REVIEW

Phospholipases: Occurrence and Production in Microorganisms, Assay for High-Throughput Screening, and Gene Discovery from Natural and Man-Made Diversity

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ABSTRACT: Various kinds of phospholipids have wide industrial applications such as in food and nutraceuticals, cosmetics, agricultural products, and pharmaceuticals. The demand for reliable biocatalysts for the production of phospholipid products, such as phospholipases A_1, A_2, C, and D, has steadily increased over the past several decades. A large number of microbial phospholipases have been isolated and characterized, and the increasing availability of these enzymes could eventually lead to the sustained development of phospholipid-related biotechnology. Although a number of reactions have been performed using phospholipases, a reliable and efficient supply of superior phospholipases in quantity is still a challenge for their practical application. High-throughput functional assay methods for phospholipases should be devised to develop superior new species from the huge diversity of phospholipases. Recent biotechnological advances in the discovery of new phospholipase genes from natural sources, such as extremophiles and metagenome libraries, and in the functional enhancement of phospholipases by protein engineering, such as directed evolution, can provide valuable means of rapidly developing practical uses of phospholipases for various applications.

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Phospholipids are naturally occurring lipids found in all organisms; their mixtures are particularly abundant in most vegetable oils, marine oils, animal fats, and other biomasses. The major resources for the industrial production of phospholipids are vegetable oils, such as soybean oil, and egg yolks. Various types of phospholipids are separated from these natural resources to be used as products and/or as starting raw materials for the production of other modified phospholipids (1–5). The properties of natural phospholipids can be differentiated from each other by their hydrophobic groups and polar head groups; a great variety of phospholipid structures are found in nature, exhibiting enormous diversity in the structures of both their polar and nonpolar moieties. Figure 1 shows a typical glycerophospholipid structure. “Phospholipid” refers to any lipid-containing phosphoric acid as a mono- or diester, and “glycerophospholipid” signifies any derivative of glycerophosphoric acid that contains at least one *O*-acyl group attached to the glycerol residue (6,7). Ether phospholipids contain either an *O*-alkyl or an *O*-(1-alkenyl) group found in, for example, plasmalogen (1-*O*-alk-1′-enyl-2-acyl glycerophospholipids) (8). In addition, some other main classes exist, such as sphingosine-containing phospholipids. Sphingosine-containing phospholipids are classified into two major classes: phosphocholine derivatives of ceramides, commonly known as sphingomyelin, and glycosylated derivatives of inositol phosphoceramides, commonly known as phytoglycolipids (9).

A number of review articles have been written on the natural occurrence, metabolism, and various functions of these phospholipids as the result of extensive research (10–14). Effective physiological functions of various phospholipids originate primarily from the role of the fundamental components of the biomembranes: the maintenance of cell shape, the support of membrane-associated proteins, and the involvement in transmembrane signaling as activators and substrates for a number of enzymes, especially for phospholipases. In addition to their physiological roles, phospholipids have many favorable functions for their application as ingredients in many lipid-related products in industrial fields. First, their zwitterionic property, with hydrophilic and lipophilic moieties in the individual molecules, allows the phospholipids to be used mainly as emulsifiers. Lysophospholipids, for example, are excellent emulsifiers and are particularly suitable for use in many industrial applications, such as food technology and the cosmetics and pharmaceutical industries. Second, the physiologically active phospholipids and their derivatives are useful as functional ingredients in foods, drugs, cosmetics, and agrochemicals. Third, liposomes composed of phospholipids such as PC and PE can be used as carriers for drugs and other physiologically active materials (13). In addition, phospholipids can be exploited to modulate biocompatibility in the manufacture of industrial coating materials and biomedical products that come into contact with biological fluids and tissues (14).

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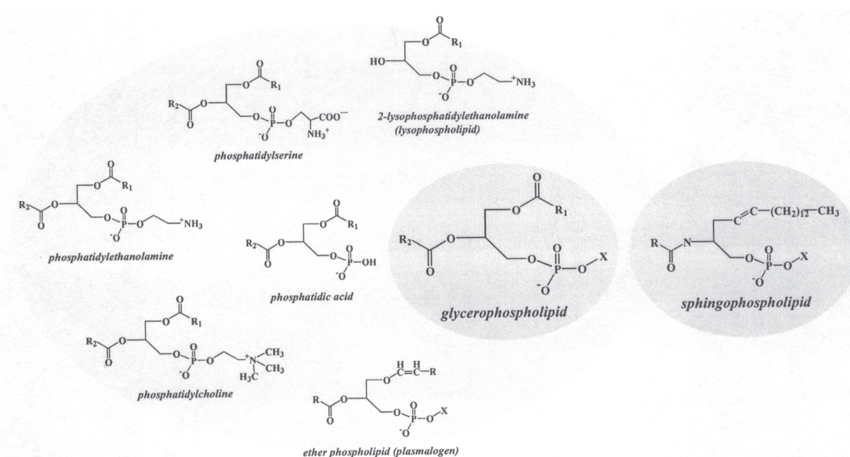

FIG. 1. Schematic representation of phospholipids such as the glycerophospholipid and the sphingophospholipid. R, R_1, and R_2, hydrocarbon tails of FA; X, a polar alcohol moiety such as choline, serine, ethanolamine, myo-inositol, glycerol, or phosphatidylglycerol.

The need for phospholipids with potential uses in a variety of industrial applications has significantly expanded the field of chemical and biocatalytic syntheses of phospholipids in recent years. Although chemical methods are well established and have been successfully employed for a long time, enzymatic transformation approaches, as complements to chemical reactions, appear to be quite promising. Phospholipases hydrolyze phospholipids, which selectively recognize each of the four individual carboxylic or phosphate ester bonds of glycerophospholipids. Based on their site of action, phospholipases can be categorized into four major classes. Acyl group-modifying phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), and lysophospholipase attack the carboxylic ester bonds of phospholipids, thus removing and replacing the acyl chain in various phospholipids *via* the reaction mechanisms of hydrolysis, esterification, and transesterification. Polar head group-modifying phospholipases C (PLC) and D (PLD), which are also known as phosphodiesterases, recognize the two sides of the phosphodiester linkage, respectively.

