Development of Phospholipases to Produce Structured Phospholipids

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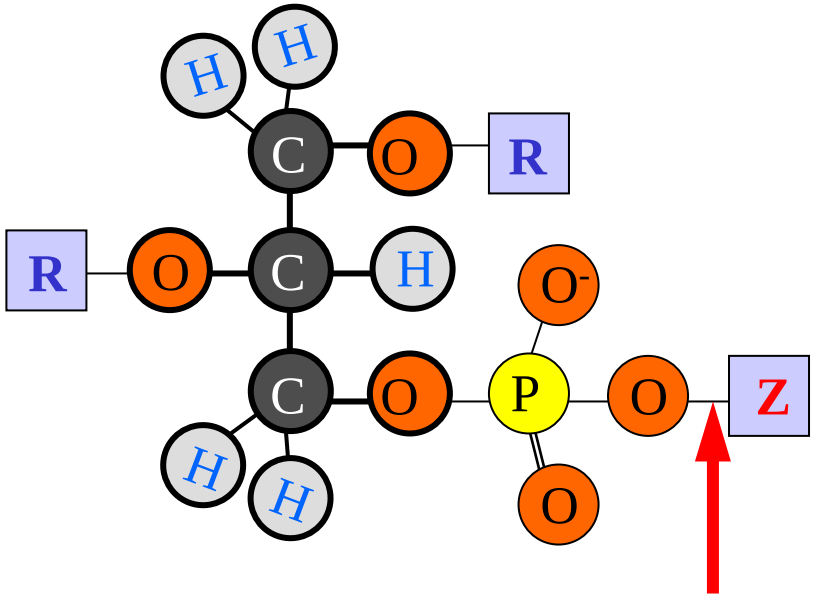
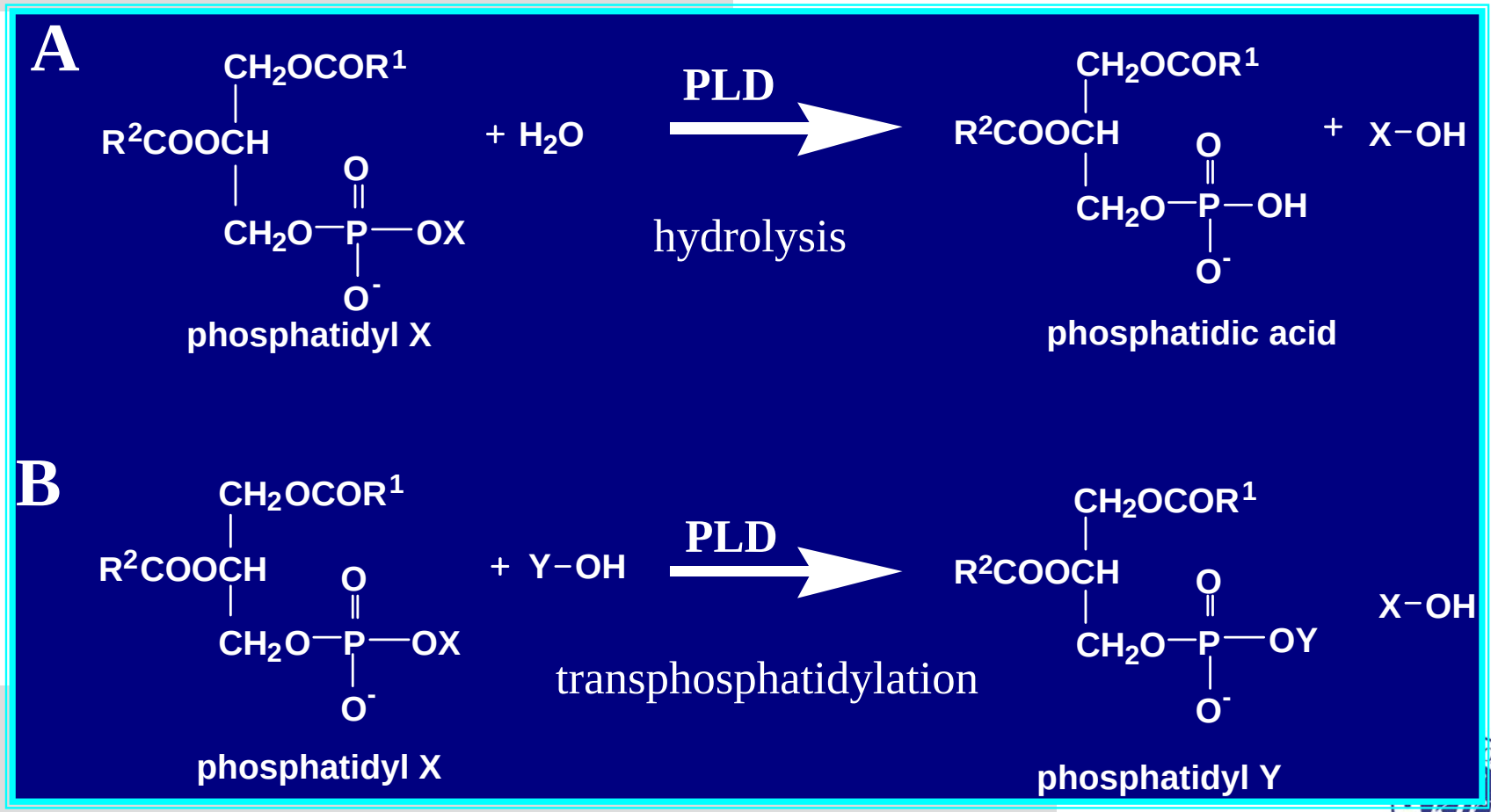
Phospholipase D



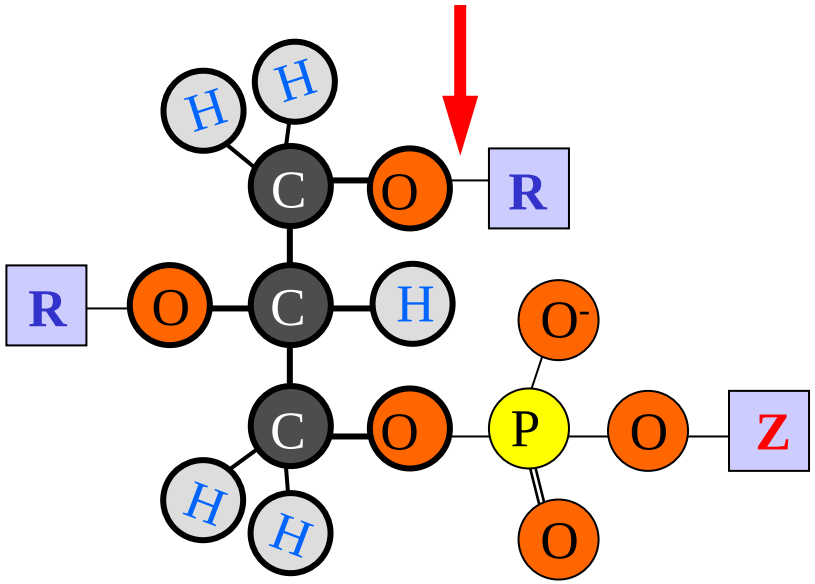
R	Fatty acyl chain
Z	- H *Phosphatidic acid (PA)*
	- CH_2 - CH_2 - NH_2 *Phosphatidylethanolamine (PE)*
	- CH_2 - CH_2 - $N(CH_3)_3$ *Phosphatidylcholine (PC)*
	- CH_2 - $CH(NH_3)$ - COO^- *Phosphatidylserine (PS)*
	- Inositol *Phosphatidylinositol (PI)*

Phospholipase D

- Hydrolysis(A), Transphosphatidylation(B)



Phospholipase A_1



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Lyso-Phospholipids

- Greater emulsion activity than PL, pharmaceutical agents, food preservatives
  - fat digestion in animal duodenum
  - cell autolysis by solubilizing bacterial membrane
  - natural surfactant with low toxicity
  - synergist with other surfactants
  - gentle solubilizing agent
  - inhibition of prostaglandin, etc.

Sources of PLD

- Carrot, cabbage
- *Actinomycetes - Streptomyces, Actinomadura, Streptoverticillum* etc

Screening from soil & Cultivation,

Enzyme Purification & Characterization

Screening of *Actinomycetes*

- 30g soil – drying overnight – dissolve in 6% yeat extract, 0.05% sodium dodecylsulfate – heat at 40°C for 20min – cultivate on HV agar plate containing nalidixic acid(20mg/l) at 30°C for 3 weeks
- Isolate colony and cultivate on Bennet agar plate

Cultivation of *Actinomycetes*

- Cultivate colony isolated in test tube containing peptone broth at 30°C for 3 days
- Fermentation using BioFlo IIC -

  working volume 1.8liter, 5% inoculation, 30°C, pH 7.2, 1 v/v/m, 600rpm, 3 days
- Centrifuge culture broth to obtain supernatent containing extracellular phospholipase D

Purification and Characterization of PLD

- Ultrafiltration – Amicon PM10
- Ion exchange chromatography –

  CM Sephadex C-50
- Gel permeation chromatography –

  Sephadex G-100
- FPLC – Mono S (HR 5/5)
- SDS-PAGE, pH, temperature, metal ions

Hydrolytic activity of PLD

- 1 unit - 1μmole choline produced per min, phosphatidylcholine(PC) emulsion in Na-citrate buffer (pH 6), enzyme solution, 37°C, 10min reaction, boiling with EDTA
- Choline oxidase, peroxidase, Tris-Cl buffer (pH 8), 37°C, 20min reaction, OD at 500nm

Transphosphatidylation activity of PLD

- Egg yolk PC, glycerol, acetate buffer (pH 5.6), enzyme solution, 25°C, 30min reaction, reaction stop by citric acid
- Extract PL using diethyl ether-EtOH(4:1), concentrate, develop TLC
- **1st TLC – $CHCl_3$:MeOH:NH_4OH(130:60:8)**

  2nd TLC - $CHCl_3$:MeOH:acetic acid:H_2O(170:25:25:6)

- Quantify PC, phosphatidylglycerol(PG), phosphtidic acid(PA)

