SUBSTRATE REQUIREMENTS OF PHOSPHOLIPASE D FROM *Streptomyces netropsis* IN THE TRANSPHOSPHATIDYLATION SYNTHESIS OF PHOSPOLIPIDS

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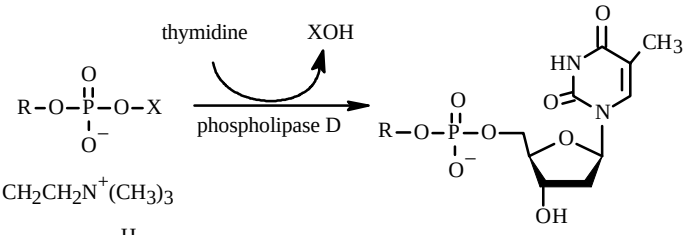
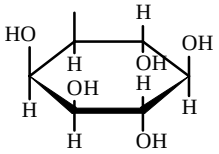
The effect of the structure of the phospholipid substrate on the yield of phosphatidyl-5′-thymidine by transphosphatidylation catalyzed by phospholipase D from Streptomyces netropsis *was studied. The reaction and product yield depended on the structures of the polar and nonpolar parts, the hydrophobic-hydrophilic balance, and the degree of unsaturation of the fatty-acids in the phospholipid substrate.*

Key words: phospholipase D, phospholipids, transphosphatidylation, thymidine, *Streptomyces netropsis*.

Transphosphatidylation catalyzed by phospholipase D (PLD) from microorganisms of the *Streptomyces* family is used widely in one-step syntheses of unique phospholipids. This reaction produces phosphatidyl derivatives of vitamins E [1] and C [2], modified nucleosides [3-6], peptides [6], hydroxyethyltrimethylarsonium [7], and various cyclic and heterocyclic alcohols [6-8]. We found previously [9] that the strain *S. netropsis* BIM B-235 produces Ca^{2+}-independent PLD with high catalytic activity in the reaction of phosphatidyl transfer from phosphatidylcholine to various natural and modified nucleosides. This opens up possibilities for using this enzyme to synthesize various analogs of phospholipids.

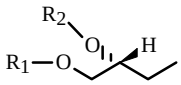


1 - 14: X = $CH_2CH_2N^+(CH_3)_3$

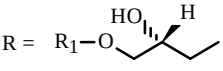
15: X = 

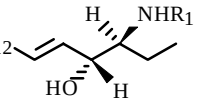
16: X = $CH_2CH(COOH)^+NH_3$

17: X = $CH_2CH_2N^+H_3$

1 - 11, 15 - 17: R = 

1, 15, 16: R_1, R_2 - natural fatty acids;
2: $R_1 = R_2 = C_3H_7CO$; **3:** $R_1 = R_2 = C_9H_{19}CO$;
4: $R_1 = R_2 = C_{10}H_{21}CO$; **5:** $R_1 = R_2 = C_{11}H_{23}CO$;
6: $R_1 = R_2 = C_{13}H_{27}CO$; **7:** $R_1 = R_2 = C_{15}H_{31}CO$;
8: $R_1 = R_2 = C_{16}H_{33}CO$; **9:** $R_1 = R_2 = C_{17}H_{35}CO$;
10: $R_1 = R_2 = C_{19}H_{39}CO$; **11:** $R_1 = R_2 = C_{17}H_{33}CO$;
17: $R_1 = R_2 = C_{16}H_{33}CO$

12: R = 
$R_1 = C_{15}H_{31}CO$ (80%)
$C_{17}H_{35}CO$ (20%)

13, 14: R = 

13: R_1 = natural fatty acids; **14:** $R_1 = C_{16}H_{33}$

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TABLE 1. Phospholipid Substrate Properties in Transphosphatidylation Catalyzed by PLD from *S. netropsis*

No.	Phosphatidyl donor	PLD activity		Maximum reaction yield, %	Reaction time, h
		nmol/min·mL of preparation	%, rel.		
1	Natural phosphatidylcholine	3260	100	71.0	1
2	Dibutyrylphosphatidylcholine	0	0	0	2
3	Dicaprinoylphosphatidylcholine	1090	33.4	50.0	1.5
4	Diundecanoylphosphatidylcholine	1060	32.5	49.2	1.5
5	Dilauroylphosphatidylcholine	1110	34.0	50.0	1.5
6	Dimyristoylphosphatidylcholine	1090	33.4	53.6	2
7	Dipalmitoylphosphatidylcholine	950	29.1	54.3	2
8	Diheptadecanoylphosphatidylcholine	940	28.8	47.4	2
9	Distearoylphosphatidylcholine	900	27.6	47.6	1
10	Diarachidoylphosphatidylcholine	730	22.4	57.5	2
11	Dioleoylphosphatidylcholine	1020	31.3	58.7	1
15	Soybean phosphatidylinositol	0	0	0	2
16	Bovine brian phosphatidylserine	0	0	0	2
17	Dipalmitoylphosphatidylethanolamine	490	15.0	29.4	1

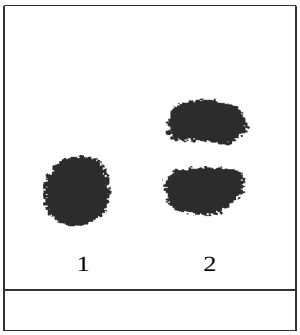

Fig. 1. Thin-layer chromatogram of a reaction-mixture extract before (1) and 2 h after (2) the start of dimyristoylphosphatidyl-5′-thymidine synthesis using PLD in system 2.