Some reviews have been written on phospholipases as biocatalysts that mainly give details of phospholipase-catalyzed reactions for the production of phospholipids with industrial relevance and/or the details of common biochemical properties of phospholipases for use as biocatalysts (15–18). For example, a fine review article recently published by Guo and coworkers (17) gave an overview of phospholipids and phospholipases, including the natural occurrence and characteristics of phospholipids, the molecular structural characteristics and catalytic properties of phospholipases, and special details regarding phospholipase-catalyzed phospholipid modification. In this review, we highlight (i) the occurrence and biotechnological relevance of four classes of mainly microbial phospholipases, including gene cloning and microbial production (see Table 1); (ii) the assay and screening methods essential for developing relevant high-throughput technologies; and (iii) the recent biotechnological approaches for obtaining superior new phospholipases through natural and man-made diversity.

PLA_1

PLA_1 (EC 3.1.1.32) and PLA_2 (EC 3.1.1.4) have a specific capacity to hydrolyze the carboxylic ester bond at the *sn*-1 and *sn*-2 positions of phospholipids, respectively. Lysophospholipase prefers an ester bond in partially hydrolyzed phospholipids (lysophospholipids), whereas an acyl-hydrolyzing phospholipase that does not discriminate between the two acyl ester bonds is generally designated as phospholipase B. The enzymes acting in the *sn*-1 and/or *sn*-2 position of glycerophospholipids constitute a very diverse subgroup and have been

TABLE 1
Examples of Sources of Biotechnologically Relevant Phospholipases[a]

Phospholipase	Source	References
PLA_1	*Serratia* sp. MK1	27–28
	S. liquefaciens	29
	Tetrahymena thermophila	34–35
	Aspergillus oryzae	36–37
	A. fumigatus	38
PLA_2	Animal pancreas (e.g., porcine)	46
	Venom (e.g., bee, snake)	40–41
	Starfish	52
	Streptomyces violaceoruber	53–54
	Streptococcus sp.	55
PLA_1/PLA_2	*Fusarium oxysporum*	48
	Aspergillus sp.	49
	Hyphozyma sp.	50
PLC	*Bacillus cereus*	63,67,72–73
	B. thuringiensis	64
	Streptomyces antibioticus	65–66
	Pseudomonas fluorescens	68–69
	Clostridium perfringens	70–71
PLD	*Streptomyces* sp. PMF	84–85
	S. antibioticus	65,89–90
	S. chromofuscus	95
	S. septatus	93,96
	S. halstedii	94

[a] PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D.

found in a large number of different organisms. These kinds of enzymes, including PLA_1 and PLA_2, can remove and replace the acyl chains in various phospholipids *via* hydrolysis, esterification, and transesterification.

The modification of phospholipids at the *sn*-1 position also has been carried out by 1,3-specific lipases (19), which can be less specific for the phosphate group-containing lipids than PLA_1 (20). The use of commercial 1,3-specific lipases to incorporate FA into the *sn*-1 position of glycerophospholipids have been reviewed (21,22). Some reports have been written on the use of PLA_1 as the biocatalyst for the modification of phospholipids. For example, ricinoleic acid was incorporated into the *sn*-1 position of egg and soybean lecithin using immobilized PLA_1 (23). The immobilized PLA_1 showed an exclusive regioselectivity toward the *sn*-1 position, which was absent from known complicated chemical methods (24). The enzymatic production of lysophospholipids from phospholipids was performed using PLA_1 from *Aspergillus niger* and others (25,26). PLA_1 is not yet widely available, providing motivation in the search for various superior PLA_1 sources for the industrial modification of phospholipids at the *sn*-1 position. An available stable PLA_1 would overcome the low yields and conversion rates obtained with lipases.

A number of enzymes with PLA_1 activity have been found in mammals, venoms, and microorganisms. PLA_1 from *Serratia* sp. MK1, *Aspergillus oryzae*, and *Tetrahymena thermophila* have been relatively well studied in industrial applications. PLA_1 was identified from the culture supernatant of *Serratia* sp. MK1 (27,28) and *S. liquefaciens* (29). *Serratia* sp. MK1, in particular, expressed a significant amount of PLA_1 in the extracellular medium. The preparation of PLA_1 from the optimized cultivation of *Serratia* sp. MK1 was then applied to the modification of phospholipids. When a 20% (w/w) phospholipid substrate was subjected to hydrolysis in a two-phase system, PLA_1 completely converted the phospholipids to lysophospholipids within 1 h (26). Choline-containing plasmalogen and ethanolamine-containing plasmalogen were also concentrated to 95% purity from an initial value of 45%, and to 99% purity from an initial value of 64%, respectively (30). To increase the potential utility of PLA_1, the authors cloned the gene encoding the secretory PLA_1 (31) and further engineered the enzyme genetically (32,33).

PLA_1 from the ciliate *T. thermophila* was secreted into the extracellular medium. *Tetrahymena* spp. generally secreted a number of lysosomal enzymes, including PLA_2, PLC, PLD, and lipase. PLA_1-hypersecretory mutants of *T. thermophila* were isolated using random chemical mutagenesis (34), and a practical purification method was designed for large-scale production of the PLA_1 enzyme (35). The purification process, based on the selective interaction of *T. thermophila* PLA_1 with its liposomal substrate, was similar to affinity chromatography but less expensive, as no substrate immobilization was required.

The genomic DNA and cDNA encoding PLA_1 from *A. oryzae* were cloned and expressed in *Saccharomyces cerevisiae* (36). The secretion of PLA_1 was increased in *S. cerevisiae* by the optimization of fermentation parameters, and in *A. oryzae* by an increase in gene dosage and the improvement of mycelial morphology (37). PLA_1 from *A. oryzae* and other *Aspergillus* spp. (including *A. fumigatus*) (38) are expected to contribute significantly to the phospholipid-related industry if a higher level of the secretory PLA_1 production can be accomplished using a recombinant *Aspergillus* host.

PLA_2

PLA_2 functions in the signal transduction of eukaryotic cells (39), the toxicity of venomous organisms (40,41), the virulence factor in bacterial infections (42), and biocatalytic applications for the modification of phospholipids (43) have been studied extensively. Most PLA_2 are systematically classified into specific groups of the PLA_2 superfamily. According to Six and Dennis (44), PLA_2 whose amino acid sequences are completely known can be classified into 11 groups (I–XI) by readily identifiable sequence homology and enzymatic characteristics. They are grouped into two large categories by their utilization of a catalytic histidine or serine. All secretory PLA_2 previously reviewed by Murakami and Kudo (45) had a catalytic histidine, the binding site of crucial Ca^{2+}, and conserved the disulfide bonds. These small secretory PLA_2 were discovered mainly in venoms and in the pancreatic juices of various animals: PLA_2 from porcine pancreas (46) and bee venom (41) are the most frequently used biocatalysts for the modification of phospholipids.