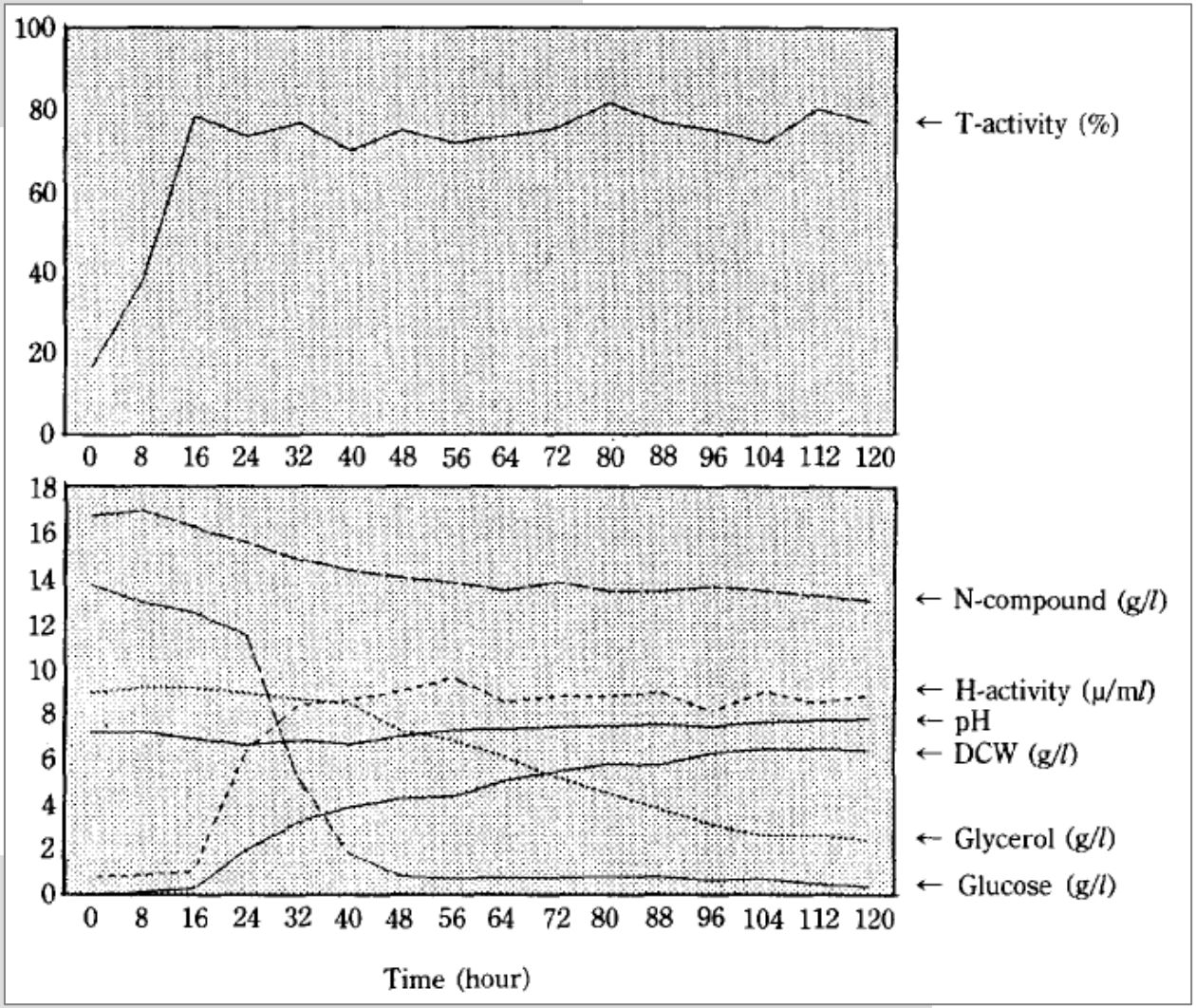
- T activity (%) = 100 x PG / (PC+PG+PA)

Selection of *Actinomycetes*

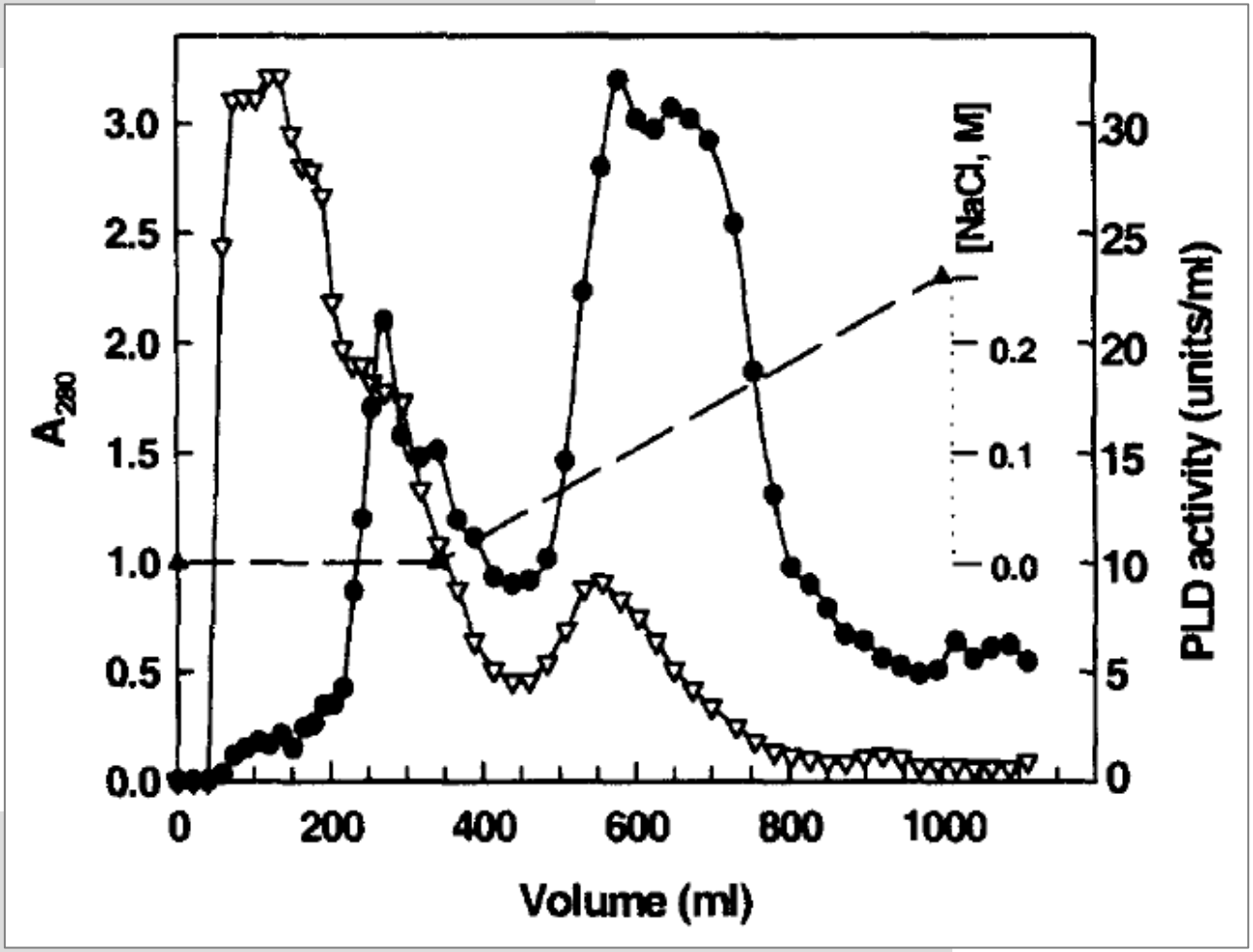
- 1,100+ soil samples – 131 HA>0.3u/ml
  23 TA>20%

