Enzymatic production of phospholipid derivatives: System design can do more for improving specificity

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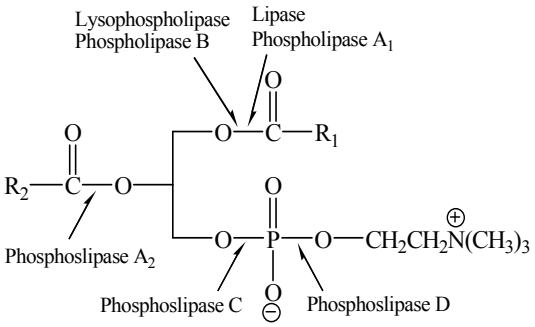
Overview

- Possibilities for enzymatic modification of phospholipids;
- Technical challenges for process realization;
- Strategies and factors — a general consideration to engender reaction and improve specificity;
- Art of optimization;
- Relevant activities in our group;
- PLD-catalyzed preparation of PC-VB6;
- Concluding remark.

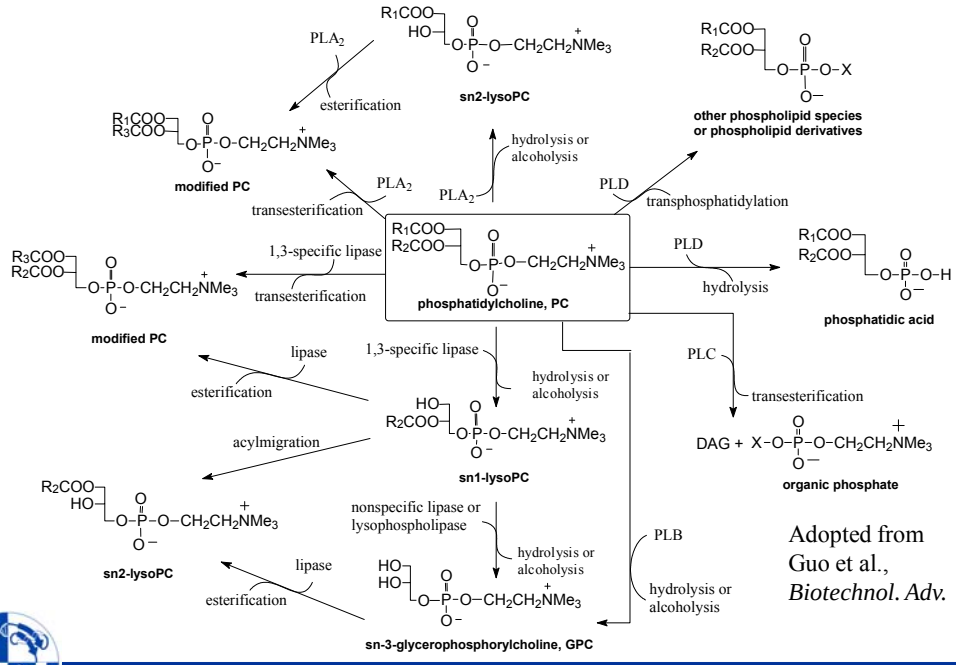
Possibility of enzymatic modification

- Selective cleavage of ester bond in phospholipids by phospholipase constitute the molecular basis for enzymatic modification;
- Knoweldge and understanding of the proper working conditions are leading the successfulness to proceed an enzymatic reaction;
- A judicious system design may maximize yields and selectivity.



Adopted from Guo et al., *Biotechnol. Adv.*

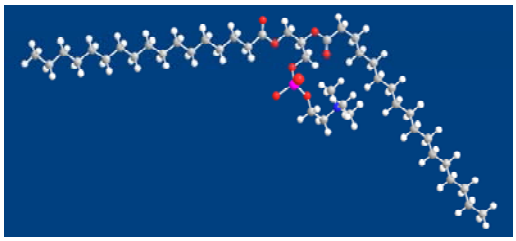
Transformation of glycerophospholipids (PC)



Adopted from Guo et al., *Biotechnol. Adv.*

Technical challenges for process realization

- PLs: solid or high viscous liquid (commercial lecithin with 400 times higher viscosity than vegetable oil).
- Lower solubility in many inert nonprotonic solvents (hexane etc.)
- Interfacial active and critical micelle concentration (CMC)
- Enzyme availability and stability



