Review

Reminiscence of phospholipase B in *Penicillium notatum*

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(Communicated by Kunihiko Suzuki, M.J.A.)

Abstract: Since the phospholipase B (PLB) was reported as a deacylase of both lecithin and lysolecithin yielding fatty acids and glycerophosphocholine (GPC), there was a question as to whether it is a single enzyme or a mixture of a phospholipase A2 (PLA2) and a lysophospholipase (LPL). We purified the PLB in *Penicillium notatum* and showed that it catalyzed deacylation of sn-1 and sn-2 fatty acids of 1,2-diacylphospholipids and also sn-1 or sn-2 fatty acids of 1- or 2-monoacylphospholipids (lysophospholipids). Further, it also has a monoacyl lipase activity. The purified PLB is a glycoprotein with m.w. of 91,300. The sugar moiety consists of M9 only and the protein moiety consists of 603 amino acids. PLB, different from PLA2, shows other enzymatic activities, such as transacylase, lipase and acylesterase. PLB activity is influenced by various substances, e.g. detergents, deoxycholate, diethylether, Fe3+, and endogenous protease. Therefore, PLB might have broader roles than PLA2 in *vivo*. The database shows an extensive sequence similarity between *P. notatum* PLB and fungal PLB, cPLA2 and patatin, suggesting a homologous relationship. The catalytic triad of cPLA2, Ser, Asp and Arg, is also present in *P. notatum* PLB. Other related PLBs, PLB/Lipases are discussed.

Keywords: Phospholipase B (PLB), complete deacylation, 1,2-diacyl- and 1- or 2-monoacylphospholipids, purification, cPLA2, catalytic triad

1. Introduction

The term “phospholipase B” was first described by Contardi and Ercoli¹ in 1933 as lecithase B, which attacked an acyl–ester bond of lysolecithin and produced one mole each of fatty acid and glycerophosphocholine (GPC). Lysolecithin was enzymatically derived from lecithin by lecithase A which was the only phospholipase known at that time. Therefore, lecithin was to be enzymatically deacylated successively by lecithase A and lecithase B. On the other hand, they reported that the lecithase B also deacylated both of the acyl chains of lecithin and produced 2 moles of fatty acids and one mole of GPC.

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Abbreviations: PLA: phospholipase A; PLB: phospholipase B; PLC: phospholipase C; PLD: phospholipase D; LPL: lysophospholipase; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; PA: phosphatic acid; GPC: glycerophosphocholine; TMS: trimethylsilyl.

do: 10.2183/pjab.90.333
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Three important developments should be noted in the history of *P. notatum* PLB. The first was the discovery of powerful LPL activity in *P. notatum* autolysate by Fairbairn in 1948. The enzyme attacked only lysolecithin but not lecithin. So Fairbairn used the name LPL instead of lecithinase B, which was proposed by Contardi and Ercoli.

The second development was the articles from the groups of Dawson and Kates between 1957 and 1965. Both groups showed that under certain conditions *P. notatum* autolysate could hydrolyze both of the acyl linkages of lecithin simultaneously, yielding 2 moles of fatty acids and one mole of GPC. In 1957, Dawson attempted to detect lysolecithin in the crude lipids of rat liver with *P. notatum* enzyme. As a result, an unexpectedly large amounts of fatty acids and water-soluble phosphate, mainly GPC, were detected, suggesting breakdown of coexisting lecithin. However, purified lecithin isolated from the same crude lipids was not hydrolyzed with the enzyme preparation. He thought that there was an additional factor in the crude liver lipids which was responsible for the *P. notatum* enzyme attacking the liver lecithin. Then two active lipids were isolated, which were monophosphoinositide and most probably a polyglycerol phospholipid. Later, among various lipids and non-lipids tested, it was found that in addition to the above two phospholipids, cardiolipin was the most effective activating lipids, and tripalmitin and tristearin were less effective. He concluded that hydrolysis occurred only when the net negative surface potential of the lecithin particles had reached a certain critical value. In 1965, Kates and coworkers reported that *P. notatum* enzyme catalyzed rapid deacylation of purified ovolecithin in the absence of lipid activators such as cardiolipin, provided if the substrate was dispersed in a sufficiently fine state. The ultrasonically dispersed lecithin was found to be rapidly hydrolyzed by the enzyme, whereas, without ultrasonication, lecithin was only slowly hydrolyzed. It therefore seems likely that the initial attack of the *P. notatum* enzyme is not primarily dependent on the substrate particles having a negative surface potential but rather on the degree of dispersion of the substrate particles.

The third development was purification of *P. notatum* enzyme in my laboratory. The enzyme preparations described above were not purified. We started to purify the enzyme in 1973 and the results were summarized in short reviews. A large scale culture of *P. notatum*, FIO 4640, yielding 51.2 kg of mycelia per 2,000 liters, was conducted by the Research Laboratories of Toyo Brewing Co., Shizuoka, Japan.