The pancreatic PLA_2 preparation Lecitase® 10L from Novozymes A/S (Bagsvaerd, Denmark) has been used in a more environmentally friendly degumming process for refining vegetable oils. The enzymatic degumming process known as Lurgi's EnzyMax process was recently introduced to the market and performed with a PLA_2 that could convert the nonhydratable phospholipids into hydratable phospholipids in a crude oil–enzyme solution (47). Because of the higher price and limited supply of PLA_2 extracted from animal pancreas, new microbial PLA_1 or PLA_2 enzymes with high levels of activity and stability are likely to be more applicable to the enzymatic degumming process (48). Therefore, a screening program for microbial PLA_1 or PLA_2 mainly for oil degumming has been carried out by many research groups, including commercial enzyme suppliers. The screening criteria were generally to develop stable and robust PLA_1 or PLA_2 with optimal degumming performance in the range of pH 4–5 and at 30–70°C. Phospholipases of type A_1 or A_2 from *Fusarium oxysporum* (49), the genus *Aspergillus* (50), and the genus *Hyphozyma* (51) have been isolated and proven to be superior to other phospholipases for oil degumming and phospholipid modification. These PLA were generally secreted into the extracellular space from their native host organisms, making them more suitable for commercial production.

In addition to pancreatic and fungal PLA_2, the number of reports on PLA_2 development for biotechnological applications has increased. The cDNA encoding starfish PLA_2 was cloned and expressed in *Escherichia coli* (52). Starfish PLA_2 hy-

drolyzed PC more effectively than PE, similar to venom PLA_2, whereas pancreatic PLA_2 hydrolyzed PC and PE almost equally. The specific activity of starfish PLA_2 was remarkably higher than that of commercially available PLA_2 from porcine pancreas, although the other enzymatic properties (e.g., Ca^{2+} requirement, optimal pH and temperature, and FA specificity) were similar. The *Streptomyces violaceoruber* PLA_2, the first PLA_2 discovered in prokaryotes, was cloned and overproduced using a *Streptomyces* host-vector system (53). The crystal structure of this bacterial PLA_2 has been determined and discussed at the molecular level (54). PLA_2 from *S. violaceoruber* is a monomeric enzyme of about the same size (12–15 kDa) as secretory PLA_2 from eukaryotic sources, and it hydrolyzed PC more effectively than PE. A prophage-encoded *Streptococcus slaA* gene was identified by genome sequencing; the SlaA had a region of conserved amino acid residues found in several secretory PLA_2. The *Streptococcal*-secreted enzyme was also confirmed as a PLA_2 from an analysis of activity against multiple phospholipids (55). These two PLA_2 from *Streptomyces* and *Streptococcus* showed that the extracellular PLA_2 were not produced exclusively by higher eukaryotes.

PLC

PLC catalyzes the hydrolysis of phospholipids to DAG and organic phosphates. PLC has been studied for its biochemistry relevant to the regulation of eukaryotic cell metabolism (56), but the industrial applications of this enzyme have been somewhat limited thus far. Moreover, most of the PLC is available only in small quantities. However, some useful biocatalytic reactions catalyzed by PLC, such as the preparation of various inositol phosphate analogs (57), enantiomerically pure DAG (58), and 1,3-cyclic glycerophosphate (59), have been proposed. PLC has been found in a variety of different bacteria as well as in eukaryotic organisms (60). Some bacterial PLC elicit toxic effects by mimicking the actions of the eukaryotic enzymes of host cells. Many PLC-producing bacteria have been identified as intracellular pathogens, and PLC has been implicated as a virulence factor in such cases (61).

Bacterial PLC can be classified according to two different substrate specificities: (i) PI-specific PLC (PI-PLC) (62), and (ii) PC-preferring PLC (PC-PLC). PI-PLC are secreted by bacteria such as *B. cereus* (63), *Bacillus thuringiensis* (64), and *Streptomyces antibioticus* (65); those of higher organisms are intracellular enzymes. Mammalian PI-PLC play a role in membrane-associated signal transduction, whereas bacterial PI-PLC are considered to be potential virulence factors. The PI cleavage of bacterial PI-PLC constitutes an intramolecular phosphotransferase step that cleaves PI to inositol-1,2-cyclic phosphate and a cyclic phosphodiesterase step that converts inositol-1,2-cyclic phosphate to inositol-1-phosphate (62). Bruzik *et al.* (57) reported the first enzymatic synthesis of inositol phosphodiesters starting from alcohols and inexpensive, readily available soybean phospholipids. The PI-PLC reactions were also successfully applied to the preparation of a number of complex inositol phosphoesters of mono- and oligosaccharides, nucleosides, and peptides. Two PI-PLC from *S. antibioticus* were characterized (65), and their genes ($PI\text{-}PLC_1$ and $PI\text{-}PLC_2$) were cloned (66). $PI\text{-}PLC_2$ was a conventional Ca^{2+}-independent bacteria-type enzyme. However, the $PI\text{-}PLC_1$ enzyme was Ca^{2+}-dependent, as mammalian PI-PLC require Ca^{2+} for catalysis; all bacterial PI-PLC known until recently have been metal independent.

PC-PLC has been detected and cloned from a variety of bacteria, including *B. cereus* (67), *Pseudomonas fluorescens* (68,69), and *Clostridium perfringens* (70,71). They have relatively broad specificity toward the head group of phospholipids. In particular, the extracellular nonspecific PLC-encoding gene from *B. cereus* was expressed as intracellular inclusion bodies in *E. coli* (72). *Bacillus cereus* PLC was also expressed in *Pichia pastoris* to overproduce PLC as an extracellular enzyme for industrial applications (73). Although the level of PLC prepared from the culture supernatant was still relatively low, the further development of an overexpression system for PLC as an extracellular protein would be necessary for the successful application of PLC. As described above, *B. cereus* produces several different PLC, including PC-PLC, PI-PLC, and nonspecific PLC. The PLC from the culture broth of an overproducing strain of *B. cereus* was concentrated and immobilized (58). Chiral 1,2-DAG was obtained with the immobilized PLC in an organic solvent at a controlled water content. The use of immobilized PLC was also expected to enhance the transesterification capacity of PLC by suppressing the otherwise prevalent hydrolysis reaction.

