Recent Progress on Phospholipases: Different Sources, Assay Methods, Industrial Potential and Pathogenicity

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Abstract Significant studies on phospholipases optimization, characterization, physiological role and industrial potential have been conducted worldwide. Some of them have been directed for biotechnological advances such as gene discovery and functional enhancement by protein engineering. Others reported phospholipases as virulence factor and major cause of pathophysiological effects. A general overview on phospholipase is needed for the identification of new reliable and efficient phospholipase, which would be potentially used in number of industrial and medical applications. Phospholipases catalyse the hydrolysis of one or more ester and phosphodiester bonds of glycerophospholipids. They vary in site of action on phospholipid which can be used industrially for modification/production of new phospholipids. Catalytically active phospholipase mainly use phosphatidylcholine as major substrate, but they can also show specificity with other phospholipids. Several accurate phospholipase assay methods are known, but a rapid and reliable method for high-throughput screening is still a challenge for efficient supply of superior phospholipases and their practical applications. Major application of phospholipase is in industries like oil refinery, health food manufacturing, dairy, cosmetics etc. All types of phospholipases can be involved as virulence factor. They can also be used as diagnostic markers for microbial infection. The importance of phospholipase in virulence is proven and inhibitors of the enzyme can be used as candidate for preventing the associated disease.

Keywords Phospholipase types · Assay methods · Industrial uses · Bacterial phospholipase · Fungal phospholipase · Pathogenicity · Virulence factor

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Abbreviations

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PL	Phospholipase
PLA	Phospholipase A
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PA	Phosphoric acid
PC	Phosphatidylcholine
PI	Phosphatidylinositol
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
SM	Sphingomyelin
LPC	Lysophasphatidylcholine
LPI	Lysophosphatidylinositol
LPG	Lysophosphatidylglycerol
PLC N	Non-haemolytic PLC
PLC H	Haemolytic PLC
DLPC	Dilinoleoyl phosphatidylcholine
DMPC	1,2-sn-Dimyristoyl phosphatidylcholine

Introduction

Heterogeneous groups of enzymes 'phospholipase' have ability to hydrolyze one or more ester linkages in glycerophospholipids. It represents a class of lipolytic enzymes, acyl hydrolyses and phosphodiesterases [1]. Although all phospholipases target phospholipids as substrates, they vary in the site of action on the phospholipids molecule, their function, mode of action and their regulation. Depending on the site of action, phospholipases are classified and qualifying letters such as A, B, C and D are used to differentiate among them and indicate the specific bond targeted in the phospholipids molecule (Table 1; Fig. 1). For example, phospholipase A_1 (PLA_1) hydrolyses the fatty acyl ester bond at the *sn*-1 position of the glycerol moiety. A complete cleavage can be achieved by all classes of phospholipases. The PLA_1 and PLA_2 enzyme actions produce free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively. The fatty acid linked to the lysophospholipid is cleaved by lysophospholipase activity of phospholipase B (PLB). Phospholipases C (PLC) are defined as phosphodiesterases that cleave the glycerophosphate bond and finally the base group of phospholipid removed by phospholipase D [2–4]. Catalytically active phospholipases share a common substrate—phospholipids and phosphatidylcholine (PC) as major substrate. However, they can also act on phosphatidyl-

Table 1 Classification of phospholipase

Type of phospholipase	Bond cleaved	Site of action
PLA_1 (EC 3.1.1.32)	Fatty acyl ester bond	*sn*-1 position of glycerol moiety
PLA_2 (EC 3.1.1.4)	Fatty acyl ester bond	*sn*-2 position of glycerol moiety
PLB (EC 3.1.1.5) (lysophospholipase)	Fatty acyl ester bond	*sn*-1 position and *sn*-2 position of glycerol moiety
PLC (EC 3.1.4.3)	Phosphodiester bond	First phosphodiester bond
PLD (EC 3.1.4.4)	Phosphodiester bond	Second phosphodiester bond

Fig. 1 The site of action of various phospholipases on phospholipid. *X* phospholipid common base (e.g. choline, ethanolamine, serine, myoinositol)
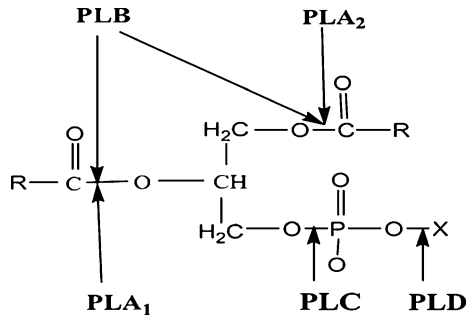
ethanolamine (PE), phosphatidylinositol (PI), sphingomyelin, lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI) in some organisms and tissues [5]. Phospholipases are used as scientific research tool for hydrolysis, synthesis and trans-phosphatidylation, which provides various kinds of phospholipids [6]. These phospholipids have wide industrial applications in food technology, cosmetics and pharmaceuticals. Phospholipases used for industrial processes in food industry are mainly produced from porcine or bovine pancreas. Microbially produced phospholipases are used for degumming of edible oils, synthesis of chemicals and production of heath food, dairy and agricultural products (Table 2) [7, 8].