2. Chemical structure of *P. notatum* PLB

2-1. Purification. As shown in Table 1, both the PLB and LPL activities copurified through several purification steps to a single spot on disc electrophoresis and on SDS-PAGE stained with Coomassie Brilliant Blue and Schiff’s reagent. The last steps of the purification (A) were later replaced by affinity chromatography (B). Throughout the purification steps the ratio of PLB activity to LPL
activity remained approximately 1 : 110. The long-held view that PLB consists simply of a mixture of PLA and LPL appeared highly unlikely, and Waite described in his book, “The Phospholipases”,\textsuperscript{20} that this crucial work stands as the true first example of PLB. Triton X-100 stimulated the PLB activity but inhibited the LPL activity. Under the optimum condition in the presence of Triton X-100 for ovolecithin and without Triton X-100 for the lysolecithin, the activity ratio was 1 : 16.\textsuperscript{18} Limited proteolysis. However, the activity ratio, 1 : 110, was variable according to culture lots. It was found later that the PLB enzyme was partially modified by endogenous protease during cultivation and autolysis. The purified preparation which showed one spot of 90 kDa on non-reducing SDS-PAGE gave three more spots, 68, 38 and 33 kDa, in the presence of 2-mercaptoethanol. As the protease activity was inhibited by phenylmethysulfonylfluoride (PMSF), the mycelia cultured at 25 °C for 70 hr were homogenized at 4 °C in the presence of PMSF and purified. The final preparation showed one spot even on the reducing SDS-PAGE.\textsuperscript{19} On the other hand, on the slab SDS-PAGE in the absence of 2-mercaptoethanol the purified enzyme showed two spots, 106 and 95 kDa, both of which showed an identical amino acid composition and peptide mapping. Each was re-analyzed on reducing slab SDS-PAGE and the 95 kDa spot showed no change but the 106 kDa gave three spots, 70, 37, and 32 kDa, indicating that the 106 kDa was a proteolytically modified form of the PLB.\textsuperscript{21} These two modified and native forms showed differences in chemical and enzymatic nature\textsuperscript{22} and the most distinct one was that the PLB activity greatly decreased in modified form but the LPL activity was the same in both forms.\textsuperscript{19} Therefore, the ratio depends on the degree of limited proteolysis. The Fairbairn’s preparation mentioned above might be the modified form because he cultured for 5 to 8 days and autolyzed for 24 hr at 25 °C.

2-2. Molecular weight. The most reliable value was found to be 91,300 kDa by mass spectrometry (Tamaki, H., Miura, R. and Saito, K. unpublished data) (Fig. 2).

2-3. Structure of the carbohydrate moiety. The sugar chain was determined to be of the high mannose type, M9, (Fig. 3) by chemical treatment with anhydrous hydrazine and confirmed by NMR and MS analyses.\textsuperscript{23}
2-4. Structure of the protein moiety. A cDNA library of *P. notatum* was screened by hybridization with two synthetic oligodeoxyribonucleotide probes, which were synthesized based on each amino acid sequence of the N-terminal region of the 95 kDa protein and of the 70 kDa peptide. The primary structure deduced from the cDNA consisted of 603 amino acids with a calculated molecular weight of 64,779 Da, consistent with the properties of the enzyme reported before. It had 8 cysteine residues and one possible disulfide bond should be between Cys-16 and one of the other cysteine residues. Seventeen potential N-glycosylation sites, Asn-X-Ser/Thr, were present. The limited proteolysis occurred between Leu-175 and Asp-176 (Fig. 4). On the hydropathy profile, the N-terminus was found to be more hydrophilic than the C-terminus. No similarity was found between the amino acid sequence of PLB and those of other proteins stored in the SWISS-PROT database, including phospholipases.

3. Enzymatic actions

3-1. Kinetics of the hydrolysis of \[^{32}P,^{14}C\]phosphatidylcholine and \[^{32}P,^{14}C\]phosphatidylethanolamine. The substrates were isolated from the labelled *Candida lipolytica* and hydrolyzed in the absence of any ‘activators’ and without being subjected to ultrasonication. The labeled PC was rapidly hydrolyzed with the concomitant release of \[^{14}C\]-labelled fatty acids and \[^{32}P,^{14}C\]-labelled GPC (Fig. 5(A)). No labelled lysoPC was detected throughout the course of the hydrolysis with either the high or low enzyme concentration, even in the early stage of hydrolysis.

The course of the hydrolysis of radioactive PE was similar to that of PC under the same conditions, except that the rate of hydrolysis was low (Fig. 5(B)). GPE was the only radioactive water-soluble product formed and no lysoPE was detected.