- *Actinomycetes* KF923, HA 12u/ml, TA 95%

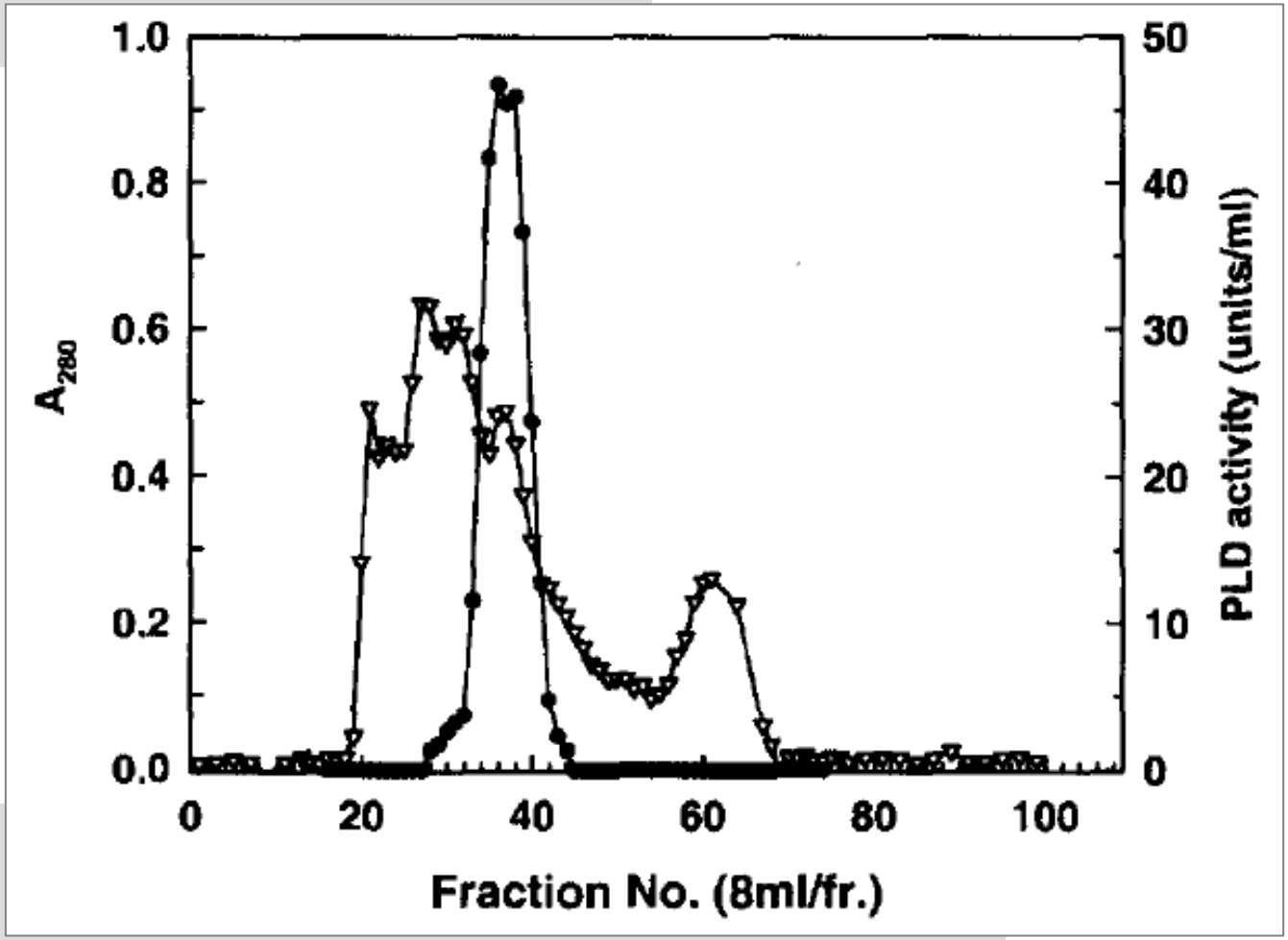
Cultivation of *Act.* KF923



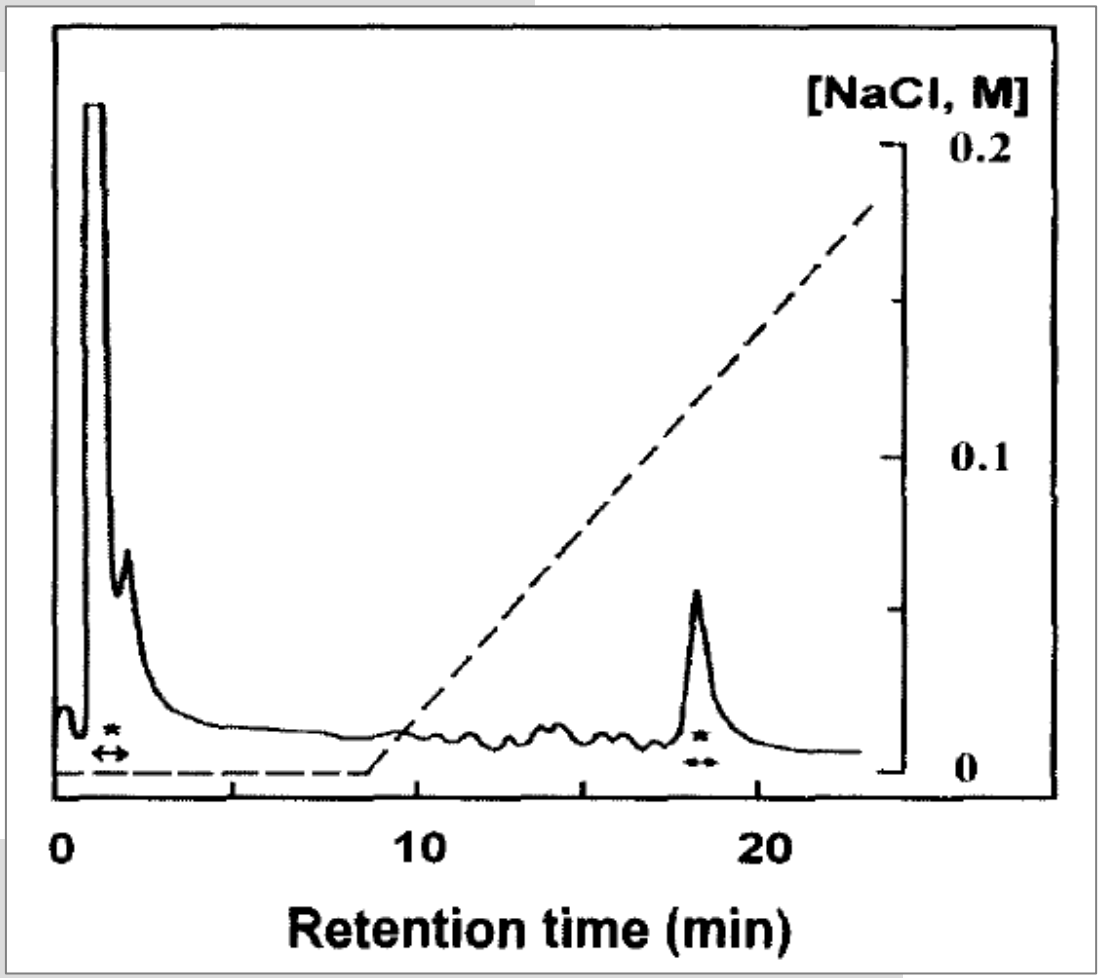
Ion exchange chromatography of PLD



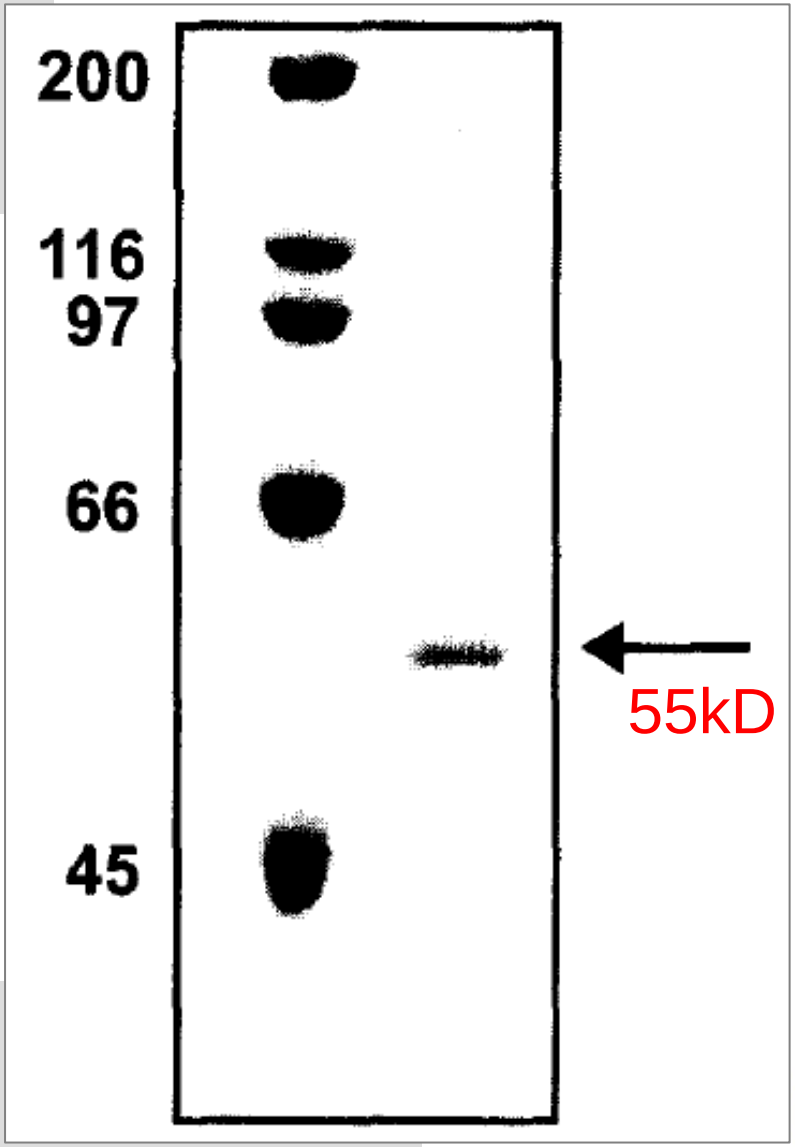
Gel permeation chromatography of PLD



FPLC of PLD on Mono S



SDS-PAGE of PLD

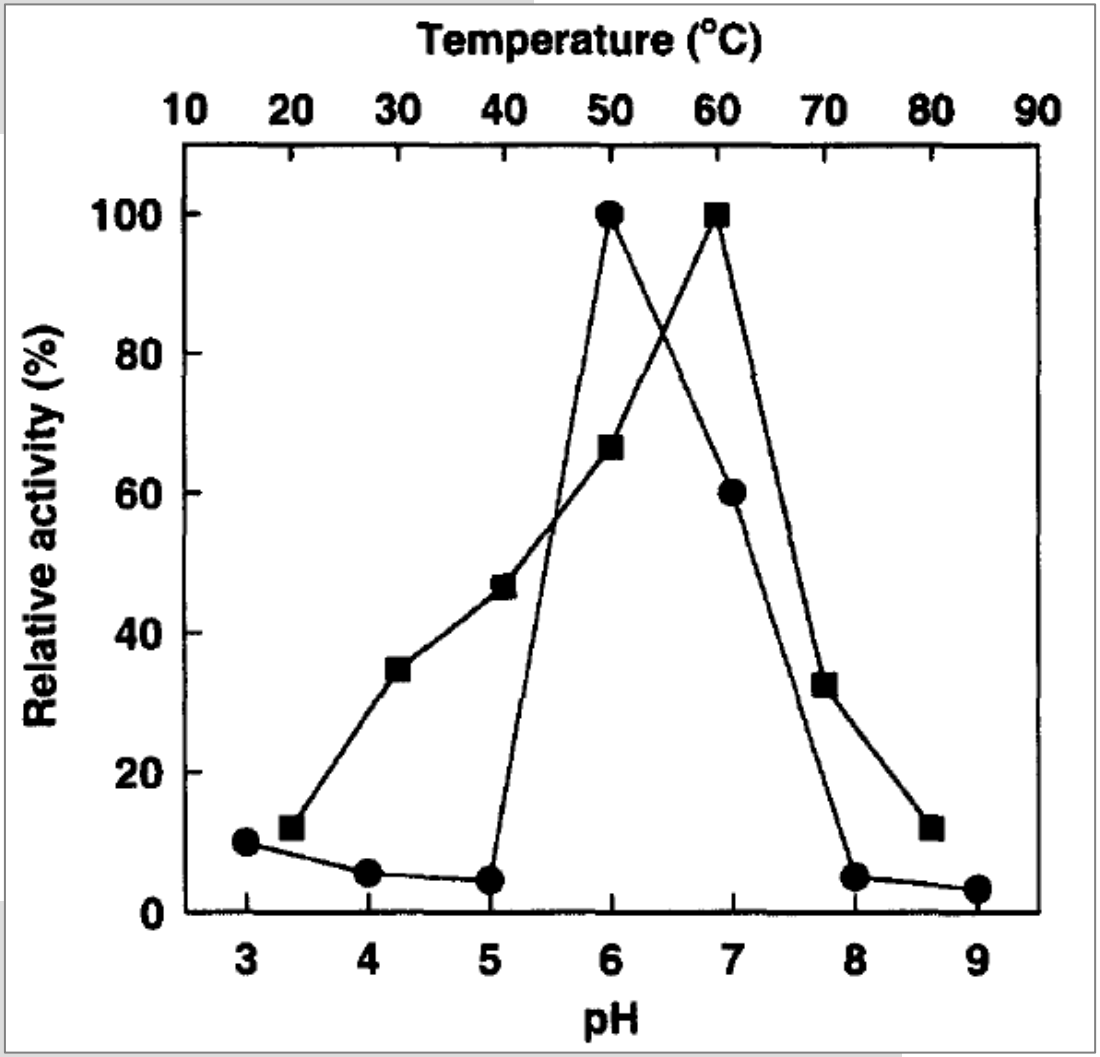


Purification of PLD from *Act.* KF923

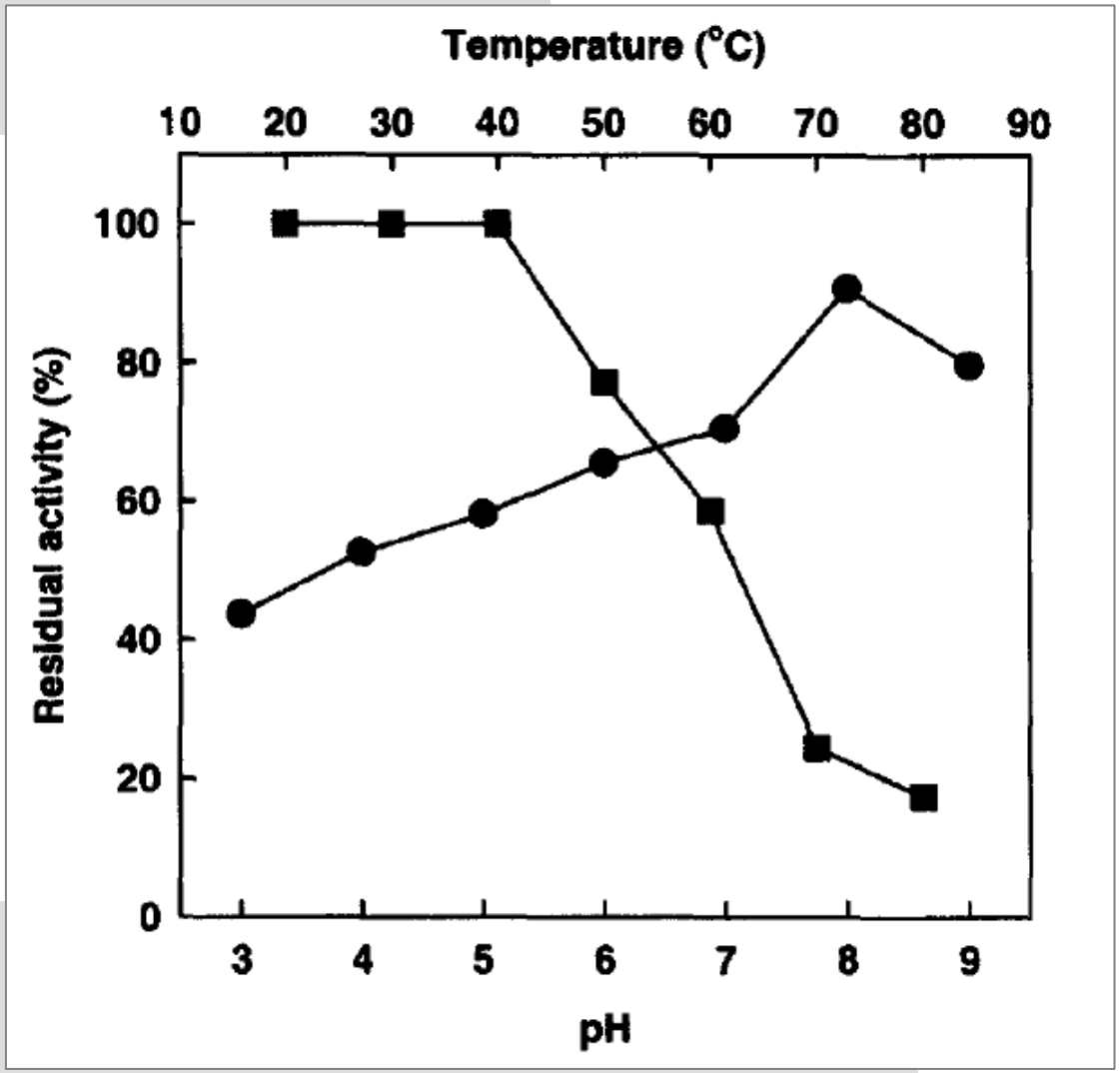
Table 1. Purification of Phospholipase D from Actinomycetes KF923.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude supernatant	25,641	24,120	0.9	1	100
Ultrafiltration	1,956	12,864	6.6	7.03	53.3
CM-Sephadex C-50	180	3,113	17.3	19.2	12.9
Sephadex G-100	22.6	1,142	50.5	56.1	4.7
Mono S	0.54	306	566.7	629.7	1.3

Effects of pH and temperature on TA of PLD



Effects of pH and temperature on stability of PLD



Effects of metal ions on TA of PLD

Ions[1]	Relative activity (%)[2]
Ba	123