The phosphatidyl donors for preparation of phospholipids using PLD are, as a rule, natural phosphatidylcholine or its synthetic analogs. It was found earlier [10-12] that the product yield from reactions using plant and microbial PLD depends substantially on the structure of the polar groups and the hydrophobic part of the phospholipid substrate.

Herein the effect of phospholipid structure on enzyme activity and reaction yield for transfer of phosphatidyl to thymidine is studied to establish the structural requirements of PLD from *S. netropsis* on phospholipid substrates.

In the first step, the compositions and structures of products formed during fermentation using phospholipid **6** as a substrate were analyzed. Figure 1 shows a chromatogram of the extract from the reaction mixture, from which it can be seen that a new product accumulated during the fermentation process. It was confirmed to be a phospholipid by the color of the spot after treating the chromatogram with a reagent specific for phospholipids [13]. The absorption spectrum of the product isolated using flash chromatography exhibited an absorption band at 267 nm, typical of thymidine. The ratio of the P content and the chromophore in the resulting phospholipid was 1:1. The PMR spectrum contained signals characteristic of phosphatidyl and thymidine protons (see Experimental).

The analytical data lead to the conclusion that the reaction product is dimyristoylphosphatidyl-5′-thymidine. The selectivity of the reaction for the primary hydroxyl was demonstrated by phosphatidylation of glycerine by PLD from *S.* species [8]. It is important to note that the hydrolysis product phosphatidic acid was not found among the reaction products. It usually accompanies the transphosphatidylation product. Such a high specificity for thymidine transphosphatidylation suggests that it is ideal for studying the structural effect of the phospholipid substrate on enzyme activity and maximum product yield.

TABLE 2. Conditions and Efficiency Parameters of Transphosphatidylation Catalyzed by PLD from *S. netropsis* using Second Group Substrates as Phosphatidyl Donors

Phosphatidyl donor	Reaction conditions	Yield, %
12, 13, 14	10 mM donor, 10 mM acceptor (1:1 ratio); 270 units/mL PLD	0
12	10 mM donor, 50 mM acceptor (1:5 ratio); 2700 units/mL PLD	30
13	5 mM donor, 25 mM acceptor (1:5 ratio); 2700 units/mL PLD	20
14	30 mM donor, 10 mM acceptor (3:1 ratio); 2700 units/mL PLD	25-30

Therefore, we selected phospholipids that could arbitrarily be divided into three groups (Fig. 1). The first group included phosphatidylcholines (PC) with different chain lengths and degree of unsaturation of the fatty-acids (**1**-**11**); the second, phospholipids with a phosphocholine group with different structures for the hydrophobic parts (**12**-**14**); the third, diacylphosphoglycerides with different polar groups (**1**, **15**-**17**). The following tendency could be seen by analyzing the results from transphosphatidylation using **1**-**11** as the phosphatidyl donor. Decreasing the length of a saturated fatty-acid from 20 C atoms to 12 in the phospholipid increases the efficiency of phosphatidyl transfer to the nucleoside (Table 1).

The same trend was found for transphosphatidylation catalyzed by PLD isolated from cabbage [10, 14]. The chain length of the fatty acids could be varied only over certain limits. For example, the enzyme was inactive toward phospholipid **2**. This was most probably due to a decrease of the concentration of the hydrophilic substrate at the water—organic phase boundary, where the biocatalytic properties of the PLD are evident. A study of the substrate properties of PLD from *Streptomyces* PMF for hydrolysis also showed a sharp decrease for the degree of hydrolysis of short-chain phospholipids compared with the long-chain analogs [12].

Apparently the presence of unsaturated bonds has a significant effect on the phospholipid substrate properties. Introduction of a conjugate bond in **11** produces a noticeable increase in the enzyme activity and the reaction yield. The presence of unsaturated fatty-acids in natural phosphatidylcholine **1** ($C_{18:1}$, 32%; $C_{18:2}$, 19%; $C_{20:4}$, 2.6%) can be explained by its high activity as a phosphatidyl donor.

Significant quantities of products were not observed if substrates of the second group (**12**-**14**) were reacted under the conditions for the first group of substrates. However, all three products could be produced for selected conditions (increased ratio of donor:acceptor concentrations and amount of enzyme in the reaction mixture) (Table 2).

Regarding the effect of the polar head of the phospholipid on its substrate properties, the best substrate for the enzyme from *S. netropsis*, like for PLD from cabbage [1], was phosphatidylcholine (Table 1). The enzyme activity in the reaction with **11** was almost half that for **7** of the same fatty-acid composition. Phosphatidylinosite **15** and phosphatidylserine **16** with natural fatty-acids did not exhibit donor properties under these reaction conditions.