3-2. Mode of deacylation of sn-1 and sn-2 ester bonds. We tried to detect intermediate lyso-compounds in the presence of sufficient Triton-X-100, which stimulates the PLB activity and inhibits the LPL activity. The system consisted of a mixture of 1-[^14]C]stearoyl-2-acyl and 1-acyl-2[^14]C]oleoyl-PCs. After 5 min, 36% of the substrates was hydrolyzed and a small but significant amount of[^14]C]lysoPC, accounting for 1.3% of the radioactivity accumulated. The ratio, \[^{14}C_{18:0}/^{14}C_{18:1}\] of the lysoPCs, was 6.6, indicating 1-acyl-2[^14]C]oleoylPC was hydrolyzed faster. This finding suggests the *P. notatum* PLB attacked sn-2 first although the enzyme might possibly prefer oleic acid to stearic acid. The similar finding was also reported before. To obtain the final answer studies using 1-acyl-2-alkyl and 1-alkyl-2-acylPCs will be required.

A possible mechanism to account for the activity of the enzyme might involve an active site and two substrate-binding sites, Site I for diacylphospholipids and Site II for lysophospholipids. The following steps might occur sequentially in the hydrolysis of PC: (1) Binding of PC to Site I; (2) Transfer of the acyl group at sn-2 to the catalytic site and simultaneous binding of the resultant lysoPC to Site II; (3) Transfer of sn-2 acyl group to H\(_2\)O; (4) Transfer of sn-1 acyl group to the catalytic site; (5) Transfer of sn-1 acyl group to H\(_2\)O. Hydrolysis of lysoPC

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**Fig. 3.** Structure of the sugar moiety of the PLB.23)

**Fig. 4.** Schematic representation of the protein moiety of the PLB.24) S-S and CHO indicate the disulfide linkage and possible glycosylation sites, respectively. R-89, S-125 and D-407 are the catalytic triads.
would involve direct binding to Site II, followed by steps 3–5 above.26)

3-3. Effects of several compounds on the activity. Many metals and reagents were tested.18) Briefly Fe$^{+2}$ and Fe$^{+3}$ showed marked inhibition on PLB activity but not on LPL activity. K$_3$Fe(CN)$_6$ activated only the LPL activity. DFP, phenylglyoxal and N-bromosuccinimide inhibited both activities. Among detergents tested Triton X-100, sodium deoxycholate and sodium taurocholate stimulated PLB activity but inhibited LPL activity. Sodium cholate, sodium dodecylphosphate, Tween 80 inhibited both activities.

Generally, in such a heterogeneous system as diacylphospholipids, some activators or treatments, e.g. diethylether, Triton X-100, or ultrasonication, are necessary to initiate the enzymatic reaction. In a homogeneous system of lysophospholipids, however, they are rather inhibitory.

3-4. Substrate specificity. 3-4-1. A homologous series of PC and its lysoPC from C$_8$ to C$_{18}$. In general it was observed as shown in Table 2 that the shorter the chain length the greater the hydrolysis rate of PC. Diethylether stimulated hydrolytic rate of long chain PC (C$_{12}$ to C$_{18}$) but inhibited the shorter chain PC (C$_8$ to C$_{10}$). The long chain substrates would be in the solid crystalline state, but would be converted to some extent into the liquid crystalline state by diethyl ether.

Table 2. Hydrolysis of an homologous series of PCs and 1-Acyl LPCs.

| Chain length | Specific activity (nmoles/min/ug protein) |
|--------------|------------------------------------------|
|              | PC                                       | LPC                                      |
|              | -ether | +ether | -ether | +ether |
| C$_8$        | 103    | 58     | -      | -      |
| C$_{10}$     | 59     | 44     | 5550   | 6620   |
| C$_{12}$     | 28     | 36     | 6550   | 5950   |
| C$_{14}$     | 15     | 25     | 6100   | 4810   |
| C$_{16}$     | 8.4    | 14     | 4300   | 4190   |
| C$_{18}$     | 5.3    | 11     | 2300   | 3510   |
| C$_{18}$ (oleoyl) | 6.3 | 14 | 5050 | 4140 |

The enzyme used was Fr. SBE (starch block electrophoresis).

Fig. 5. Kinetics of hydrolysis of [32P,14C]PC (A) and [32P,14C] PE (B) by purified P. notatum PLB.26)
state in the presence of ether, thereby resulting in increasing hydrolysis rate. The shorter PCs and lysoPCs are readily soluble in buffer and the enzyme would have high affinity for the substrates in the liquid crystalline state. The hydrolysis rates for the lysoPCs were much higher than for the corresponding PCs.26)

3-4-2. Hydrolysis of various dipalmitoylphospholipids. The order of hydrolysis in the presence of diethyl ether: PS > PI > PA > PC > PE > cardiolipin. It was observed that the optical clarity of the substrates in the presence of ether followed closely the order of the hydrolysis rates. The PS dispersion is almost water-clear: when ether was added drop by drop to the PS suspension in a stoppered test tube, the suspension suddenly became clear at some point but others did not even when the amount of ether was changed. The suitable amount of ether depended on the nature and quantity of the substrates.26)