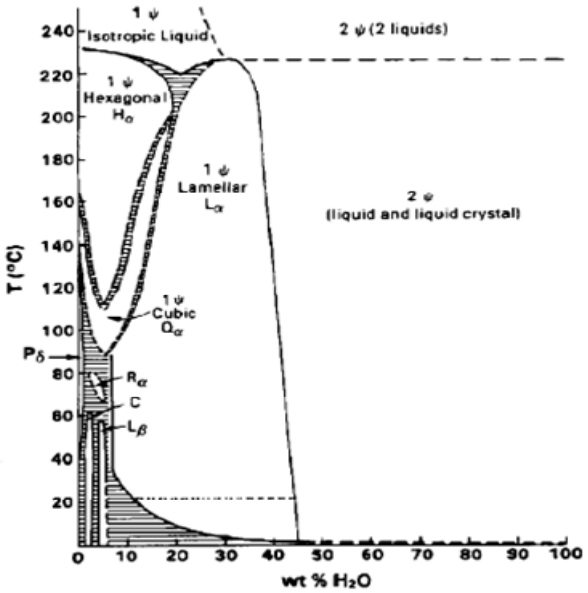
Zwitter ion structure

Technical challenges for process realization

Influence of temperature and water content on phases of egg lecithin

(modified after Small [1986])

C = Crystalline
H = Hexagonal liquid crystalline
La = Lamellar liquid crystalline
Lb = Lamellar gel
Q = Cubic disordered
R = Rhombohedral disorde

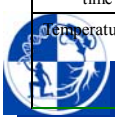


Strategies and factors — a general consideration to engender reaction and improve specificity

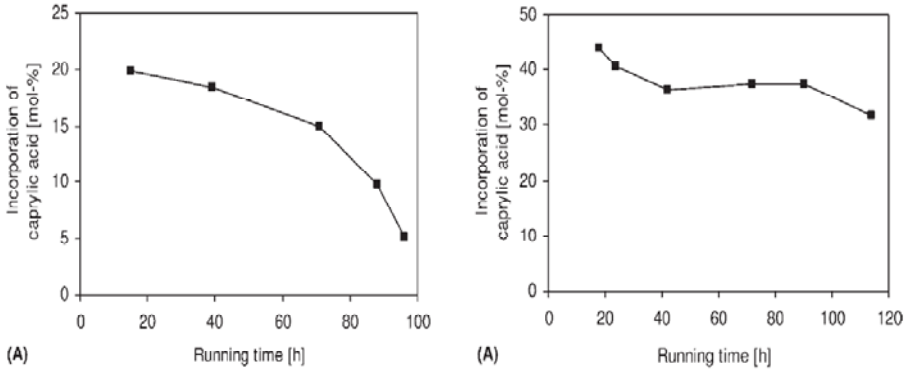
- Lysophospholipids
  - hydrolysis → lower cost reaction, complex post-processing
  - alcoholysis → fast reaction, high productivity and simple product separation
  - esterification of GPC → for high purity lyso-PC preparation
- Modified or Structured phospholipids
  - positional recognition → Enzyme selection
  - reaction routes → hydrolysis-reesterification or interesterification
- Phosphatidyl derivatives (headgroup exchange by PLD) → in-situ removal of choline and minimize hydrolysis

Factors **affect** reaction equilibrium and efficiency (synthesis of structured PLs