Ca	123
Co	119
Cu	117
K	113
Mg	123
Mn	128
Na	110
Zn	111
Control	100

Sources of PLA_1

- Intracellular PLA1 – rat liver, brain & heart of human & bovine, *E.coli, B. megaterium*
- Extracellular PLA1 – *Serratia sp.*

Screening from soil & Cultivation,

Enzyme Purification & Characterization

Production of LPL & Optimization

Experimental methods

- Cultivation of *Serratia* sp. for PLA1 production
  - M9 minimal salt medium for seed culture
  - 3 liter fermentor, nutrition broth
  - aerobic, 14hrs at 30°C
- Enzyme preparation
  - crude extract, precipitation, dialysis
  - QAE-Sepharose ion exchange column
  - Sephacryl S-200 gel filtration column

- Two-phase system

  - 10ml Phospholipon 90G in solvents, 10ml PLA1 (32units) and $CaCl_2$ in aqueous phase, pH 8, 40C

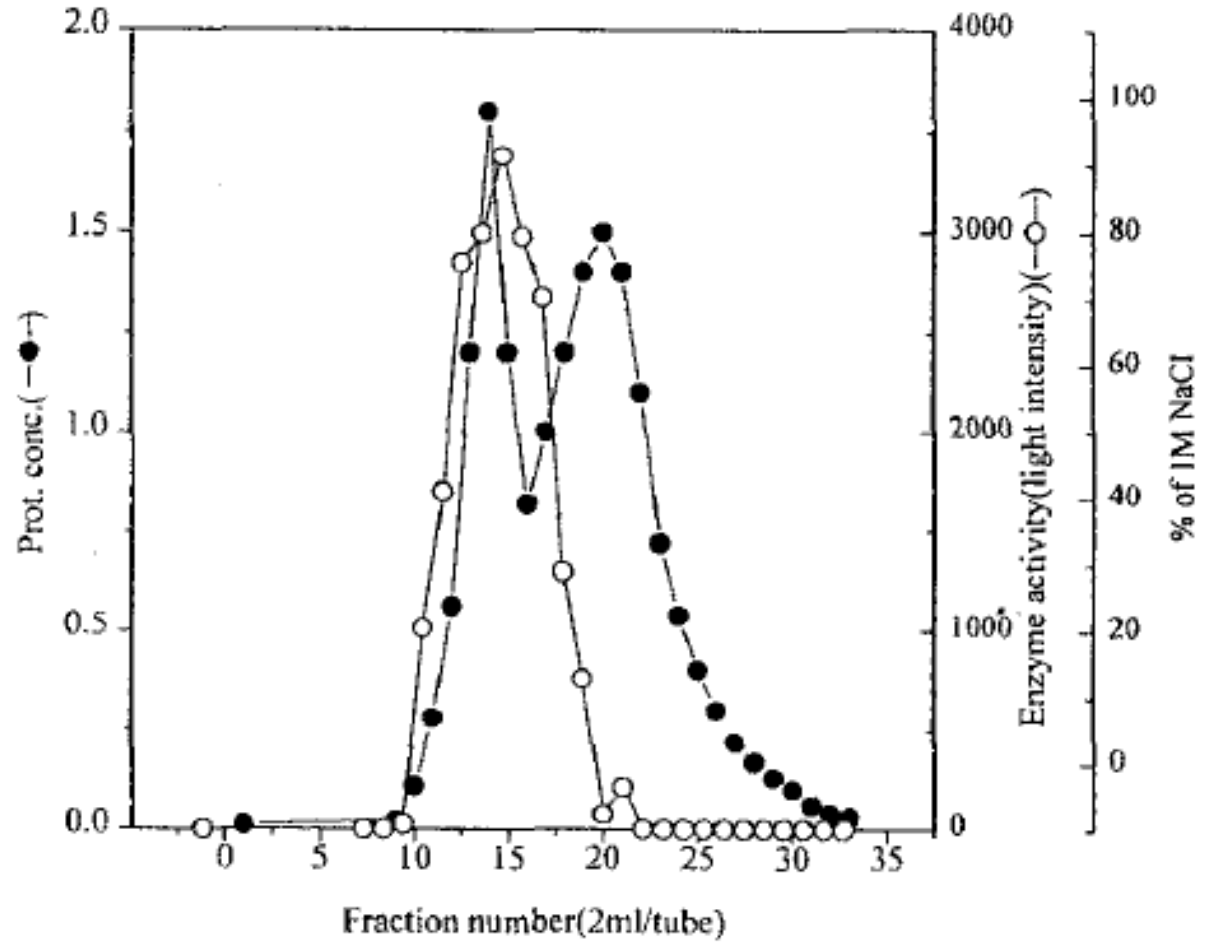
- Emulsion system

  - Phospholipon 90G, sodium deoxycholate in Tris-HCl buffer, add PLA1 and $CaCl_2$ solution