PLD

PLD hydrolyzes the terminal phosphodiester bond of phospholipids to generate PA and a hydrophilic constituent. PLD can also substitute the polar head of phospholipids *via* transphosphatidylation reactions in the presence of a reactive hydroxyl group. The catalytic mechanism and interfacial regulation of the PLD superfamily have been reviewed in detail (18,74). PLD and other superfamily members have a highly conserved HxKxxxxD (HKD) motif that is considered to play an important role in the catalytic function. The catalytic potential of PLD is not restricted to the phosphatidyl backbone of natural glycerophospholipids but allows modifications in a very diverse spectrum of chemical structures (75). In view of its hydrolysis activity, PLD is considered to be a general phosphodiesterase with a very wide substrate specificity (76) and generally requires Ca^{2+} for catalytic activity. There are two different explanations for the Ca^{2+} requirement of bacterial PLD: One is the direct binding of Ca^{2+} to PLD, causing a change that enhances substrate binding, optimizes catalysis, and so on, and the other is the physical effect of Ca^{2+} on PA, relieving product inhibition (77).

PLD-catalyzed transphosphatidylation reactions have been most commonly used in the preparation of modified phospholipids, including the industrial preparation of less-abundant natural phospholipids (e.g., the conversion of PC to PS) (78) and the synthesis of phospholipid conjugates for use in the food,

cosmetics, and pharmaceutical industries (e.g., phosphatidyl nucleotides, phosphatidyl ascorbic acid, phosphatidyl sialic acid, etc.) (15,79). Transphosphatidyl modifications of the polar head group of phospholipids were highly interdependent on the enzyme source, the reaction medium, and the nature of the primary or secondary alcohol (80,81). Transphosphatidylation reactions were usually favored in aqueous or organic two-phase systems, although the reaction could occur in the presence of a bulk water phase (82). The PLD used for these biocatalytic purposes were from plants, particularly cabbage, and microbes, mainly *Streptomyces* species. The PLD from cabbage and *Streptomyces* sp. are commercially available and also can be prepared from cabbage homogenates and extracellular PLD-hyperproducing microbial strains, respectively (83). In addition, many researchers are still trying to isolate PLD-hyperproducing strains and to produce large amounts of PLD in their culture media.

Microbial PLD, including *Streptomyces* PLD, are known to show generally broader substrate specificity and relatively higher transphosphatidylation activity than their eukaryotic counterparts. PLD from *Streptomyces* sp. strain PMF was purified (84) and the crystallographic structure was determined (85). The 54-kDa PLD consisted of a single polypeptide chain that was folded into two domains, and its active site was located at the interface between the two domains. Furthermore, the gene of this strain encoding PLD was cloned in *E. coli* and expressed in the form of a soluble, active PLD (86). The expression system that is suitable for generating variants of the PLD enzyme by mutagenesis and the 3-D structure that helps our understanding of the molecular properties of PLD will greatly accelerate the development of superior PLD for biotechnological purposes.

Although many *Streptomyces* strains that secrete large amounts of PLD into their culture media have been isolated, the productivity of the isolated strains has not been high enough to permit industrial applications. Moreover, overexpression of the proteins in *E. coli* often yields problematic inclusion bodies that require complicated and costly denaturation and refolding processes to recover the biologically active proteins. Extracellular PLD from *Streptomyces* sp. was expressed as an inclusion body in *E. coli* (87). Although the PLD-encoding gene of *S. antibioticus* that fused with the pectate lyase B signal sequence was expressed in the culture supernatant from recombinant *E. coli*, only about 3 mg/L of PLD was obtained under optimal conditions (88). Therefore, *Streptomyces* host strains were used to enhance the secretion of active PLD: The secretory production of active heterologous proteins has been reported using a *Streptomyces* expression system (89,90). The *Streptomyces lividans* expression system was markedly more efficient in producing PLD from an *Actinomycetes* strain, *Streptoverticillium cinnamoneum* (91,92). In contrast with the original strain that secreted only a small amount of PLD (2 mg/L), the *S. lividans* transformant released much more PLD (118 mg/L) into the medium.

Many strains secrete a significant amount of PLD, including *S. antibioticus* (65), *S. septatus* (93), *S. halstedii* (94), and *S. chromofuscus* (95). The transphosphatidylation activities of *Streptomyces* species were screened by the spectrophotometric method using an artificial substrate, phosphatidyl-*p*-nitrophenol. When PLD-producing strains were isolated from among 6,000 *Streptomyces* strains, PLD from *S. septatus* TH-2 showed the highest ratio of transphosphatidylation activity to hydrolytic activity (96). *Streptomyces septatus* TH-2 produced approximately 34 mg/L extracellular PLD, and the PLD content was more than 7% of the total protein content. In view of the amount and ratio of PLD produced, the authors claimed that the production of PLD using *S. septatus* TH-2 might be industrially feasible. In addition, the gene of *S. septatus* encoding PLD TH-2 was cloned and compared with a commercially available PLD from a *Streptomyces* species (93). The authors also cloned the PLD genes from *S. halstedii* K1 and then constructed chimeric PLD genes using those from *S. septatus* TH-2 and *S. halstedii* K1 to compare the thermostability of *Streptomyces* PLD (94).

ASSAY AND SCREENING METHODS FOR PHOSPHOLIPASES

Rapid and reliable methods for determining enzyme activity are essential for screening, characterizing, and improving enzymes. A number of accurate phospholipase assay methods are known, but they are not amenable to high-throughput screening. Most require discontinuous manipulation and serial instrumental analysis of individual enzyme reactions. To screen a large number of samples, high-throughput screening is required. To facilitate screening, large numbers of whole microorganisms or enzyme preparations from natural and synthetic sources are needed. Feasible assay methods for measuring and screening phospholipase activities in a reliable, high-throughput manner are discussed in the following paragraphs (Table 2).

