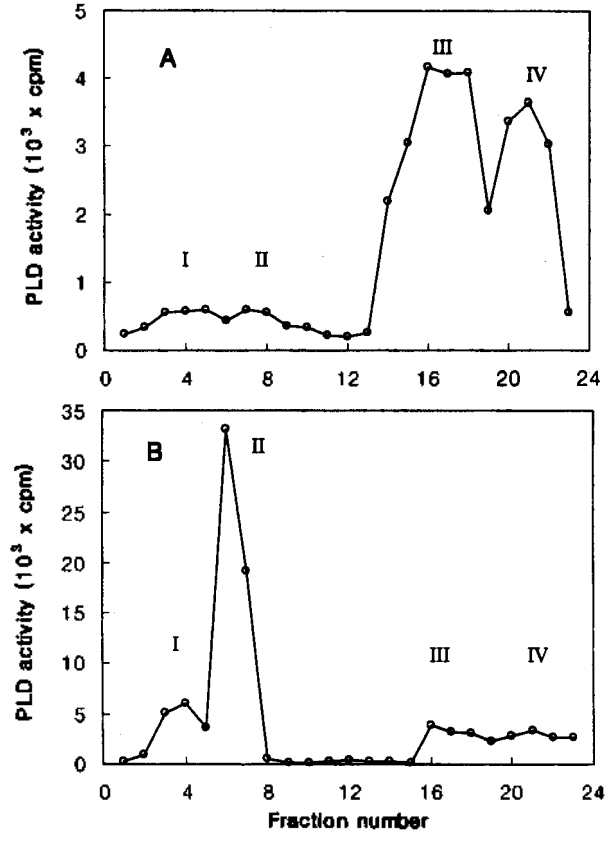

Figure 1. Profile of Sephacryl S-300 chromatography of the solubilized PLD by MEGA-10. Oleate-activated PLD activities of 25 μL aliquot of each fraction were assayed with 2 mM oleate. A, immediately after chromatography; B, after one week storage at 4 °C.

The activity of PLD III was stimulated by Ca^{2+} and Mg^{2+} at the concentration of millimolar range. The PLD IV enzyme also had similar stability on storage to compare with that of PLD III. The optimum pH of PLD IV was 6.5 and the PLD IV activity was optimal at 2 mM oleate and 0.6 mM PA. The activity of PLD IV was stimulated by Ca^{2+} but not by Mg^{2+}.

It has been shown that the activational effect of oleate on PLD depended on substrate concentration of PC as well as amounts of membrane protein used in assay tubes.[4,5] The optimal concentration of oleate shifted to a higher concentration along the increasing amounts of PC or membrane proteins. Therefore we investigated whether the oleate effect was dependent on the concentration of detergent MEGA-10. It turned out that the oleate dependency was drastically affected by the detergent. Figure 2 shows the effect of MEGA-10 on the oleate-activated PLD in the presence of varying amount of oleate. At higher concentration of MEGA-10 higher oleate concentration was required for an optimal activity of PLD. This correlation was confirmed further in the fractions of Sephacryl S-300 (Figure 3). In the concentration of 2 mM oleate the profile of PLD activity was similar to that of Figure 1A. However when oleate concentration increased to 8 mM, the activity of PLD I and II increased markedly but those of PLD III and IV decreased slightly. Thus the profile changed similar to that of Figure 1B which was obtained aft-

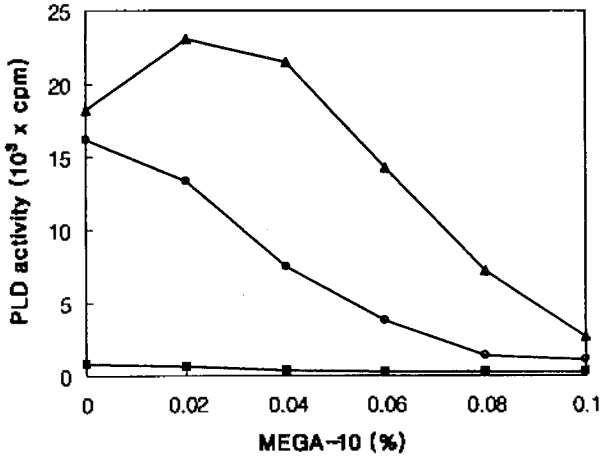

Figure 2. Effect of MEGA-10 concentration on microsomal PLD activity in the presence of varying amount of oleate. The assay mixtures maintained constant ionic strength with 375 mM NaCl. -■-, 2 mM; -○-, 5 mM; -▲-, 8 mM.

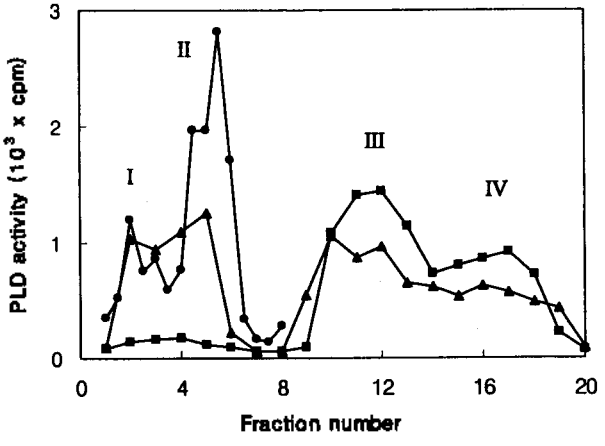

Figure 3. Dependence of oleate-activated PLD profile of Sephacryl S-300 on varying amount of oleate or period of storage time. Activity of each fraction was assayed immediately after column work with 2 mM oleate (-■-); 8 mM oleate (-▲-); or assayed after one week storage at 4 °C with 2 mM oleate (-●-). In this set of PLD assay, the radioactive phosphatidylcholine used was 0.05 μCi.

er phase separation. This observation can be interpreted as a mutual correlation between MEGA-10 and the oleate-activation. PLD I and PLD II fractions contained higher concentration of MEGA-10, therefore they required higher concentration of oleate. The inhibitory effect observed in PLD III and PLD IV was probably due to the excess amount of oleate in the assay tube. At lower concentration of MEGA-10 after phase separation, 2 mM oleate was enough to exert an optimal effect on oleate-PLD. For standard condition, therefore, amount of substrate, detergent, protein, and oleate should be cross-examined each other to obtain an optimal activity.