3-4-3. 1-0-Alk-1
\[\text{B}\]
B-enyl-2-[1-14C]palmitoyl (or oleoyl) and 1-0-alkyl-2-[1-14C]palmitoyl (or oleoyl)-sn-GPCs. These substrates were hydrolyzed with concomitant release of [1-14C] palmitic (or oleic) acid and 1-0-alk-1
\[\text{B}\]
B-enyl- and 1-0-alkyl-sn-glycero-3-phosphocholine. Triton X-100 stimulated the reaction greatly. The rate of hydrolysis of the alkanyl form was faster than that of the alkyl form. 1-0-Alkyl-sn-glycero-3-phosphocholine is a lyso-form of platelet activating factor (lyso-PAF).27)

The substrates were prepared as follows. Briefly, the choline glycerophospholipid fraction obtained from bovine heart muscle was hydrolyzed with 0.25 M methanolic KOH and the resulted lysopalmitalogen was purified and used for (1) acylation for 1-alk-1
\[\text{B}\]
B-enyl-GPC, (2) hydrogenation and acylation for 1-alkyl-2-acyl GPC, and (3) after removal of phosphocholine with PLC the conversion to ditrimethylsilyl (diTMS) derivative for analysis of the molecular species.28)–30) The molecular species of 1-0-alk-1
\[\text{B}\]
B-enylglycerol consisted of C18 = 0 (1.5%), C19 = 0 (88.8%), C20 = 0 (3.6%), C21 = 0 (1.1%), and C22 = 0 (6.2%).

Platelet activating Factor (PAF) was hydrolyzed (personal communication, Professor Junko Sugitani, University of Shizuoka). She used [3H]acetyl-PAF as the substrate and purified P. notatum PLC, which consisted of about 70% native and 30% modified forms, and the enzyme activity was 1.52 nmol/min/mg protein.

3-4-4. 2,3-Dipalmitoyl-sn-1-GPC. It was hydrolyzed and produced fatty acid and lysoPC, but no water-soluble GPC.31) The point was whether the lysoPC was 2-palmitoyl GPC or 1-isomer. The lysoPC was hydrolyzed with B. cereus PLC and the resulted monopalmitoylglycerol was converted to diTMS derivative, which was analyzed by GC-MS. One peak appeared and the mass spectrum is in Fig. 6(A) where the main fragment ions are m/z 459 [M-15]⁺, m/z 218 [CH₂OSi(CH₃)₂CH=CHOSi(CH₃)₂]⁺ and m/z 129 [CH₂OSi(CH₃)₂CH=CH]⁺. On the other hand, the spectrum of the authentic 1-isomer (Fig. 6(B)) shows m/z 459 and m/z 371 [CH₂OOC(CH₃)₂H₁₁=CHOSi(CH₃)₂]⁺. The diTMS derivative analyzed was identical with 1,3-diTMS-2-palmitoyl glycerol and the original lysoPC was found to be 2-palmitoyl-sn-glycero-3-phosphocholine. It was concluded therefore, that PLB could not attack the sn-2 ester linkage of unnatural enantiomeric PC but attacked the sn-3 ester linkage non-stereospecifically. Thus, PLA₁ activity of PLB can be determined by using this compound.

2,3-Dipalmitoyl-sn-1-GPC was prepared as follows: rac 1,2-dipalmitoyl-sn-3-GPC was hydrolyzed with Crotalus adamanteus PLA₂ and resulting 2,3-dipalmitoyl-sn-1-GPC was isolated by silicic acid chromatography. It showed an optical activity value, [α]₂, of −7.0 ± 0.2 in chloroform, whereas 1,2-isomer exhibited an optical activity, [α]₂, of +6.8 ± 0.2.

3-4-5. 1-[1-14C]polytolyl- and 2-[1-14C]palmitoyl-sn-glycero-3-phosphocholines. The former was hydrolyzed about 15 times faster than the latter.27),32)
3-4-6. Substrates for lipases and other acylesterase.
Among the various acylglycerols tested, only monooleoyl- and monopalmitoyl-glycerols were hydrolyzed at a low rate. Cholesterol oleate and p-nitrophenyl acetate were not hydrolyzed.18) 

3-5. Partially deglycosylated PLB.
The PLB is a glycoprotein and a decrease in sugar content would be favorable for the PLB activity. This prediction was confirmed by the following experiment. The PLB, both the native and modified forms, were incubated with endoglycosidase H for 180 min. The recovery rate of the enzyme was about 70%, in both forms and those of the sugar were 70% and 50% in the native and modified forms, respectively and no more decrease occurred. The PLB activity, units/mg protein, of the deglycosylated native form increased from 750 to 1220 and that of modified one from 112 to 386 in the presence of the detergent.33) To identify each activity of PLB, PLA1, PLA2, LPL1, and LPL2 the substrates shown in Table 3 are very useful.