Synthetic phospholipids containing a ^{14}C-, ^{32}P-, or ^{3}H-labeled FA or head group have routinely been used to quantify the enzymatically liberated products by TLC and liquid scintillation counting. Radiochemical assays, however, are discontinuous, time-consuming, and expensive, and are inadequate for rapid assays and high-throughput screening. A variety of synthetic thio-labeled phospholipid analogs can be used for nonradioactive and spectrophotometric phospholipase assays. For example, 1-thiodecanoyl-2-decanoyl PC (97), 1-palmitoyl-2-thiopalmitoyl PC (98), and 1-hexadecyl-2-arachidonoylthio-2-deoxy-*sn*-glycero-3-phosphorylcholine (99) can be used for a sensitive spectrophotometric detection of PLA_1 or PLA_2 activity. Hydrolysis of the thioester bond by PLA_1 or PLA_2 releases free thiol groups from the synthetic phospholipids. Thiophosphate analogs of 1,2-dioctanoyl-PC (100) and 1,2-dimyristoyloxypropane PI (101,102) also have been synthesized for hydrolysis by PLC to produce a free thiol group. These reactions could be monitored by thiol-reactive chromogenic agents such as DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)] and DTP (4,4′-dithiobispyridine) at 412 and 324 nm, respectively (Fig. 2). Phospholipase assays using thio-labeled phospholipid analogs are likely to be suitable for a microtiter plate format, as described by the Dennis group (99,103).

TABLE 2
Assay Methods Probable for High-Throughput Screening of Phospholipases[a]

Assay and substrate	Product liberated by (phospholipase) reaction	Principles involved	References
Spectrophotometry			
Thio-labeled phospholipid	1-Thiol-phospholipid (PLA_1)	Sequential formation of colored product by thiol-reactive agents (e.g., DTNB, DTP).	97–103
	2-Thiol-phospholipid (PLA_2)		
	Thiophosphate analogue of phospholipid (PLC)		
4-Nitro-3-(octanoyloxy)-benzoic acid	4-Nitro-3-hydroxy-benzoic acid (PLA_2)	Colored product measured at 425 nm.	109–114
Phosphatidyl-*p*-nitrophenol	*p*-Nitrophenol (PLD)	Colored product measured at 405–410 nm; phosphatase-coupled conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol.	115, 119–121
	p-Nitrophenyl phosphate (PLC)		
Linoleoyl phospholipid (unlabeled)	Linoleic acid (PLA_1, PLA_2)	Sequential formation of hydroperoxide by the coupled lipoxygenase reaction.	122–124
PC (unlabeled)	Choline (PLD)	Coupled detection of choline using choline kinase or choline oxidase; alkaline phosphatase-coupled conversion of phosphocholine to choline.	125–128
	Phosphocholine (PLC)		
Fluorescence assay			
Phospholipids with alkyl group with a fluorescent group, e.g., pyrenyl group, BODIPY, NBD	Fluorescent free prenyl groups or pyrene-labeled lysophospholipid (PLA_1, PLA_2)	Shift in fluorescence emission after phospholipid hydrolysis or detection of the fluorescence of monomeric pyrene label. Use depends on the availability of various inexpensive substrates.	104–108
	Pyrene-labeled phospholipid (PLC, PLD)		
Plate assay			
Natural phospholipid, e.g., egg yolk, soybean	FFA and lysophospholipids (PLA_1, PLA_2)	Transparent or cloudy halo around microbial colony on agar plates; less quantitative, but still most commonly used for microbial cell-based primary screening.	31–33, 129
	Free organic phosphates and DAG (PLC)		
Phosphatidyl-2-naphtol	2-Naphtol (PLD)	Formation of water-insoluble azo dye by coupling with a coexisting diazonium salt; can be used for various solid materials.	130
Specific phospholipid (+ pH indicator)	FFA	Color change of pH indicator supplemented in agar plates; the use is not yet reported for phospholipases, but is expected possibly in agar plate- and solution-based screening.	131–136

[a]DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); DTP, 4,4′-dithiobispyridine; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-*S*-indacene; NBD, 7-nitrobenzo-2-oxa-1,3-diazol-4-yl; for other abbreviations see Table 1.

Phospholipases also could hydrolyze pyrene-containing phospholipids such as *sn*-2-pyrenedecanoyl-labeled phospholipids (104–106), *sn*-1-pyrenedecanoyl-labeled phospholipids (107), and *sn*-1-pyrenesulfonyl-labeled phospholipids (105); the progress of hydrolysis can be monitored in real time as an increase in fluorescence emission at 380 nm (Fig. 3). The continuous fluorometric assay for PLA_2 could measure picogram amounts of phospholipases using mixed liposomes and mixed micelles of substrate phospholipids. The hydrolysis of pyrene-containing phospholipids and the subsequent displacement of pyrene moieties by BSA has led to the fluorescence of a pyrene label (104,107). In addition to the pyrene-labeled phospholipids, there are a number of fluorescent-labeled phospholipids such as BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*S*-indacene)-labeled phospholipids (Molecular Probes, Invitrogen Co., Eugene, Oregon), dansyl (5-dimethylamino-naphthalene-1-sulfonyl)-labeled phospholipids, and NBD (7-nitrobenzo-2-oxa-1,3-diazol-4-yl)-labeled phospholipids. The sensitive and continuous fluorescence-based assays may be applicable to all types of phospholipases if the suitable substrates and instrumentation are readily available (108).

The chromogenic substrate analogs have been used to assay phospholipase activity. PLA_2 activity from various sources was assayed using 4-nitro-3-(octanoyloxy)-benzoic acid that had originally been synthesized by Cho *et al*. (109) and Cho and Kezdy (110). The appearance of the nitrophenolate ion, 4-nitro-3-hydroxy-benzoic acid, by the action of PLA_2 could be detected spectrophotometrically at 425 nm [Fig. 4(a)]. In addition to the original kinetic assay, high-throughput screening methods capable of large numbers of samples have been developed (111,112). This assay has been used successfully to detect PLA_2 activity from venoms (113,114), protein purification