In current study we were able to solubilize oleate-activated PLD from rat brain microsomes by 0.4% MEGA-10 at high ionic strength. The solubilization condition suggests that the PLD in microsomal fraction appeared to be an integral membrane protein. The solubilized PLD source can be fractionated at least into 4 fractions by gel filtration. Each fraction exhibited subtle differences in their properties such as pH optimum, PA dependence, or cation effects. These multiple activities observed are different from the single peak of oleate-activated PLD activity reported in heparin-Sepharose column.[17] Although the multiple activities are apparent, further evidence is required to clarify whether each activity corresponds to new or known isoforms of PLD. Nonetheless the activational effect of PA observed on PLD in all four fractions reinforces the previous PA effect on oleate-activated PLD.[21]

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A Convenient Preparation of (1S)-(+)-10-Mercaptoisoborneol from (1S)-(+)-10-Camphorsulfonyl Chloride with High Diastereoselectivity

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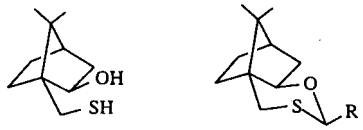
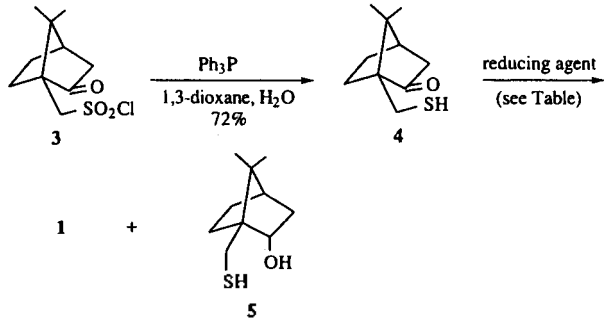
Chiral auxiliaries and catalysts derived from (+)-10-mercaptoisoborneol (exo-2-hydroxy-10-mercaptonorbornane, **1**) are used in various asymmetric syntheses.[1-5] Recently, 1,3-oxathianes **2** prepared from **1** have been used as highly effective chiral catalysts for the asymmetric epoxidation of aldehydes.[6]



1 **2**

According to the literature,[1] thiol **1** was obtained by reduction of (+)-10-camphorsulfonyl chloride (**3**) with excess lithium aluminum hydride (chloride : hydride=1 : 4 molar ratio) in ether, followed by chromatographic separation of a 4 : 1 mixture of **1** and 10-mercaptoborneol (**5**). Because a large excess of pyrophoric reducing agent is employed, handling of this reagent and acidic workup need a careful manipulation. Also, the stereoselectivity in the ketone reduction is not so high. In this paper, we wish to describe a highly stereoselective method of obtaining **1**, which does not use $LiAlH_4$, rendering this method applicable to a large scale preparation, as shown in Scheme 1.



Scheme 1.

First, sulfonyl chloride **3** was converted to (+)-camphor-10-thiol (**4**) with triphenylphospine in dioxane-water co-solvent.[7] Thiol **4** could be easily separated from the reaction mixture by extraction with 5 M NaOH solution followed by acidification and extraction of the resulting thiol with hexanes. Sublimation of the crude product at reduced pressure gave the crystalline ketone **4** in 72% yield. Next, we studied the diastereoselective reduction of ketone **4** (Table 1). Reduction with $LiAlH_4$ or *i*-Bu_2AlH gave *exo*-isomer **1** in high selectivity (>95% de). Similarly, $NaBH_4$ in EtOH showed a high *exo*-selectivity.[8] After reduction, removal of the minor *endo*-isomer **5** (polar) by silica gel column chromatography (hexanes : ethyl acetate=20 : 1) gave the diastereomerically pure *exo*-thiol **1**. This thiol is slowly oxidized to the disulfide when exposed to the air. Therefore, it should be kept under an inert atmosphere in a cold place.

Experimental Part

The ^1H and ^{13}C NMR spectra were recorded with a Vari-

Table 1. Reduction of ketone **4** with reducing agents[a]

Reducing Agent	Solvent	Temp. (°C)	Time	1/5[b]
$LiAlH_4$	THF	−78	3 h	≥95/5
$LiAlH_4$	THF	0	3 h	≥95/5
$LiAlH_4$	ether	−78	3 h	92/8
LiAlH4	ether	0	3 h	≥95/5
$NaBH_4$	ethanol	rt	2 day	95/5
i-Bu_2AlH	toluene	−78	3 h	95/5

[a] Chemical yield was >95% in all cases. [b] The ratio was determined by the integration of two sets of peaks around 3.97 ppm (*exo*) and 4.35 ppm (*endo*) in ^1H NMR spectrum. It should be noted that the ratio determined by the ^1H NMR method can have an error of ±5%.