4. Occurrence of phospholipase B
The available literatures are rather limited, because the definition of PLB was not clear. As for the criteria for PLB the following points were postulated. The enzyme should be pure and it has both the PLB activity and LPL activity, and lysophospholipids are not end products. The occurrence of PLB has been found ubiquitously from bacteria to mammals as described below, although some of them were not purified.

4-1. PLB in bacteria. Hayaishi and Kornberg34) reported breakdown of phospholipids by a strain of Serratia polymuthicum which was isolated from soil by the enrichment culture technique. The assay system was based on the rate of choline liberation from purified egg lecithin and lysolecithin under the presence of excess pure GPC diesterase. The result showed that both substrates produced choline, indicating the presence of the PLA and LPL activities, or a PLB, in the cell homogenate.

Nojima and coworkers35),36) reported that the membrane-bound PLA1 of Mycobacterium phlei was purified and they isolated one of the final fraction, P-1α, 45 kDa, by SDS-PAGE. It showed LPL1 and LPL2 activities at about the same rate in addition to the namesake PLA1 activity, or it was a sort of PLB. On the other hand, the detergent-resistant PLA of E. coli K-12, purified near homogeneity, hydrolyzed both sn-1 and sn-2 acyl chains of PE and PC, and also attacked 1-acyl and 2-acyl lysoPE. This enzyme had not only PLA1 and LPL2 activities but also PLA2 and LPL1 activities, or PLB1 and PLB2.

Van den Bosch37) indicated in Table 4 that some of the enzymes which once considered to be PLA1 was found to be really a part of PLB. His example of bovine pancreas PLA1 will be discussed later.

4-2. PLB in Saccharomyces cerevisiae. Witt and coworkers41)–43) reported that there were three forms of PLB in S. cerevisiae H1022, two of which were found in plasma membrane and solubilized by cholate or SB 12 (a zwitterionic detergent) and the other was secreted from the cells. The purified membrane-bound enzymes were glycoproteins and showed two bands on SDS-PAGE, 220 kDa and 145 kDa. The LPL activity was stronger than the PLB activity and the transacylase activity was also detected. The secreted form gave a broad band between 200 and 280 kDa on SDS-PAGE.

Ichimasa and coworkers44) solubilized the membrane fraction of baker’s yeast (Saccharomyces cerevisiae) with sodium deoxycholate (DOC) and purified the PLB. Two glycoprotein bands, one major and one minor, were evident on SDS-PAGE in the absence of 2-mercapethanol. The m.w. was found to be 330 kDa by gel filtration.

Tamai and coworkers45),46) also purified the membrane-bound PLB from Torulaspora delbrueckii (old name, Saccharomyces rosei). The final prepara-
compared to the native one. and more susceptible to proteolysis by V8 protease PLB activity and less stable upon incubation at 50 °C. The carbohydrate-depleted enzyme was higher in and liberated about 75% of the total carbohydrate. PLB was digested with endoglycosidase H for 24 h contained about 50% carbohydrate and the soluble PLB with a m.w. 170 kDa was not hydrolyzed. Later, they isolated a water-soluble PLB with a m.w. 170–200 kDa. These PLBs contained about 50% carbohydrate and the soluble PLB was digested with endoglycosidase H for 24 h and liberated about 75% of the total carbohydrate. The carbohydrate-depleted enzyme was higher in PLB activity and less stable upon incubation at 50 °C and more susceptible to proteolysis by V8 protease compared to the native one.

4-3. PLB in intestinal mucosa. The intestinal mucosa has long been known to contain various phospholipases. Subbaiah and Ganguly\(^7\) reported subcellular distribution and characteristics of different phospholipases in rat intestinal mucosa. It was also reported by Ottolenghi\(^9\) that the phospholipase in small intestine of mouse which infected with the tapeworm *Hymenolepis nana* attacked diacylphospholipids with liberation of free fatty acids and corresponding decrease in lipid-phosphorus without accumulation of lysophospholipids. Pind and Kuksis\(^{47-50}\) reported that the purified brush-border membrane of rat jejunum contained Ca\(^{2+}\)-independent PLA\(_2\) and LPL activities. A cDNA encoding the PLB/Lipase was also distributed in oesophagus and testis. A cDNA encoding the PLB/Lipase was cloned from an ileac mucosa cDNA library using a probe amplified by the polymerase chain reaction and a hydrophobic domain near the C-terminus. All the enzyme activities were located in the second repeat. The catalytic triad (Fig. 7) is Ser-404, Asp-656 and His-659. The Ser-404 is not present in the usual pentapeptide sequence G-X-S-X-G but in G-D-S-L sequence.