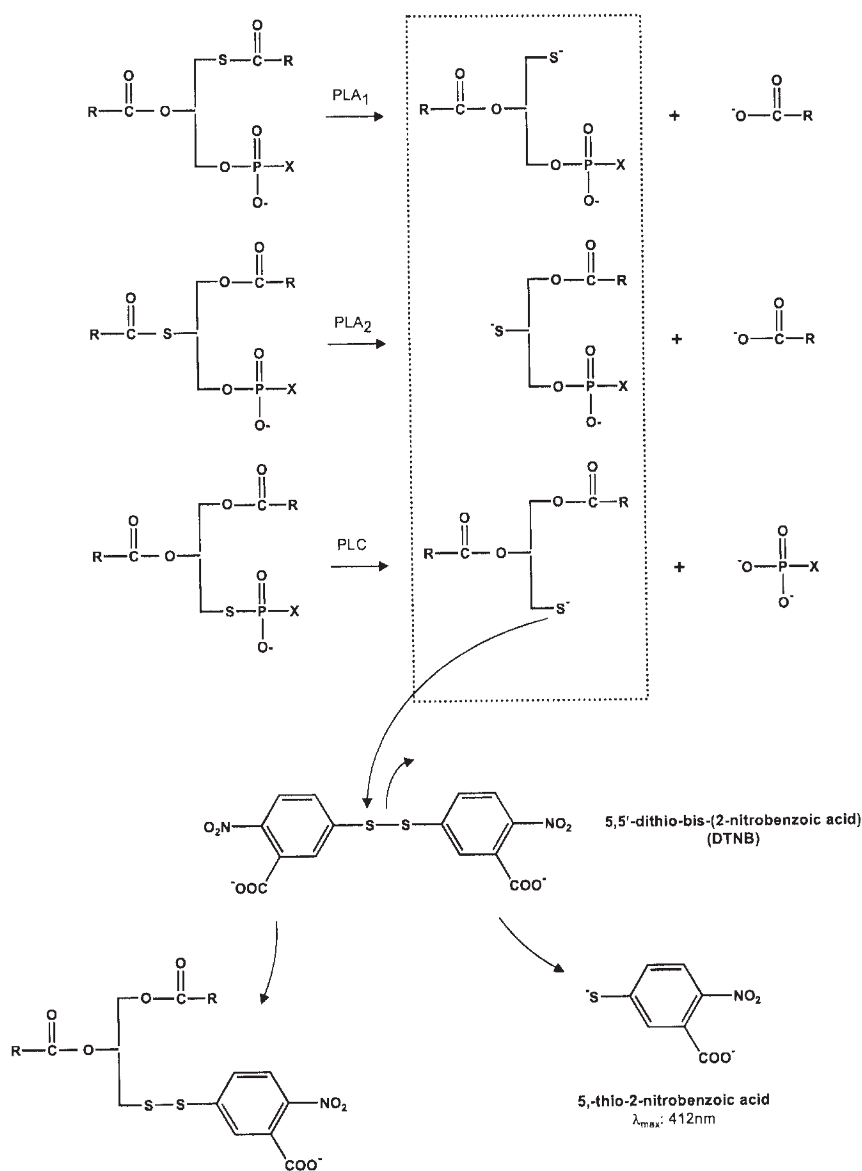

FIG. 2. Reaction scheme for the assay of phospholipases (PLA_1, PLA_2, and PLC) using a thio-labeled phospholipid as the substrate. PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C.

fractions (111), and human serum fractions (112). D'Arrigo *et al.* (115) described the preparation of phosphatidyl-*p*-nitrophenol and its application in the assay for PLD-catalyzed hydrolytic activity [Fig. 4(b)]. The authors also proposed that, if *p*-nitrophenyl phosphate liberated by the PLC-catalyzed hydrolysis could be hydrolyzed by a coupled phosphatase, the assay also could be used to measure PLC activity. Although the release of *p*-nitrophenol from another chromogenic substrate, *p*-nitrophenylphosphorylcholine, has often been ascribed to PLC (116–118), phosphatidyl-*p*-nitrophenol is likely to be a more suitable chromogenic substrate for PLC than *p*-nitrophenylphosphorylcholine (119). The release of *p*-nitrophenol could be also applied to the PLD-catalyzed transphosphatidylation reaction of phosphatidyl-*p*-nitrophenol and ethanol in an aqueous–organic emulsion system (120). The PLD and PLC assay using *p*-nitrophenol-linked phospholipids highly similar to their natural substrates are likely to be more reliable than the lipase assay using *p*-nitrophenyl esters (121) and thus more suitable for microtiter plate-based high-throughput screening.

PLA_1 and PLA_2 activities can be assayed using a continuous spectrophotometric method based on a coupled enzymatic assay. Jimenez *et al.* (122) reported a lipoxygenase-coupled spectrophotometric assay using dilinoleoyl PC as a nonradiolabeled and natural substrate of phospholipase. The linoleic acid released by PLA_2 was oxidized by soybean lipoxygenase, and the phospholipase activity was then followed spectrophotometrically by measuring the increase in absorbance at 234 nm attributable to the formation of the corresponding hydroperox-

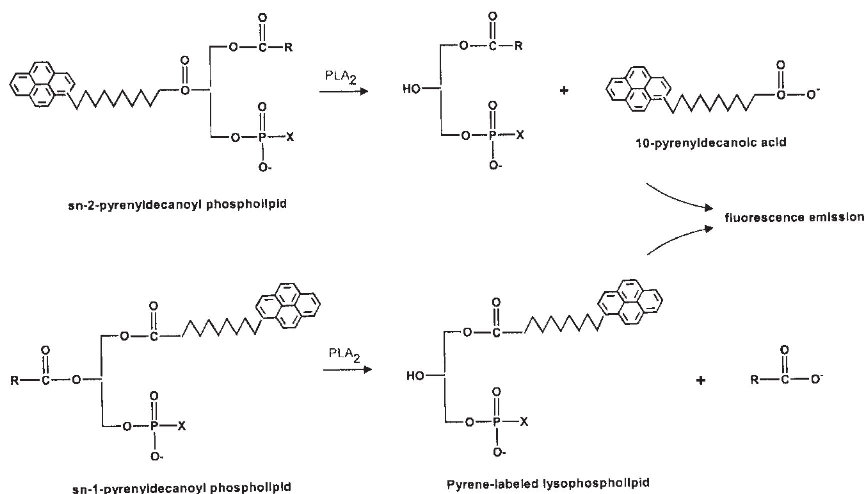

FIG. 3. Reaction scheme for the assay of PLA_2 using a pyrene-containing phospholipid as the substrate. For abbreviation see Figure 2.