Factors	Remarks
Enzyme	• Phospholipase A1 and A2 or lipases can be used. • Increasing enzyme dosage results in higher incorporation. • Increase of enzyme may result in increased hydrolysis. • Enzyme can have different reactivity on PLs with different head groups.
Water	• A certain dynamic water environment should be maintained in order to have high enzyme activity. • Reaction time to reach equilibrium increases with decreasing water activity. The increase in reaction time is caused by a decreased enzyme activity. • Yield increases when water activity is low. • The possibility of using low water activity depends on individual enzymes. For most enzymes the catalytic activity increases with increasing water activity. However many lipases are active at low water activity and this makes it possible to obtain high yields. • Water activity influences the molecular organization of phospholipid substrate. The packing density of PL molecules increases with decreasing water activity.
Acyl donor	• By using a large excess of free fatty acids, hydrolysis reaction is inhibited. • Usually it is not a problem to use a high concentration of acyl donors although a slight decrease in reaction rate has been observed for very high concentrations. • Generally free fatty acids are more efficient acyl donors than their esters. • Reactivity relates to chain length and degree of saturation.
Solvent	• Solvent is not necessarily needed; however, solvent medium reduces viscosity of the substrates and as a consequence the reaction rate is increased through mass transfer increase of substrates. • Reaction is solvent type dependent, the rate being inversely proportional to solvent polarity. • Polar solvents should be avoided since it competes with enzyme on available water, which is required for three-dimensional structure of the enzyme and may disrupt the enzyme activity. • The solubility of the substrate depends on solvent types.
Reaction time	• The longer the reaction time the higher the incorporation of acyl donors into phospholipids can be expected. • Long reaction time however may also result in increased acyl migration.
Temperature	• Optimal temperature changes with enzyme source and type. • Increased temperature may result in higher acyl migration. • Higher temperature lowers viscosity of reaction medium. • Activity, stability and type of selectivity of enzyme are temperature.



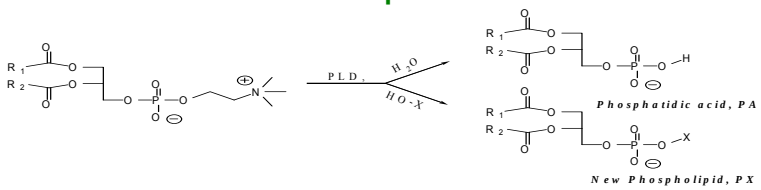
Operation stability of Lipozyme RM IM catalyzed incoporation of caprylic acid into PC in solvent-free / solvent system by PBR

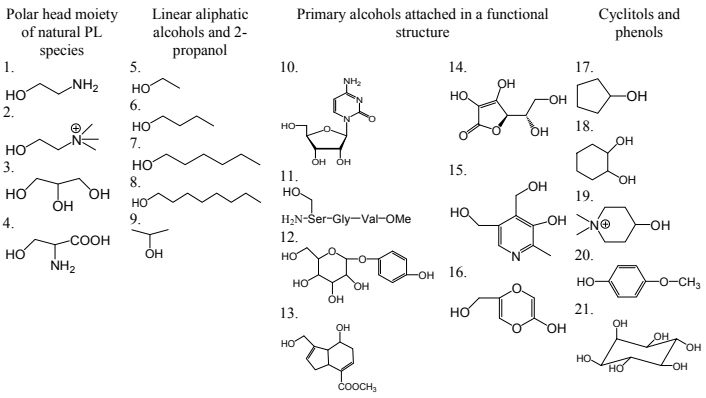


Solvent-free system Hexane system

Adopted from **Anders F. Vikbjerg et al.,** *Eur. J. Lipid Sci. Technol.* **108** (2006) 802–811

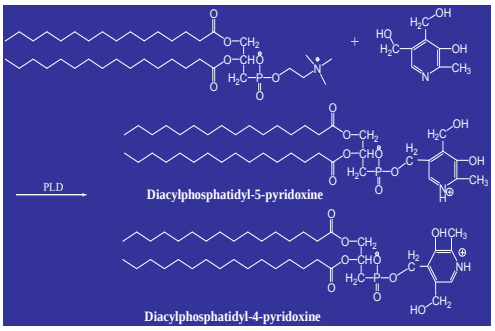
PLD-catalyzed transphosphatidylation of PC and representative structures of reactive alcohol acceptors