Analytical methods

- Activity determination of PLA1
  - Hydrolysis, FFA by pHstat (egg yolk lecithin) or TLC-FID
  - Activity staining, water-insoluble FA
- Substrate specificity of PLA1
  - TLC-FID, 2 developments, monopalmitin
- Molecular mass by Matrix Assisted Laser Desorption Ionization (MALDI) mass spec

Ion exchange chromatography of PLA_1



Gel permeation chromatography of PLA_1

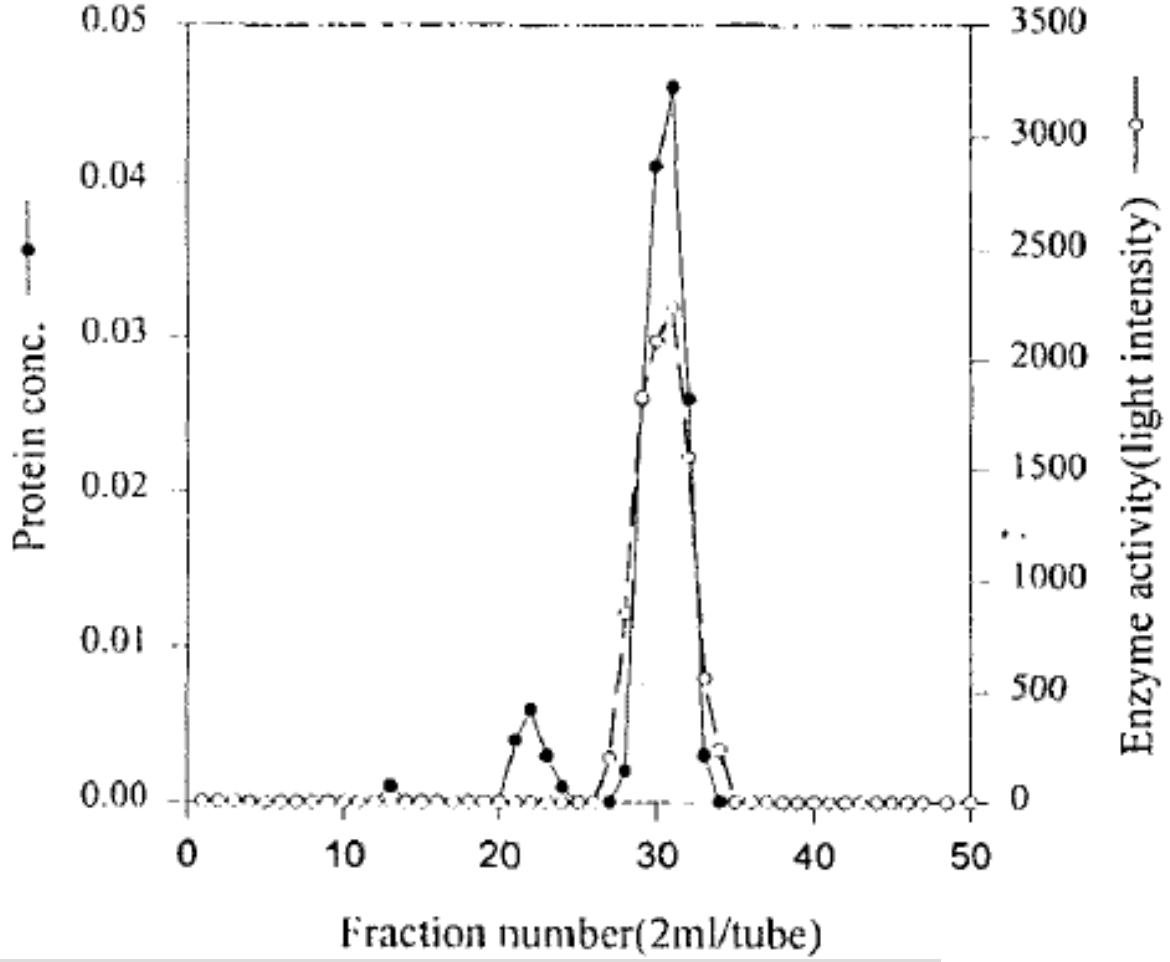
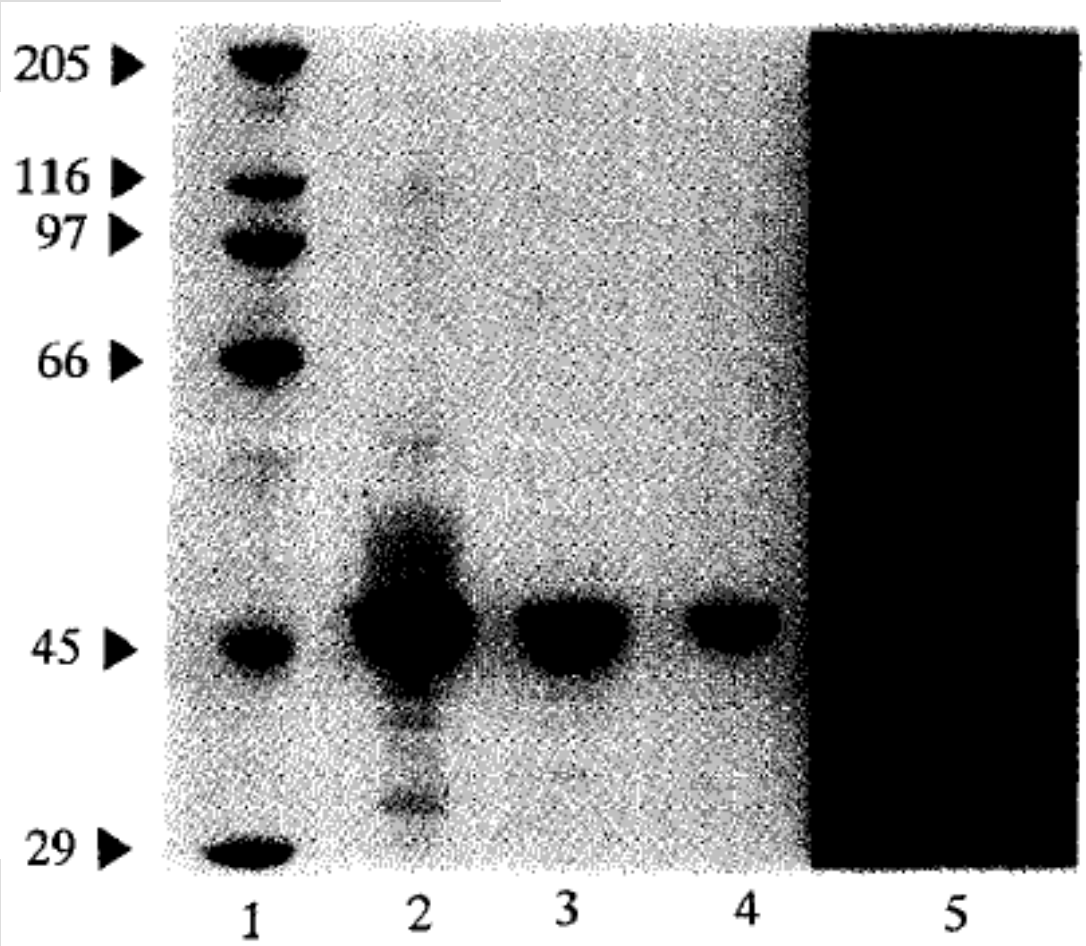


Table 1. Summary of the procedure for the purification of extracellular phospholipase A_1 from *Serratia* sp. MK1.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Fold purification
Enzyme extract	324	3480	10.7	100	
75% Ammonium sulfate	178	2040	11.5	58.6	1.1
Ion exchange	113	1480	13.1	42.5	1.2
Gel filtration	34	714	21	20.5	2.0