ide from the linoleic acid. Soybean lipoxygenase was able to oxidize dilinoleoyl PC and free linoleic acid (123). The phospholipase activity of patatin, a family of glycoproteins that accounts for 30–40% of the total soluble protein in potato (*Solanum tuberosum* L.) tubers, was also assayed using the lipoxygenase-coupled method (124). PLD and PLC activities could also be detected using the subsequent conversion of choline and phosphocholine, respectively, released by the hydrolysis of PC. Choline was converted either by choline kinase coupling the oxidation of NADH (125) or by choline oxidase coupling the formation of hydrogen peroxide. In the latter case, hydrogen peroxide could be detected spectrophotometrically by the peroxidase-catalyzed formation of a dye compound (126,127) and fluorometrically with an automated microtiter plate fluorometer (128). In PLC reactions, alkaline phosphatase was used to convert the primary product phosphocholine into choline (127). We expect that phospholipase assays coupled with secondary enzymes will continue to improve.

Phospholipase activity on solid substrates such as agar plates, membranes, and protein gels may also be used to identify positive clones from mutant libraries grown on agar plates in a high-throughput manner. The hydrolytic activities of PLA_1, PLA_2, and PLC produce a clear or turbid zone on an agar plate containing egg yolk or soybean lecithin. Based on halo formation around the site of phospholipase activity, the screening on agar plates has been used to isolate PLA_1-producing microorganisms from soil (129) and to clone PLA_1-encoding genes from a chromosomal DNA library (31). This method is also very convenient for rapidly

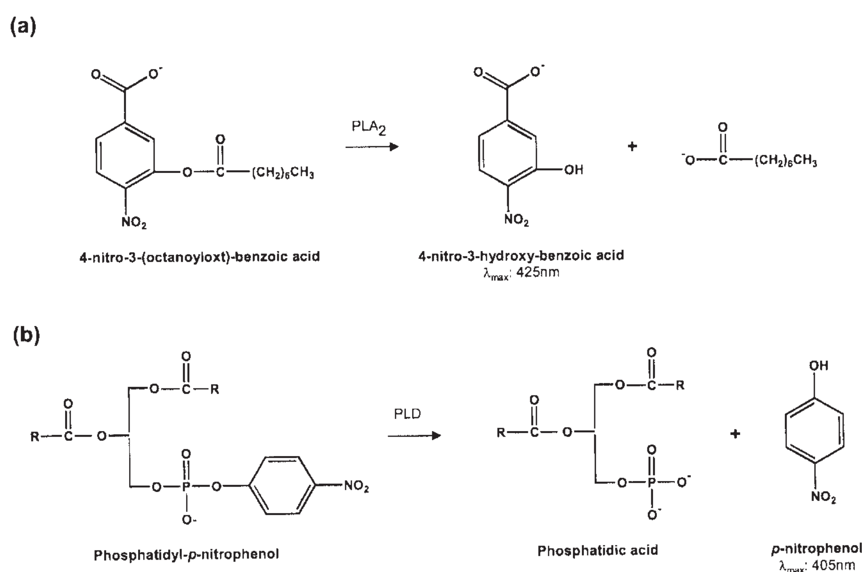

FIG. 4. Reaction scheme for the assay of (a) PLA_2 and (b) PLD using a chromogenic substrate. PLD, phospholipase D; for other abbreviation see Figure 2.

screening a library of enzyme variants blotted on nylon membranes and microbial colonies grown on agar plates (32,33). However, PLD activity on solid media cannot be detected by halo formation. Iwasaki *et al*. (130) recently reported a colorimetric method for detecting PLD activity on solid media using a synthetic substrate, phosphatidyl-2-naphthol. The water-insoluble azo dye was generated from the 2-naphthol liberated by PLD, and the color of the azo dye was stable on a nitrocellulose membrane, polyacrylamide gel, and agar. As suggested by the authors, the synthetic substrates resulting in a water-insoluble colored product will be useful for screening a number of PLD enzymes, e.g., from mutant libraries, in a high-throughput manner.

The well-known pH-stat method using egg yolk lecithin as a substrate can measure PLA_1 and PLA_2 activity quantitatively by titrating the acid products released with time by adding NaOH in order to maintain a constant pH value (131). The original pH-stat method, however, requires the use of an autotitrator, autoburette, and data recorder. A pH indicator-containing assay using the same method was more suitable for high-throughput screening even though it was less quantitative. The drop in pH attributable to the FA or other acid products released during esterase-mediated (132) or PLA_2-mediated (133) hydrolysis caused a change in color of the appropriate pH indicator. The formation of acids from a true substrate of interest was monitored either in liquid media (134,135) or in solid media (136) supplemented with an indicator. Because the phospholipases, especially PLA_1 and PLA_2, also release acid products in hydrolyzing their substrates, an assay employing a pH indicator may be a good choice for high-throughput screening (133).

In addition to the solution- and agar plate-based methods described, some remarkable methods for high-throughput screening of enantioselective biocatalysts, lipases in particular, have recently been developed using chromatographic (GC and HPLC), mass-spectrometric, IR-thermographic, and circular dichroism analyses (137,138). High-throughput methodology, combined with a proper instrumental configuration, will also be effective in detecting and screening for phospholipase-catalyzed reactions.