Table 4. Purified phospholipase A\(_1\) and phospholipase B\(^{37}\)

| Source | Main activity | m.w. (kDa) | Ca\(^{2+}\) effect | Reference |
|--------|---------------|------------|---------------------|-----------|
| *E. coli B* | PLA\(_1\)+LPLase | 29 | + | Scandella & Kornberg (38) |
| *E. coli K-12* | PLA\(_1\)+PLA\(_2\)+LPL | 28 | + | Nishijima et al. (36) |
| *M. phlei* | PLA\(_1\)+LPL | 45 | - | Nishijima et al. (35) |
| *B. megaterium* | PLA\(_1\) | 26 | - | Raybin et al. (39) |
| *P. notatum* | PLA\(_2\)+LPL | 116* | - | Kawasaki & Saito (17) |
| Brain | PLA\(_1\) | 75 | - | Woelk et al. (40) |
| Pancreas | PLA\(_1\)+LPL | 60 | - | Van den Bosch et al. (56) |

*91.3 is the value determined mass spectrometrically as seen in this text. The abbreviations are unified in this review.*

Chap and coworkers\(^{51,52}\) purified the enzyme 1800-fold to about 90% purity from papain-solubilized guinea pig intestine. Upon SDS-PAGE the main band with an apparent m.w. of 97 kDa was detected, where both PLA and LPL activities were observed. The PLB activity was Ca\(^{2+}\)-independent and insensitive for bromophenacylbromide. As for the substrate specificity, 1,2-dipalmitoylGPC and its enantiomeric isomer were hydrolyzed at the same rate, and 1-palmitoylGPC, triacyl-, diacyly- and monoacylglycerols were also hydrolyzed, whereas cholesteryl olate was not. The *P. notatum* PLB did not attack the enantiomeric dipalmitoylPC but the reason for the difference is not clear.

Tojo and coworkers\(^{53-55}\) solubilized a brush border membrane-associated PLB/Lipase with 1% Triton X-100 from rat small intestine and autolyzed at -35 °C for more than one month. The enzyme showed one band, 35 kDa on the non-reducing SDS-PAGE but the enzyme was separated into a 14 kDa peptide and a 21 kDa glycosylated peptide under reduction with 2-mercaptoethanol. It showed PLA\(_2\), LPL, lipase and esterase activities and favored hydrolysis at the sn-2 position of diacylphospholipids and diacylglycerols. It was Ca\(^{2+}\)-independent and DFP sensitive. PLB/Lipase was also distributed in oesophagus and testis. A cDNA encoding the PLB/Lipase was cloned from an ileac mucosa cDNA library using a probe amplified by the polymerase chain reaction based on the sequence of the purified enzyme. The PLB/lipase consisted of an N-terminal signal peptide, four tandem repeats of about 350 amino acids each and a hydrophobic domain near the C-terminus. All the enzyme activities were located in the second repeat.
4-4. PLB in bovine pancreas. In 1974, Vanden Bosch and coworkers\(^\text{56}\) purified PLA\(_1\) from bovine pancreas to near homogeneity, m.w. 60 kDa. The pancreas contains many lipolytic activities and in the assay system 1\-[9,10-^3\text{H}_2\]-palmitoyl-2\-[1^4\text{C}] linoleoyl-sn-glycero-3-phosphoethanolamine was used in the presence of deoxycholate. However, during purification of this activity it was noticed that the responsible protein behaved on several chromatographic columns exactly as a LPL which had previously purified from the same source.\(^\text{57}\) It strongly suggested that the amount of deoxycholate that they had used to obtain optimal PLA\(_1\) activity towards the diacylphospholipids was sufficient to inhibit completely the LPL activity of the protein. Therefore, at intermediate concentrations of deoxycholate, high enough to allow degradation of the diacylphospholipids and low enough to prevent complete inhibition of LPL activity, the protein catalyzed the deacylation of PC and lysoPC. Under these conditions this single protein exhibited PLB activity, i.e. catalyzed the complete deacylation of diacylphospholipids. They discussed that the classical example of such enzymes was that of \(P.\ notatum\)^\(\text{17}\) and the question whether this activity was an unresolved mixture of PLA and LPL activities was solved. Their pancreas PLB, however, showed some differences from that of \(P.\ notatum\) e.g. it had esterase activity for \(p\)-nitrophenylacetate and optimal pH value was 7.0.

4-5. PLB in potato tubers. It had been found that the homogenization of potato tuber tissues resulted in a rapid enzymatic breakdown of endogenous phospholipids and galactolipids to form free fatty acid and fatty acid hydroperoxides. In 1971 Galliard\(^\text{58}\) reported that the partially purified enzyme preparation catalyzed deacylation of diacyl- and monoacyl-phospholipids, galactosyl-diglycerides, monoacyl- and diacyl-glycerols, and methyl- and \(p\)-nitrophenyl-esters of long chain fatty acids but did