SDS-PAGE of PLA_1



MALDI-TOF Spectrum of PLA_1

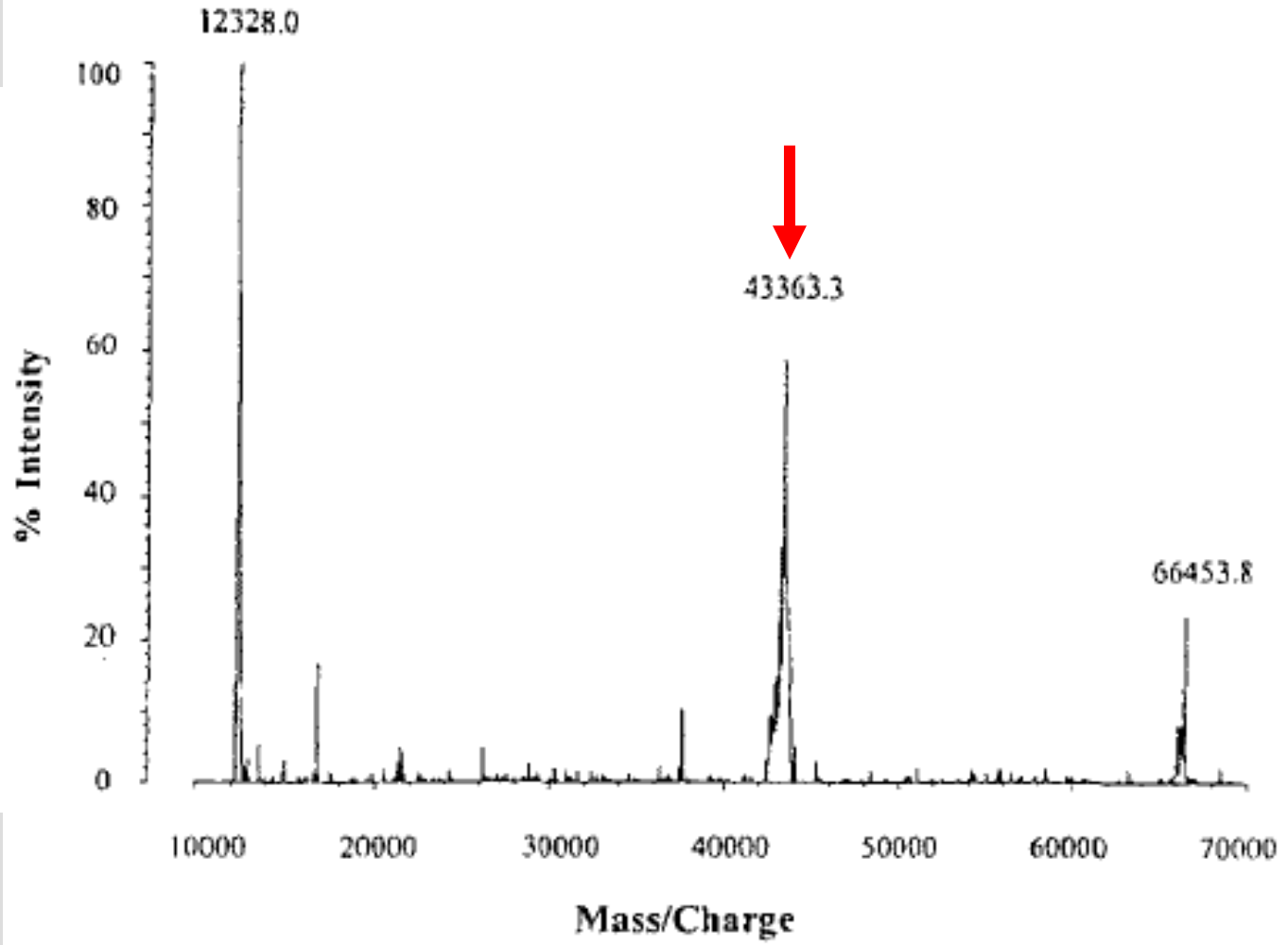


Table 2. Hydrolysis rate of various phosphoglycerides by the phospholipase A_1 from *Serratia* sp. MK1.

Substrate	Concentration (mM)	Activity (units/mg protein)
Phosphatidylcholine (PC)	3.4	2.4
Phosphatidylethanolamine (PE)	3.4	0.6
Phosphatidylglycerol (PG)	3.4	3.2
Phsphatidylserine (PS)	3.4	14.5
Phosphatidylinositol (PI)	3.4	8.2
Phosphatidic acid (PA)	3.4	10.2
Lyso-phosphatidylcholine (Lyso-PC)	3.4	3.3
Lyso-phosphatidylethanolamine (Lyso-PE)	3.4	2.8
Sphingomyelin (SP)	3.4	7.2
Cardiolipin (CA)	3.4	0.2

Rat liver, brain

Table 3. Effect of inhibitors on phospholipase A_1 activity.

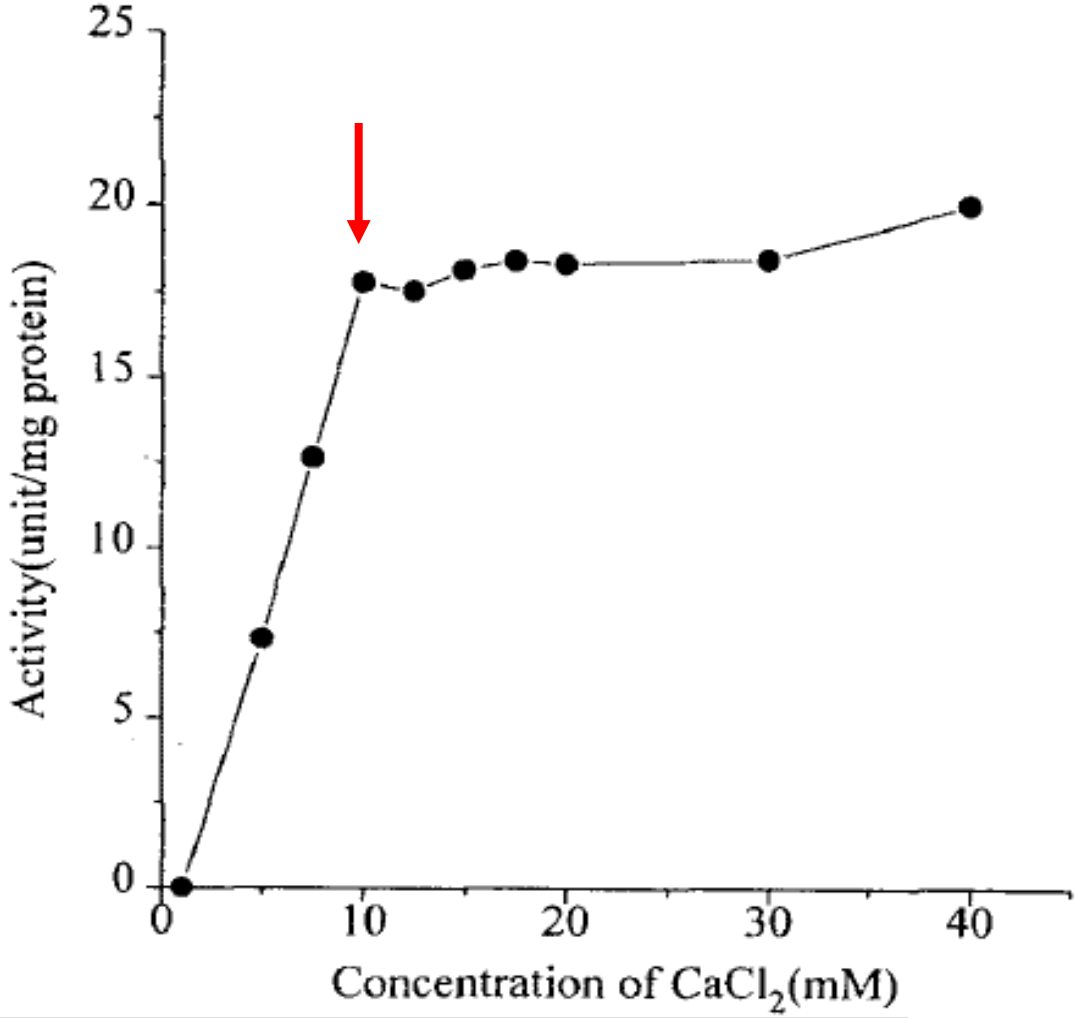
Inhibitors	Residual activity	
	Concentration	
	1 mM	10 mM
Control	100	
PCMB	104±2	63±0.4 ← His, Cys
BPB	98±3	73±4
ME	112±2	95±9
SDS	102±1	126±1 ←
DEP	103±0.4	86±0.6
PMSF	112±1	34±2 ←
IA	101±0.4	95±7
NEM	108±7	78±5

PCMB, *p*-chloromercuribenzoate; BPB, 4-bromophenacyl-bromide; ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; DEP, diethyl-pyrocarbonate; PMSF, phenylmethyl-sulphonyl fluoride; IA, iodoacetamide; NEM, N-ethylmaleimide.

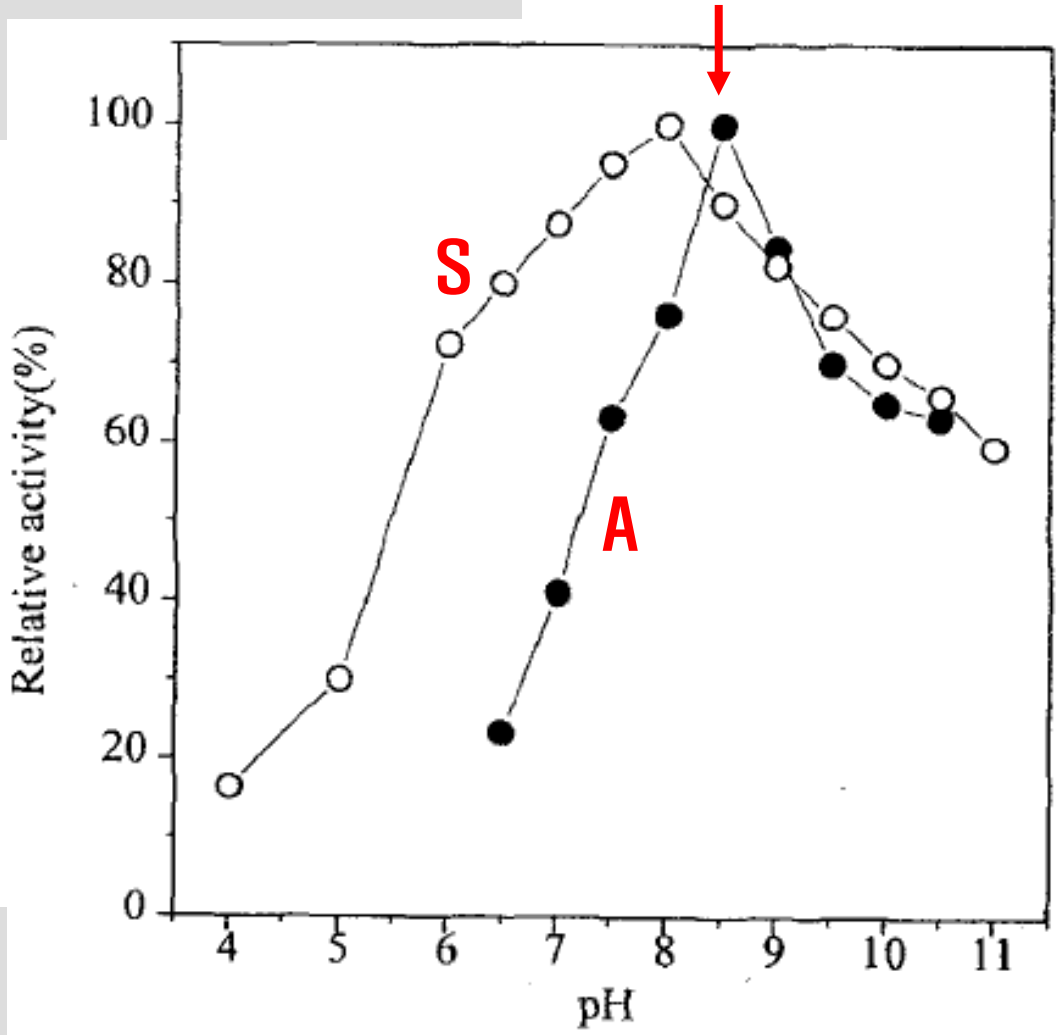
Table 4. Effect of metal ions on phospholipase A_1 activity.