Thus, the activity of PLD from *S. netropsis* and the transphosphatidylation product yield depend on the chemical nature of the phosphatidyl donor. The length, degree of unsaturation, and structure of the fatty acid in addition to the structure of the polar head are also important. Natural phosphatidylcholine and its synthetic diacyl analogs are the best substrates under transphosphatidylation reaction conditions. Decreasing the length of the fatty-acid chains within certain limits and the presence of unsaturated bonds have a positive effect on the efficiency of phosphatidyl transfer to the acceptor molecule.

EXPERIMENTAL

Phospholipids **2**, **10**, **15**, and **17** were purchased (Sigma, USA); **3**-**9** and **11**, synthesized by the literature method [15]; hexadecylphosphocholine, as before [16]. Phosphatidylcholine from chicken-egg yolks (**1**), sphingomyelin, and phosphatidylserine from bovine brain were isolated by the previously described methods [17]. Lysophosphatidylcholine was prepared from **1** by hydrolysis using phospholipase A_2 [17].

TLC was performed on Silufol-UV_{254} plates (Serva, Germany) using the solvent systems $CHCl_3$:CH_3OH:NH_4OH (14 M) (14:4:0.15, system 1) and $CHCl_3$:CH_3OH:NH_4OH (7 M) (13:5:1, system 2). Compounds were detected on chromatograms using fluorescence in UV light, a reagent specific for phospholipids [13], and H_2SO_4 (10%) in CH_3OH with subsequent heating to 200°C. The P content in the samples was determined by the Vaskovsky method [13].

Absorption spectra were recorded on a UV-1202 spectrophotometer (Shimadzu, Japan). PMR spectra were recorded on an Avance 500 instrument (Bruker, Germany). Chemical shifts are given relative to an internal standard of TMS.

Preparation of Phospholipase D. *S. netropsis* BIM B-235 was cultivated in a biological rocker at 28°C for 24 h in 250-mL Ehrlenmeyer flasks containing nutrient medium (100 mL) of the following composition (%): glucose, 1.0; yeast extract, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1 (pH 7.2). The innoculating material was an 18-hour culture (10% by vol.). The filtrate of the culture liquid after biomass separation was used as the PLD source.

Determination of Activity and Maximum Transphosphatidylation Reaction Yield. A mixture containing phospholipid (10 mM), thymidine (20 mM), Na-acetate buffer (0.33 mL, 50 mM, pH 6.0), and $CHCl_3$ (0.67 mL) was treated with enzyme preparation (0.06 mL for activity determination or 0.18 mL for maximum reaction yield determination) and incubated at 37°C. The course of the reaction was followed using TLC (system 1). Compounds were detected in UV light and eluted: thymidine, by distilled water; phospholipid derivatives, by ethanol. The concentrations of the compounds were calculated taking into account the fact that modifying the carbohydrate part of the thymidine had practically no effect on the magnitude of the molar extinction coefficient of the nucleoside chromophore ($\varepsilon = 9650\ M^{-1} \cdot cm^{-1}$). The PLD activity was determined from the time by which the product yield was less than 15-20% and was calculated as the product amount in nmoles formed by reaction for 1 min in enzyme preparation (1 mL).

Dimyristoylphosphatidyl-5′-thymidine. A mixture of thymidine (24.2 mg, 100 μmol) in Na-acetate buffer (3.3 mL, 50 mM, pH 6.0) was treated with **6** (34 mg, 50 μmol) in $CHCl_3$ (6.7 mL) and enzyme preparation (2 mL), stirred at 37°C for 2 h, and treated with NaCl solution (1.7 mL, 1 M) and CH_3OH (3.3 mL). The layers were separated by centrifugation (3000 g, 5 min). The lower $CHCl_3$:CH_3OH layer was collected and evaporated in vacuo. The solid was dissolved in $CHCl_3$ (2 mL). The solution was placed on a column of densely packed silica gel (5-40 μm particle size) and eluted with $CHCl_3$ (20 mL) and $CHCl_3$:CH_3OH mixtures with the volume content of CH_3OH increasing by 10% in each subsequent mixture. Fractions containing product according to TLC were collected and evaporated. Yield of dimyristoylphosphatidyl-5′-thymidine, 19 mg (45%), TLC (system 2): R_f 0.40. PMR (500 MHz, $CDCl_3/CD_3OD$ = 2:1, ppm): 0.88 (6H, t, $2\underline{CH_3}CH_2$), 1.26 [40H, s, $2CH_3(\underline{CH_2})_{10}$], 1.59 (4H, m, $2\underline{CH_2}CH_2CO$), 1.93 (3H, s, CH_3-5), 2.25 (2H, m, 2′-CH_3), 2.32 (4H, m, $2\underline{CH_2}CO$), 3.90-4.20 (8H, m, CH_2OCO, CH_2OP, H-3′,4′,5′), 5.23 (1H, m, CHOCO), 6.28 (1H, t, H-1′), 7.62 (1H, s, H-6). Found [13], %: P 3.65 (Na^+-form of dimyristoylphosphatidyl-5′-thymidine). Calc., %: P 3.70.

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