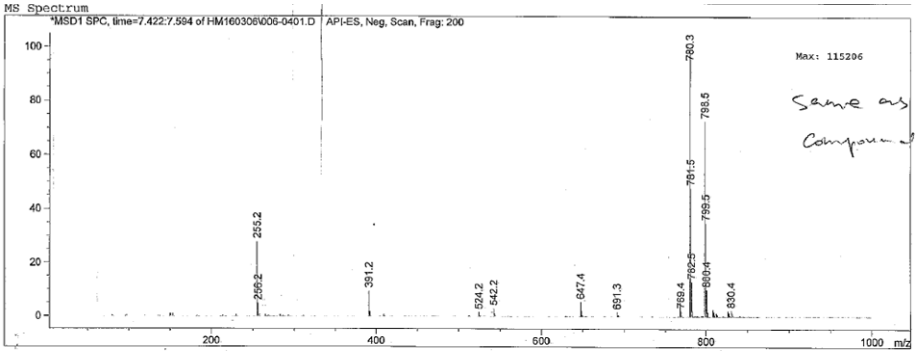


Lipophilic derivation of VB_6

- Introduction of acyl or phosphostidyl group to block the hydroxyl group of VB_6 is an alternative to increase its lipophilicity
- Enzymatic approaches may be better alternatives due to the sensitivity to oxygenation and heat
- Phospholipase D provide an efficient tool to catalyze transphosphatidylation of phospholipid with the compounds possessing hydroxyl group.
- The desired product has similar structure to PL (major component of biomembrane) and is believed to have high affinity to cell membrane.
- Transphosphatidyl derivatives of VB_6 may be slow released under the attack of phospholipase and have a different metabolic path.

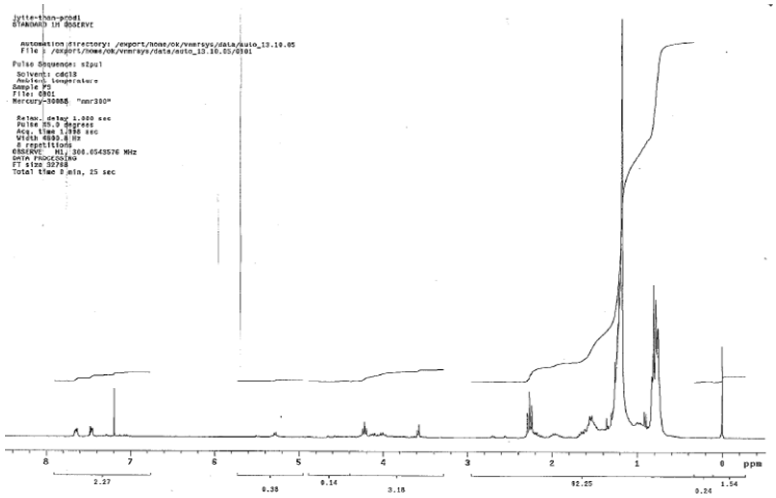


HPLC-MS of product



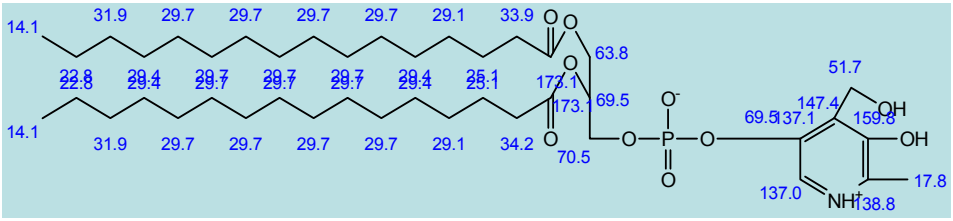
Molecular ionic peak $[M-1]^-$, 798.5; $[M-H2O-1]^-$, 780.5; [M-pyridine (VB6)-1]$^-$, 647.4; [M-palmityl-1]$^-$, 542.2; [M-pyridine (VB6) palmityl-1]$^-$, 391; and [palmityl]$^-$, 255.2.