The enzyme phospholipase has considerable physiological significance. In eukaryotes, phospholipases are involved in some stages of lipid metabolism like fat digestion, reconstitution, lipoprotein metabolism etc. The phospholipases also initiate the signal transduction cascade in response to cell surface receptor activation and function as lipid second messengers [5, 9, 10]. In microbes, they are commonly believed to have a role in virulence factor. Destruction of phospholipids by pathogenic bacteria/fungal phospholipases and subsequent change of membrane constituents lead to cell damage which is regarded to be a major virulence mechanism in infection [4]. Different classes of PLs are reported from bacteria and fungi and studied for its significance in virulence and pathogenesis (Table 3) [2, 4, 9, 11–104].

Table 2 Present fields of applications of phospholipases

Scientific research	PL are used as research tools for hydrolysis, trans-phosphatidylation, synthesis, cell biology and molecular studies, role in virulence and pathogenesis
Therapeutic application	Anti-inflammatory agents, diagnostic markers for microbial infections. PL as drug (design inhibitor and/or vaccine)
Cosmetic application	Preparations for skin moisturizers and denture cleansers
Industrial application	Oil refinery industry (removal of mucilage and content of phosphorus component or degumming in plant oil)
	Health food industry
	Production of modified phospholipids
	Bread making
	Dairy industry
	Animal feed additives
	Synthesis of surfactants
	Detergent

Table 3 Sources of different phospholipase

Source	Enzyme	Substrate specificity	PL as virulence factor	References
Bacteria				
Clostridium perfringens	PLC	PC, SM, PS, PLC	Yes	[11, 12]
Clostridium novyi	PLC	PC, SM, LPC, PE, PI, PG	Yes	[13, 14]
	PI-PLC	PI		
Clostridium bifermentans	PLC	PC	Yes	[12]
Listeria monocytogenes	PLC-A	PI	Yes	[15–19]
	PLC-B	PC, PE, PS, SM		
Pseudomonas aeruginosa	PLC-H	PC, LPC, SM	Yes	[20–22]
	PLC-N	PC		
Pseudomonas cepacia	PLC	PC	Yes	[23]
Staphylococcus aureus	beta-Toxin PLC	SM, LPC	Yes	[24–26]
	PI-PLC	PI, LPI		
Bacillus cereus	PC-PLC	PC, PE	No	[27–29]
	SMase	SM		
	PI-PLC	PI, LPI		
Bacillus thuringiensis	PI-PLC	PI, LPI	Yes	[30, 31]
Mycobacterium tuberculosis	PLD	PC	Yes	[32]
	PLA	PC		
Rickettsia prowazekii	PLA	PC	Yes	[33–35]
Corynebacterium pseudotuberculosis	PLD	PC	Yes	[36, 37]
Arcanobacterium haemolyticum	PLD	PC	Yes	[37]
Legionella pneumophila	PLC, PLA	PC	Yes	[38–40]
Leptospira interrogans	PLC	PC	Yes	[41]
Ureaplasma urealyticum	PLC	PC	Yes	[42]
Streptomyces genus	PLA_1/PLA_2	PC	Yes	[42]
Salmonella newport	PLA1	PC	Yes	[43]
Streptomyces sp.	PLD	PC	No	[44, 45]
Streptomyces chromofuscus	PLD	PC	No	[46]
Actinomycetes species	PLD	PC	No	[47]
Streptomyces antibioticus	PLD	PC	No	[48]
Streptomyces hiroshimensis	PLB	PC	No	[49]
Streptomyces cinnamomeus	PLA_1/PLA_2	PC	No	[50]
Trypanosoma brucei	PLC	PC	Yes	[51]
Mycobacterium phlei	PLA_1	PC	No	[52]
Escherichia coli	PLA_1, PLA_2 lysophospholipase	PC	No	[53, 54]
Moraxella bovis	PLB	PC	No	[2]
Helicobacter pylori	PLA_1, PLA_2, PLC		Yes	[55, 56]
Yersinia enterocolitica	PLA	PC	Yes	[57, 58]
Fungus				
Candida albicans	PLB1	PC, 1-palmitoyl-LPC, 1-arachidonyl-LPC, 1-olejoyl-LPC, palmitoyl-LPE, palmitoyl-LPI	Yes	[4, 59–66]
	PLB2	PC	Yes	
	PLD	PC	No	