APPROACH TO NATURAL BIODIVERSITY: EXTREMOPHILES AND METAGENOMES

It is now generally agreed that only a small part of the global microbial community has been identified, and a few microorganisms could be grown in pure cultures. Advanced culture techniques, including the microbial enrichment culture technique, have recently been extended to extremophiles, which can live and reproduce in harsh environments (139,140). The microbial enrichment culture technique, which is necessary to accumulate culturable microorganisms producing enzymes of interest, involves controlling growth conditions to favor the development of a specific organism or group of organisms and screening microorganisms capable of growing in those conditions. Microbial genome sequencing also has been driven by the need to exploit extremophiles and their enzymes in industry (141,142). Most notably, the properties of enzymes from extremophilic microorganisms (i.e., their outstanding stability and/or activity under extremes of temperature, pH, pressure, salt concentration, etc.) are generally adapted to environmental growth conditions and thus nearly always correspond to the most important criteria in selecting an enzyme for biocatalysis (143). For example, some lipases from the thermophilic *Bacillus* sp. and from psychrophiles could be obvious candidates in detergent formulations for high-temperature and low-temperature processes, respectively (144,145). Hydrolases such as amylases, cellulases, xylanases, proteases, and lipases have been isolated from thermophiles, psychrophiles, alkaliphiles, and halophiles (146). The hyperthermophilic archaea *Pyrococcus horikoshii* (147) and *Aeropyrum pernix* (148) produced PLA_2 with optimal activity at 90°C in their cytosolic fraction. Moreover, the PLA_2-encoding gene of the hyperthermophilic archaeon *A. pernix* was cloned by PCR with primers prepared by an analysis of genome sequence data (149). This first cloned thermophilic PLA_2 was expressed in *E. coli* and showed extreme thermostability: Its half-life at 100°C was about 1 h. As shown in PLA_2 produced by hyperthermophilic archaea, we believe that a variety of phospholipases will be identified from extremophiles that will offer new opportunities for processing phospholipids under harsh conditions, e.g., in relatively high-temperature degumming processes.

Although a large number of valuable biocatalysts have been developed from culturable microorganisms, the traditional screening approaches for isolating microorganisms of interest miss opportunities to explore 95–99% of the existing microbial resources (150). Novel approaches in biocatalyst screening technology are emerging through a cultivation-independent strategy of directly cloning “environmental DNA” comprising the collective genomes of all microorganisms in a given habitat, which is called a “metagenome” (151–153). The metagenomic approach involves extracting total microbial community DNA from a variety of natural environments (154), constructing DNA libraries in plasmids (for small-length fragments) and in cosmid, fosmid, or bacterial artificial chromosome vectors (for large-insert fragments), and then screening for biotechnologically relevant enzymes by either activity- or sequence-based methods. In particular, activity-based screening has been applied successfully to various environmental libraries, resulting in many biotechnologically important enzymes, including lipases and esterases (151,155,156). In particular, Knietsch *et al*. (157) constructed metagenomic DNA libraries after enriching them on different soil and sediment samples with glycerol and 1,2-propanediol, and finally identified 16 positive clones conferring the oxidation of short-chain polyols or a reduction of the corresponding carbonyls. Because both cultivation-based and metagenome-based approaches have their own advantages, they can be combined effectively to isolate new genes from environmentally derived microbial consortia (157,158). Although no reports of phospholipase activity in metagenome libraries exist thus far, these relatively new and exciting approaches to discover novel phospholipases in metagenomes retrieved from uncultured microbial treasures will provide useful biocatalysts with better properties.

PROTEIN ENGINEERING USING MAN-MADE ENZYME DIVERSITY

One of the recent approaches for broadening the use of enzymes in biocatalysis is the protein engineering approach, involving the creation of new proteins by genetically modifying already existing ones. It has been used to address the structure–function relationships of enzymes and confer on them desirable industrial properties. With advances in rational design and directed evolution tools, enzymatic features such as substrate specificity, stability, catalytic efficiency, and pH optima can be improved. In particular, the introduction of directed evolution has had an enormous impact on the field of protein engineering (159–161). Briefly, directed evolution techniques generally involve iterative rounds in which libraries of mutated proteins with various gene-diversification techniques are created, and subsequent intensive screening for the desired properties, leading to remarkable changes or improvements in the properties of the starting proteins (162).

Three residues in the head group-binding pocket of *B. cereus* PLC were randomly mutated to find the mutants with altered specificity profiles and high catalytic activities. The authors developed a novel protocol suitable for screening PLC activity that was conducted in a microtiter plate format and found that the substrate specificity of *B. cereus* PLC could be modulated by varying only three of the amino acid residues that constitute the head group-binding pocket (163). *Bacillus thermocatenulatus* lipase, which appeared to have relatively high stability and activity, was subjected to the directed evolution processes to create a lipolytic enzyme with both high phospholipase type-A activity and high stability (164). A similar experiment was carried out with a *Staphylococcus aureus* lipase, and the best variant, containing six amino acid mutations, displayed an 11-fold increase in phospholipase activity as compared with the parent lipase (165). Although it is still unclear whether the two experiments are suitable for the preparation of versatile biocatalysts to be used in phospholipid-related bioprocesses, they are regarded as probable approaches to efficient phospholipase preparation.

High levels of activity and stability are generally regarded as the most important traits of a biocatalyst. A chimeral-gene library between thermostable and thermolabile PLD was constructed to identify key amino acid residues for thermostability (166,167). Site-directed mutagenesis and *in vivo* DNA shuffling were applied to *Streptomyces* PLD, and their thermostability-related residues were analyzed successfully. The directed evolution strategy was applied to PLA_1 from *Serratia* sp. to improve its thermostability. In fact, only two rounds of random mutagenesis and filter-based high-throughput screening yielded two mutants of PLA_1 with six and seven amino acid substitutions each (32). The mutants showed increases in their temperature of half-inactivation of 7 and 11°C, respectively. When the catalytic activities were measured at a number of temperatures, not only were the mutants stable at the temperatures examined, but they also exhibited high levels of catalytic activity. The solvent tolerance of phospholipase was also increased by directed evolution (33). Both the stability and catalytic activity of PLA_1 in the presence of DMSO were enhanced by error-prone PCR and DNA shuffling. The first screening for PLA_1 activity of individual library members was performed following a 10-h exposure to 30% DMSO, and the second, harsher screening was done following a 36-h exposure to 50% DMSO. Three resultant mutant PLA_1 had half-life values up to fourfold longer in the presence of 30% DMSO and were more tolerant of other solvents tested than was the wild-type enzyme. Thus, directed evolution is a powerful technique for further improving the properties of biocatalysts of industrial relevance, including phospholipases.

CONCLUDING REMARKS

Because of their unique nature, phospholipids have been used as ingredients in the formulations for foods, cosmetics, agricultural products, and pharmaceuticals. Various industrial applications of phospholipid products will be realized quickly as active research reveals more about the various physiological and biological functions of phospholipids. The number of superior phospholipases that can be made available from academic and commercial sources will also increase to accelerate the industrial use of phospholipids by phospholipases. Recent progress in genomic and proteomic research, including new technologies for the discovery and improvement of enzymes (e.g., extremophiles, metagenomes, and directed evolution), will continue to inspire many industrial and academic researchers to develop new and superior phospholipase biocatalysts.

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