Metal salts (10 mM)	Relative activity (%)
Control	100
EDTA	0
$CaCl_2$	89±5
$FeSO_4$	60±3
$MnCl_2$	16±0.8
$AlCl_3$	18±0.9
$CoCl_2$	5±0.3
$MgCl_2$	3±0.2
$BaCl_2$	4±0.2
$CuCl_2$	1±0.1
NaCl	0
KCl	0
$AgCl_3$	0
$ZnCl_2$	0

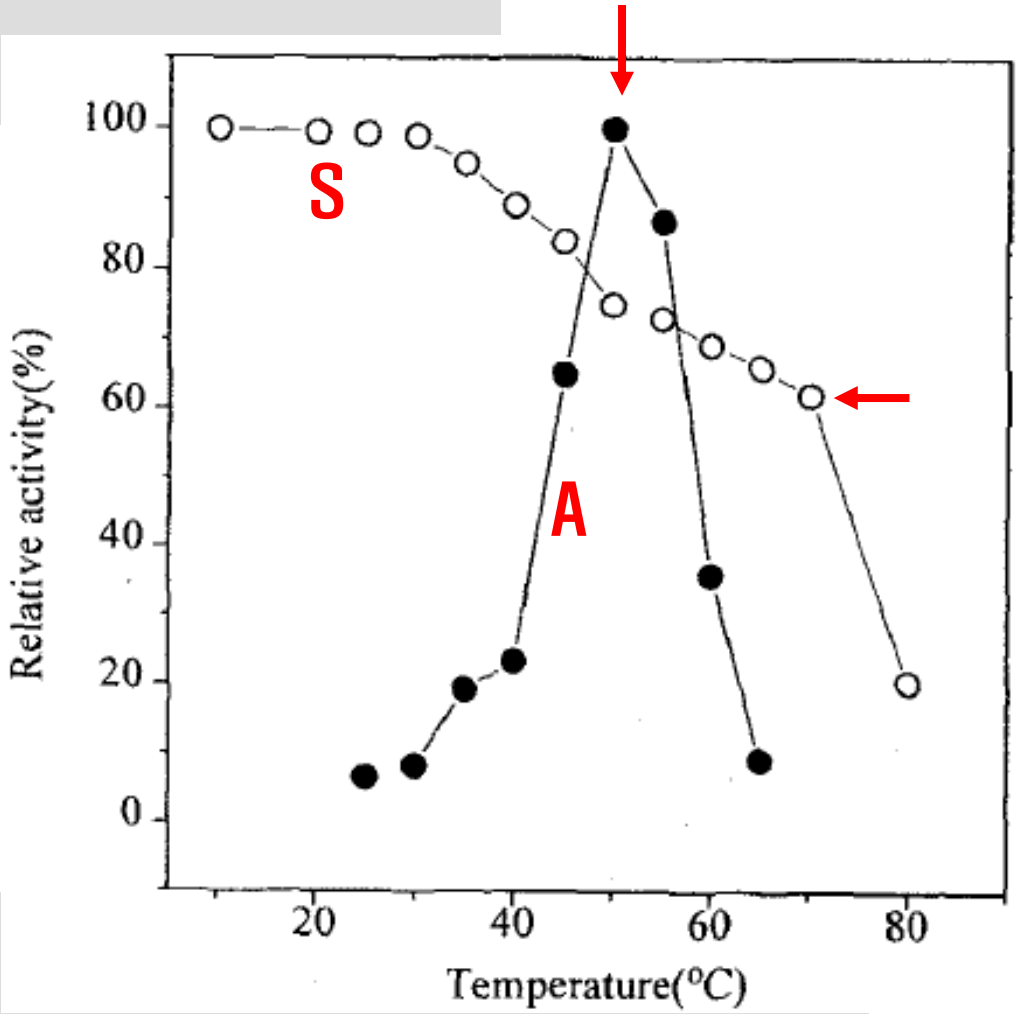
Effects of calcium conc'n on PLA_1



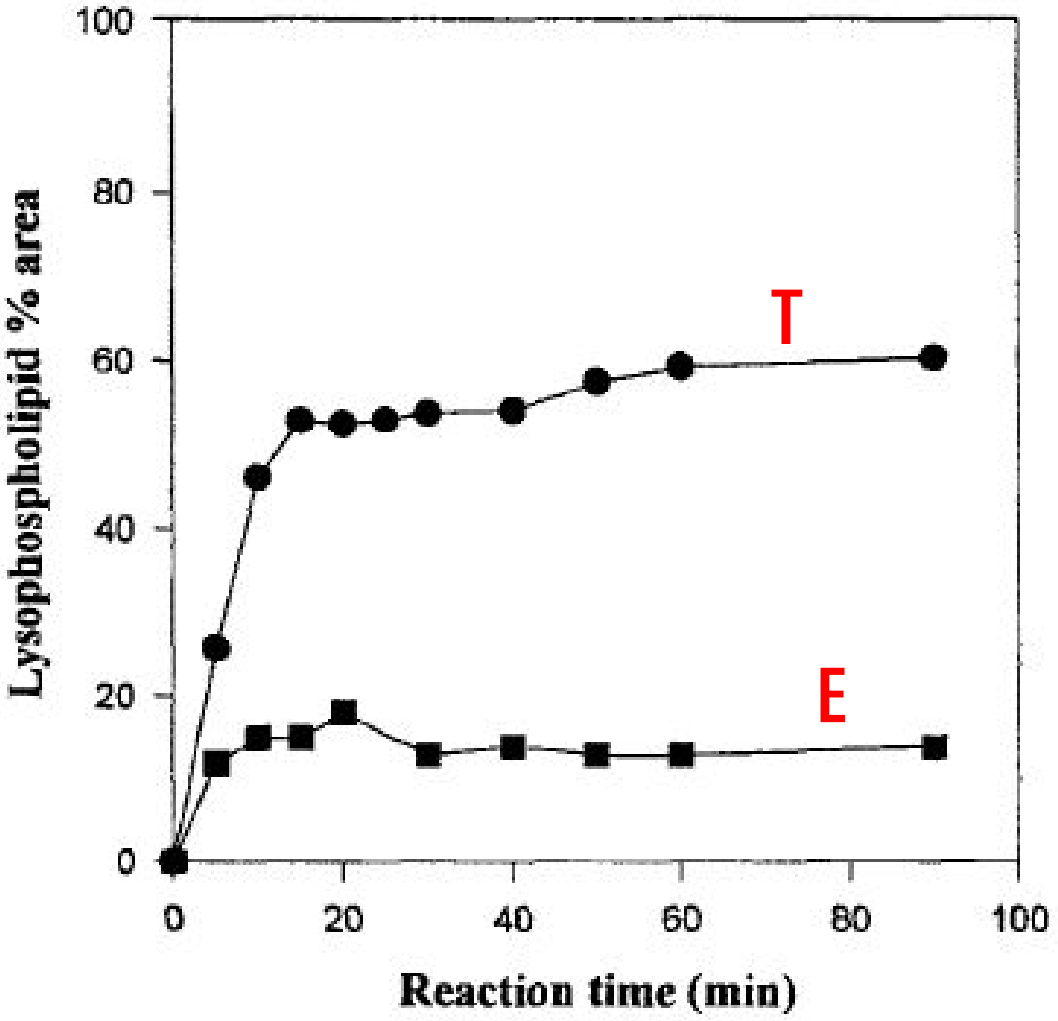
Effects of pH on PLA_1 activity and stability