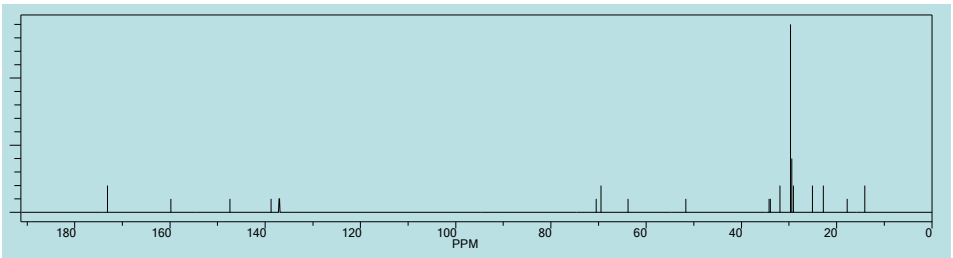
^{1}HNMR spectrum of product



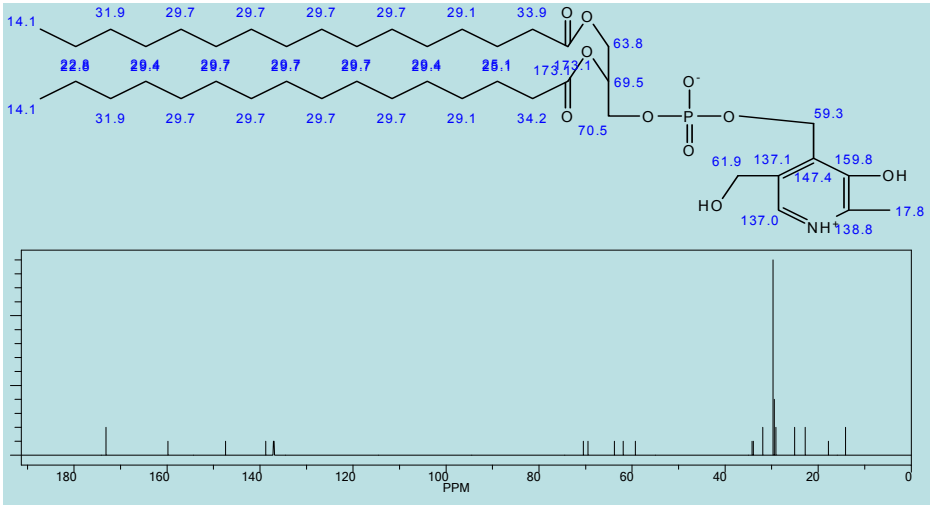
Characteristic peaks from pyridium: δ 5.5 (CH2), 5.3 (aromatic C-OH), δ 2.0 (CH2-OH*) and 4.65 (-CH2*-OH). Peaks from glycerol backbone δ 4.59 (methine), δ 3.95-4.22 (methylene, CH2). Peaks from acyl moiety: 2.2-2.3 (α-CH2), δ 1.5-1.6 (β-CH2), δ 1.22 (methylene) and 0.80-0.9 (end CH3).

Predicted ^{13}CNMR spectrum of product (5-substituted)

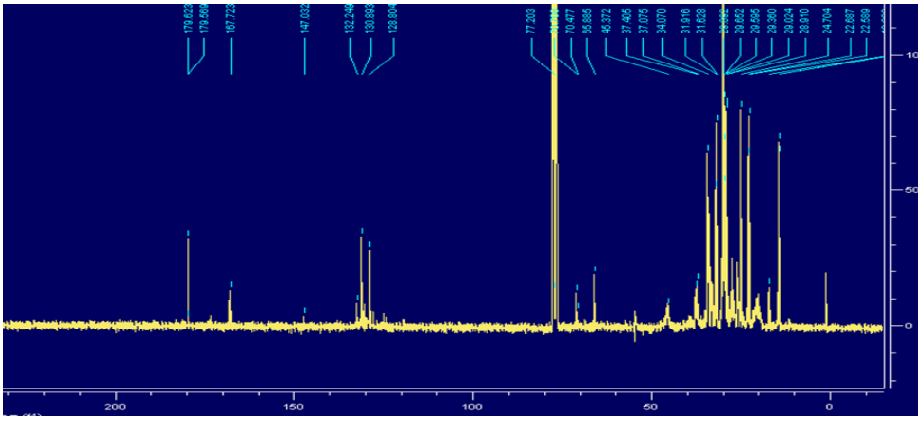




Predicted ^{13}CNMR spectrum of product (4-substituted)