| Enzyme               | Sequence                        |
|----------------------|---------------------------------|
| \(P.\ notatum\)      | 115-\text{GLLQSATYISGLSGSWLLGSIYI-139} |
| cPLA\(_2\)           | 215-\text{SGILDCATYVAGLSGTWYMSTLYS-239} |
| \(Aspergillus\)      | 154-\text{GLLQSATYLAGLSSGWLVGSTYI-178} |
| \(Saccharomyces\)    | 134-\text{LGLLQGATYLAGLSSGWNLTSTLAWN-159} |
| PLB/Lipase           | 399-\text{GAMGDSLTAGN-409} |

| Enzyme               | Sequence                        |
|----------------------|---------------------------------|
| \(P.\ notatum\)      | 399-\text{HVDIFAVDSSADTDYF-415} |
| cPLA\(_2\)           | 541-\text{KSKIKHVDSGLTFNLPY-558} |
| \(Aspergillus\)      | 433-\text{HVDIFAVDSSADTTYS-454} |
| \(Saccharomyces\)    | 421-\text{REDVFALDNADTDYD-428} |
| PLB/Lipase           | 653-\text{FAPDCFHFNVK-663} |

| Enzyme               | Sequence                        |
|----------------------|---------------------------------|
| \(P.\ notatum\)      | 83-\text{VSGGGWRALMNGAGAV-98} |
| cPLA\(_2\)           | 194-\text{GSGGFRAMFGDSVM-209} |
| \(Aspergillus\)      | 122-\text{VSGGGYRALMNGAGAI-137} |
| \(Saccharomyces\)    | 103-\text{CSGGGVRAMLSGAGML-119} |

![Fig. 7. Comparison of the catalytic sites of PLBs.](image-url)
not attack triacylglycerols, wax esters and sterol esters. It also catalyzed acyltransferase reaction in the presence of alcohol and the synthesis of wax esters from long chain alcohols and fatty acids. DFP was inhibitory.

From the articles mentioned above the PLB exists evidently. Different from PLA2, the activities and substrate specificities of PLB are not so strict and sometimes it shows lipase, esterase, and/or transacylase activities at different degree of activity. Furthermore, the apparent specificity is influenced by such substances as Triton X-100 for diacyl- and monoacyl-phospholipids, deoxycholate for PLA1 activity, the effects of diethyl ether and the endogenous protease for PLB activity. Therefore, PLB would be adjustable to the environmental circumstances and might be possible to play wider roles than PLA2 in vivo.

5. Sequence similarity and catalytic sites

Two decades have passed since my retirement. In 2013, I searched the NCBI database and found high sequence similarities among P. notatum PLB and fungal PLB, cPLA2, and patatin. A fairly large number of unnamed proteins also showed high similarity. The identity between P. notatum PLB and that of Aspergyllus oryzae R140 was 69%. The Aspergyllus oryzae was used as an enzyme source by Contardi and Ercoli in 1933. The PLB encoded by Saccharomyces cerevisiae gene showed 45% similarity.59)

As to the cPLA2, many important papers are available including those of Clark et al.60) Lin et al.61) and Uozumi et al.62) but in this review the subjects were limited mainly to phospholipase activity. The cPLA2 was first characterized in RAW 264.7 mouse macrophage cells by Leslie et al. in 198863) and in 1991 it was found from human monoblast U937 cells by Kramer et al.64) The 85-kDa cPLA2 (cPLA2α) consists of 749 amino acid residues and the essential amino acid residues for catalytic function are Ser-228, Asp-549 and Arg-200, which are quite different from the classical Ser-His-Asp mechanism of well-known secretory PLA2.65)–68) It has PLA2 and LPL activities, and PLA1, transacylase, lipase and acylesterase activities are also found, all of which are inhibited by DFP. Ca2+ is not essential for the activity but stimulates at µM level. The receptor-mediated cPLA2 leads to preferential release of sn-2 arachidonic acid of membrane phospholipids, thus initiating biosynthesis of the potenti inflammatory mediators, prostaglandins, leukotrienes and lysoPAF.

The amino acid alignments of catalytic sites of the related PLBs is shown in Fig. 7. As for the catalytic serine, aspartic acid and arginine, cPLA2, P. notatum, Aspergyllus oryzae, and Saccharomyces cerevisiae show high sequence similarity. A four-amino-acid motif (G*—T*—L*—Y*) is also found.

Patatin which originally means potato69) is a family of potato tuber proteins and serves not only as a storage protein but also exhibits many enzymatic activities which seem to be complicated.70) Galliard’s enzyme mentioned above,58) the cPLA2 reported by Senda et al.71) and a novel phospholipase A reported by Hirschberg et al.72) belong to patatin but the relationship between they and PLB is unclear.

As is obvious from the above findings the phospholipases of cPLA2, Aspergyllus oryzae, and Saccharomyces cerevisiae are the same as P. notatum PLB, but PLB/lipase is different.