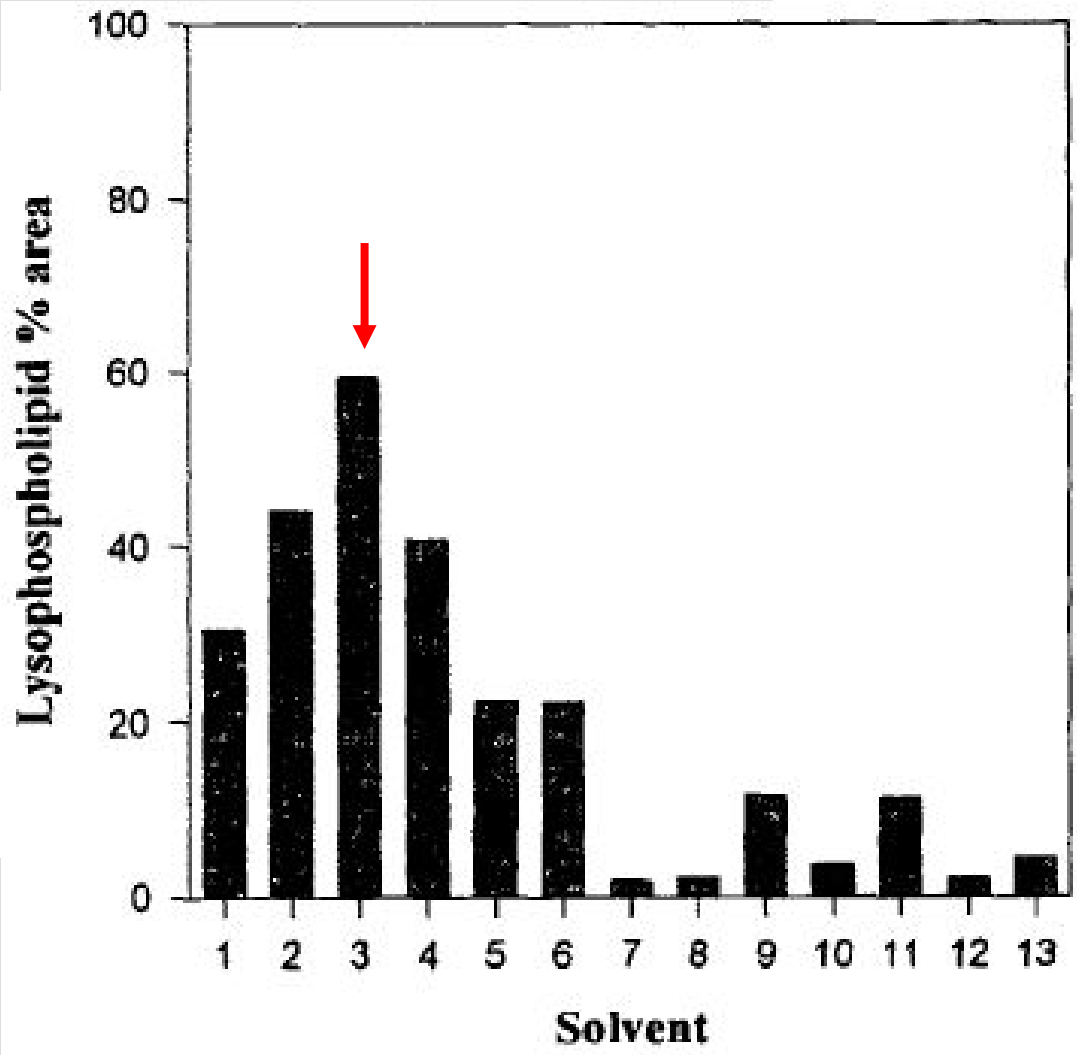
Effects of temp on PLA_1 activity and stability



Production of LPL in two-phase and emulsion systems

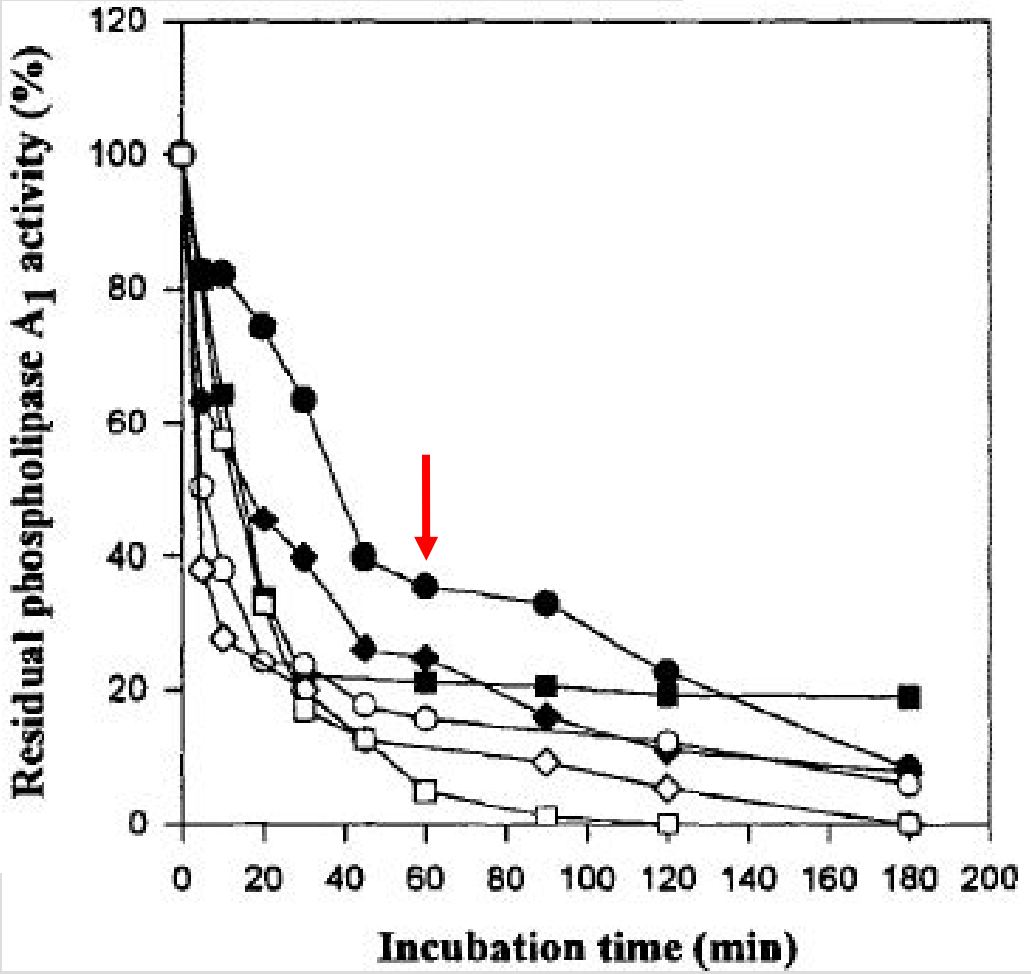


Effects of organic solvents in two-phase system



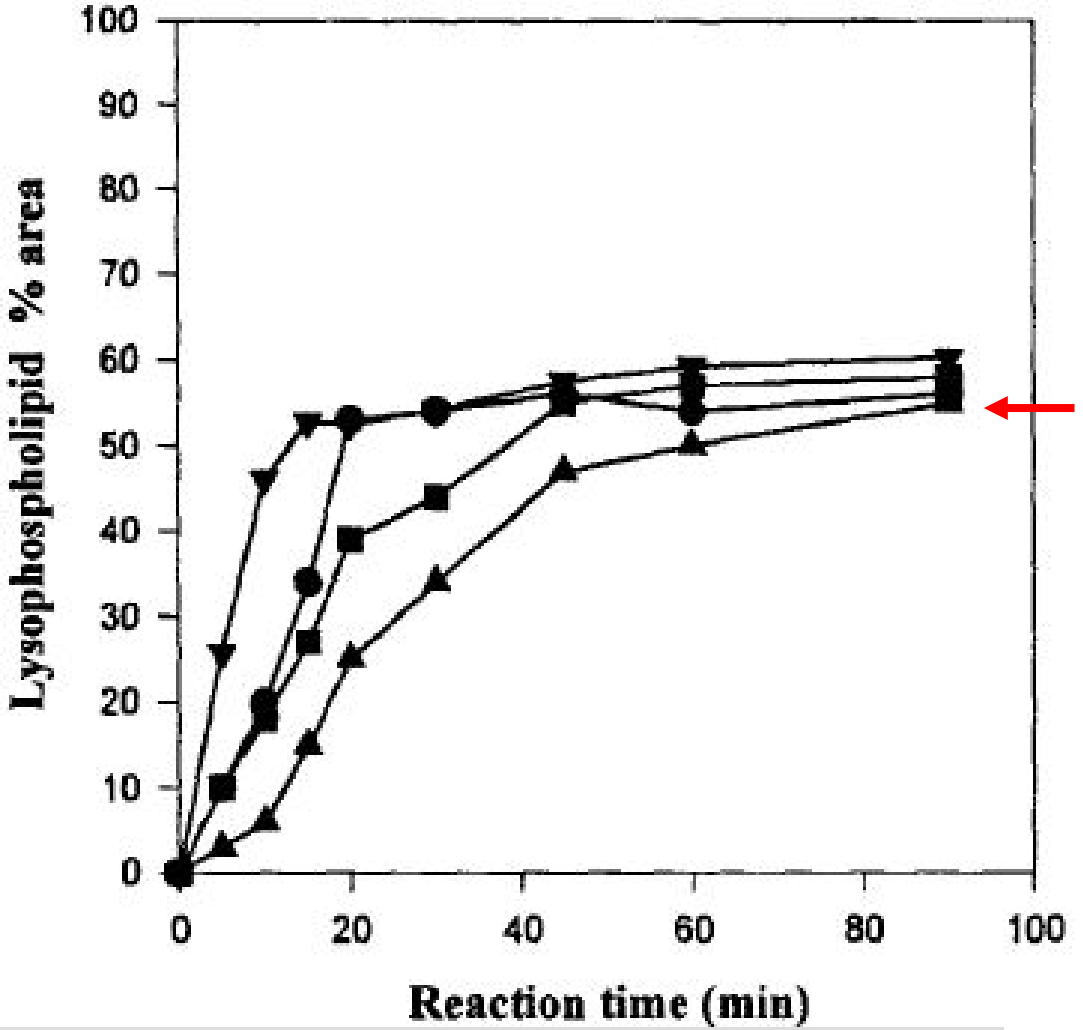
1. Ethyl acetate
2. Diethyl ether
3. Butyl acetate
4. Isopropyl ether
5. Benzene
6. Chloroform
7. Butyl ether
8. Cyclohexane
9. Hexane
10. Heptane
11. Isooctane
12. Decane
13. Dodecane

Effects of organic solvents on stability of PLA_1



○ Ethyl acetate
■ Diethyl ether
● Butyl acetate
◆ Benzene
□ Butyl ether
◇ Cyclohexane

Effects of PL conc'n on LPL production in butyl acetate



Conclusion

- PLD - specific activity of 567 unit/mg, MW 55kD, optimum pH 6.0, temperature 60°C, no essential requirement for special metal ions
- PLA1 – extracellular MW 43kDa (monomer)
  - Optimum pH 8.5, temperature at 50°C, stable up to 70°C
  - Enzyme activity inhibited by EDTA, and recovered by Ca^{++}
  - High lipolytic activity for phosphatidylserine
  - Reaction yield of LPL in two-phase system higher than that in emulsion system
  - Highest catalytic activity and stability of the enzyme in butyl acetate 20% PL completely hydrolyzed into LPL in two-phase system