^{13}CNMR spectrum of product

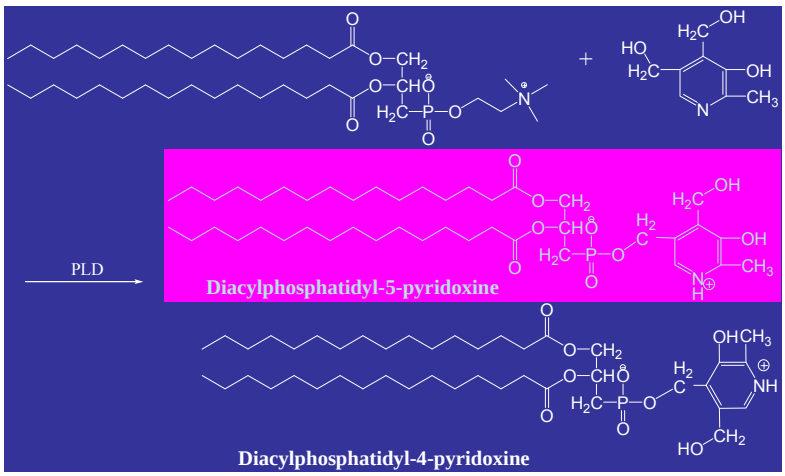


13C-NMR (CDCl3, 300 MHz): δ 179.6, 179.6, 167.7, 147.0, 132.2, 130.9, 128.8, 77.2, 70.7, 70.5, 65.9, 52,1, 45.4, 37.4, 37.1, 34.1, 31.9, 31.6, 29.7, 29.7, 29.6, 29.4, 29.0, 28.9, 24.7, 22.7, 22.6, 16.9, 14.1, 14.1.

^{13}CNMR spectrum report of product

	Dipalmitoyl glycerol moiety	Pyridoxine moiety
CH_3	14.1, 14.1	
CH_2	37.4, 37.1, 34.1, 31.9, 31.6, 29.7, 29.7, 29.6, 29.4, 29.0, 28.9, 24.7, 22.7, 22.6	
C=O	179.6, 179.6	
CH_2O	65.9	
CHO, CH_2OP	70.7, 70.5	
POC^*H_2-C		77.2
$POCH_2C$		130.9
C-C*H-N		128.8
N-C*-CH_3		132.2
CH_3		16.9
C*-OH		167.7
C=C*-C		147.0
CH_2OH		45.4

^{13}CNMR spectrum of product



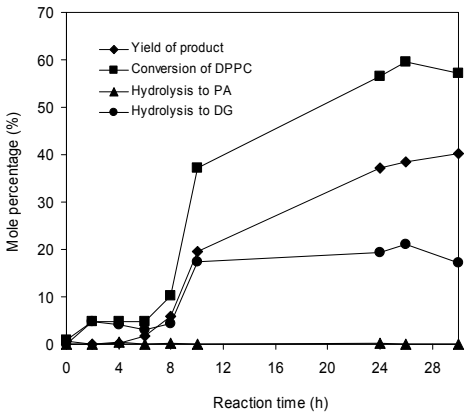
Parameter optimization

☆ Solvent system
Reaction time
pH profile
Enzyme dosage
Substrate concentrations

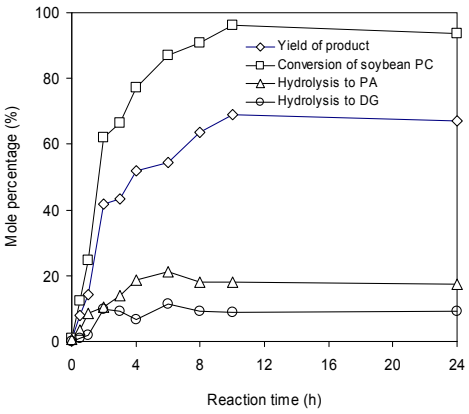
(Use commercial soybean PC as substrate)