6. Conclusion and prospect

Now, it is clear that a PLB exists from bacteria to mammals. Different from the PLA2, it catalyzes deacylation of both sn-1 and sn-2 ester linkages of diacylphospholipids. Mechanistically, as shown in Fig. 8, which follows McMurray and Magee, three ways are thought successively, 1) from sn-2 to sn-1 ester bond, 2) from sn-1 to sn-2, and 3) simultaneously, both sn-1 and sn-2 bonds. They could be named PLB2, PLB1 and PLB3, respectively. PLB3 has not yet been used but it is a better nomenclature than PLB1,2 which was previously reported.16) Therefore, PLB1 and PLB2 have intrinsically PLA1, PLA2
and LPL activities. As typical examples, PLBs of *Mycobacterium phlei* and bovine pancreas belong to PLB1, and those of *P. notatum* and cPLA2 to PLB2. Here, cPLA2 is classified as a PLB2 because it has a LPL activity and same catalytic sites as *P. notatum* PLB. Although once disused or disappeared, the PLB is classified as a member of the deacylases of phospholipids in addition to PLA and LPL.

The biochemical roles of a PLB could be, 1) complete deacylation of phospholipids, which means even though temporarily a disappearance of the glycerophospholipids, and then the remodeling of their fatty acids, 2) the fate of sn-2 fatty acids liberated is well known but that of sn-1 fatty acids is not clear, and 3) it does not produce lysophospholipids, which are thought to be cytotoxic in general, although lysophosphatidic acid is an autacoid and lysoPAF is a precursor of PAF. As seen above, *Penicillium notatum* produces a powerful PLB but its biological or biochemical meaning is unknown.

Some of the characteristics of a PLB are: a relatively large molecular mass, the catalytic triad, Ser-Asp-Arg, Ca$^{2+}$-independent, DFP-sensitive, and in addition to the namesake PLB, LPL, transacylase, lipase and acylesterase activities. The activity or the apparent substrate specificity is variable with various substances, and so in other words, a PLB could be amenable to the circumstances.

Now, our knowledge of the PLB including cPLA2 has shown big progress in biochemistry, molecular biology as well as in pathophysiology with the use of such modern techniques as transgenic and knockout mice, and I hope they will contribute to progress of the biological and medical sciences in the future.

**Acknowledgements**

I sincerely thank all of my coworkers over many years for their efforts. They are chronologically Ms. K. Sato-Mukoyama, Drs. N. Kawasaki, J. Sugatani, K. Satouchi, T. Okumura, M. Kates, Y. Takeuchi, K. Ikeda, K. Hamaguchi, N. Kitamura, N. Masuda, S. Fujii, S. Umezaki and R. Miura. In preparing this review I greatly appreciate the valuable advices by Drs. A. Tokumura, K. Miyazawa, H. Tojo, N. Kawasaki and K. Satouchi. We were indebted to Research Laboratories of Toyo Brewing Co., for culturing *P. notatum* and to the Ministry of Education, Science and Culture of Japan for financial support. Finally, I would like to thank Dr. Tamio Yamakawa, M.J.A., for his kind offer to submit this review.

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(Received July 31, 2014; accepted Sep. 2, 2014)

Profile

Kunihiko Saito was born in Tanba-City, Hyogo-Prefecture, Japan in 1926 and was graduated from Kobe Medical College in 1951. In 1952 he started his research carrier in the Department of Medical Chemistry, Graduate School of Medicine, Kyoto University. The chairman was Prof. S. Utzino. Then he became an assistant in the Department of Biochemistry, Nagoya City University under Prof. S. Akashi and studied bacterial fatty acids from 1953 to 1958, including the structures of subtilopentadecanoic and subtiloheptadecanoic acids. In 1959 he was a Research Associate in Prof. D. J. Hanshan’s laboratory, Department of Biochemistry, School of Medicine, University of Washington, Seattle, U.S.A. and studied phospholipase A of snake venom until 1961. He became Associate Professor, Department of Medical Chemistry, Kansai Medical University in 1961, was promoted to Professor in 1969 and retired in 1993. His main research projects were biochemical studies of odd-numbered fatty acids, phospholipids and PAF. Methodologically, the mass spectrometry was used preferentially. Phospholipases particularly the phospholipase B in Penicillium notatum were also the objects of his research interest. In 1972 he was invited by Prof. M. Kates as Visiting Professor, Department of Biochemistry, University of Ottawa. He was invited to the First FASEB Symposium on Phospholipases organized by M. Waite and held in Vermont, U.A. in 1988, and in 1989 to the Second International Symposium on Mass Spectrometry in the Health and Life Sciences, San Francisco, U.S.A. organized by A.L. Burlingame and J.A. McCloskey. He organized a satellite symposium of the 3rd International Conference on PAF, in Nara, Japan in 1989 and edited its proceedings, Platelet-Activating Factor and Diseases, together with D.J. Hanahan. Since 1992 he has been a Consulting Professor at the Harbin Medical University, Harbin, China. After retirement he has been enjoying a life of a farmer, a gardener and an oil painter.