Comparison of DPPC and Soybean PC

DPPC



Soybean PC

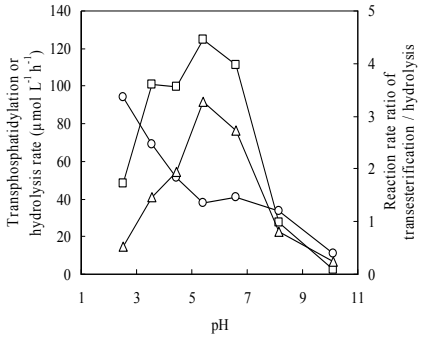


Reaction performance in normal solvents —Comparison in different solvents

	Yield of PC-VB6	PC conversion	Hydrolysis to PA	Hydrolysis to DG	Log P
Water	19.2	79.1	52.7	7.7	-
tert-Butanol	5.6	10.5	3.5	1.4	0.35
n-Hexane	3.6	5.8	1.8	0.4	3.5
t-Butylmethyl ether	*41.5*	*89.6*	*39.6*	*8.5*	*2.6*
Ethyl acetate	*50.8*	*97.0*	*35.1*	*11.1*	*0.68*
Diethyl ether	**67.2**	**96.9**	**22.7**	**7.0**	**0.85**
Chloroform	**78.9**	**97.2**	**11.1**	**7.2**	**2.0**
Dichloromethane	**80.7**	**97.7**	**10.9**	**6.1**	**2.2**

- Apart from water, the solvents could be classified into 3 groups. One is lower conversion of PC, yield of product and hydrolysis degree; Another is: the conversion of PC is higher, but the hydrolysis degree is also higher; The third has a desirable result: higher yield of PC-VB6 and lower hydrolysis.
- No direct relationships between the log P of solvent and its reaction performance.
- Good and stabile emulsification in the third group has been observed.
- Dichloromethane, chloroform and diethyl ether are preferable solvents

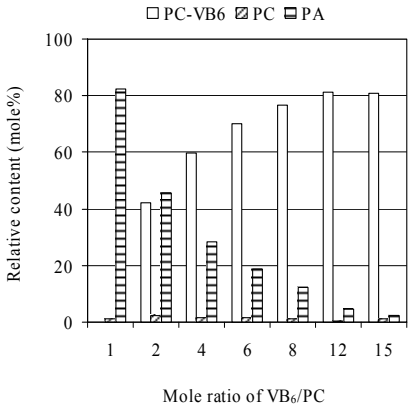
Reaction performance in normal solvents —pH effect



Transphosphatidylation rate (□), hydrolysis rate (○) and ratio of transphosphatidylation / hydrolysis (△)

- The optimum pH is at around 5.4. At the range of pH 4.5-6.6, high reaction rate for both transphosphatidylation and hydrolysis were observed.
- PLD from Streptomyces sp. seems to be more sensitive to base than to acid.
- The preference of PLD for transphosphatidylation to hydrolysis declines with the increase of pH values. This might be related to the nature of enzyme and the ionization state of VB_6 as well as the transfer of choline.

Reaction performance in normal solvents —Effect of VB_6 concentration



Reaction conditions: 10h, 30°C, 12.5mM PC in dichlorometane-buffer system, pH 5.6.

- In all reactions higher conversion of PC is observed. However, the hydrolysis of PC is faster than transphosphatidylation of VB_6 at lower VB_6 concentration.
- The results indicate higher concentration of pyridoxol could suppress the hydrolysis of PC.
- Higher concentration of pyridoxol than 10 (molar ratio of VB_6/PC) is needed to obtain higher yield of the desired product.

Optimized conditions for VB6-PC preparation

- Optimized conditions based on the balance among productivity, efficiency, reaction time and cost, etc.
- Ditheyl ether, chloroform and dichloromethane are preferable normal solvents.
- 4.5-6.6 is the better working pH range for PLD from *Streptomyces sp.* catalyzed transphosphatidylation of VB_6.
- Excessive VB_6 could inhibit the hydrolysis of PC to PA. 10 times excessive VB_6 is needed to efficiently suppress PC hydrolysis.
- 70-100 mM of PC with 5-10 U/mL PLD loading is the recommended conditions.
- With optimized conditions, 70-85% yield of product and over 90% conversion of PC could be achieved.

Concluding remark

- System design is of paramount importance to engendar reaction or govern reaction specificity;
- Solvent may not only be limited to dissolving PLs, but also be capable to change equilibrium;
- Water is absolutely essential for phospholipase, but "how much" to be controlled is a "know-what";
- Other people's work may initiate your idea, but never let it limit your thinking, because you always can do better;
- New developments in other diciplines may help in phospholipids.

Thank for your attention

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