Table 3 (continued)

Source	Enzyme	Substrate specificity	PL as virulence factor	References
Candida glabrata	PLB	PC, LPC	Yes	[61]
Cryptococcus neoformans	PLB	PC	Yes	[67, 68]
Cryptococcus gattii	PLB	PC	Yes	[69]
Aspergillus fumigatus	PLB	PC	Yes	[70–72]
	PLC			
	PLD			
Aspergillus flavus	PLB	PC, LPC	Yes	[71]
	PLA			
	PLC			
Saccharomyces cerevisiae	PLBc	PC	No	[73–75]
	PLB1d PLB2d			
	PLD			
Penicillium notatum	PLB	PC and LPC	No	[76, 77]
Schizosaccharomyces pombe	CaPLD1	PC	No	[78]
Torulaspora delbrueckii	PLB	PC	No	[79]
Protozoa				
Toxoplasma gondii	PLA	PC	Yes	[80, 81]
Entamoeba histolytica	PLA	PC	Yes	[82, 83]
Dictyostelium discoideum	PLB	PC	No	[84, 85]
Mammals				
Bovine pancreas	PLA_2	PC	No	[86]
Rabbit brain	Lysophospholipase	PC	No	[87]
Porcine pancreas	PLA_2	PC	No	[73, 88]
Human non-pancreas	PLA_2	PC	No	[89]
Human neutrophils	PLA_2	PC	No	[90]
Beef pancreas	PLA_1	PC	No	[91]
Human cells	PLD1 and PLD2, PLA_1 and PLA_2	PC	No	[92–94]
Rat brain	PLD	PC, PE	No	[95]
Rat intestine	PLD	PC	No	[96]
Invertebrates				
Snake venom	PLA_2	PC	No	[97]
Cobra venom	PLA_2	PC	No	[98, 99]
Bothrops jararacussu snake	PLA_2	PC	No	[100]
Plant				
Savoy cabbage	PLD	PC	No	[101]
Plant phospholipases	PLD	PC, PI, PE	No	[9, 102]

PC phosphatidylcholine, *PI* phosphatidylinositol, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *SM* sphingomyelin, *LPC* lysophosphatidylcholine, *LPI* lysophosphatidylinositol, *LPG* lysophosphatidylglycerol, *PLC N* non-haemolytic PLC, *PLC H* haemolytic PLC

Phospholipases has been the subject of some reviews that give details of specific physiological role, pathological potential, biotechnological advances and industrial significance. For example, review articles have been published on biological and pathological functions of secretory phospholipase A_2 and their receptor [10], specific class of phospholipases and their role as virulence factor [4, 105, 106], their industrial

applications [7], phospholipase-catalysed reactions for the production of phospholipids with industrial relevance [107, 108], occurrence, gene discovery and biotechnology [8]. The demand of new reliable and efficient new phospholipase has steadily increased over the past several decades. In this review, we will give a general overview on phospholipase classification, screening and assay methods, example of sources of different classes of phospholipases, their substrate specificity and key role to the industrial process, and finally, we will draw attention to bacterial and fungal phospholipases for pathogenicity. This will make possible the valuable means to rapidly develop their industrial and medical applications.

Phospholipase A

PLA comprises a diverse family of lipolytic enzyme produced by bacteria as well as eukaryotic cells, used to catalyse the removal of a fatty acid from phospholipids to generate lysophospholipids [104]. PLA_1 hydrolyzes the *sn*-1 fatty acid ester bond of glycerophospholipids and PLA_2 hydrolysis at the *sn*-2 fatty acid ester bond and reactions are shown in Fig. 2a, b. The enzyme subgroups can remove and replace the acyl chain in various phospholipids via hydrolysis, esterification and trans-esterification.

PLA_1 activities have been detected in many cells and tissues from mammals, venoms and microorganisms. However, a limited number of PLA_1 were purified and cloned so far [109]. PLA_1 enzymes are present in both the outer membrane and cytoplasmic stage. Outer membrane PLA (OMPLA; EC 3.1.1.32) has broad substrate specificity, hydrolyzes all phospholipids, neutral glycerides and cytoplasmic PLA_1, with a high degree of specificity for phosphatidylglycerol. Cytoplasmic PLA_1 can be inactivated by heat and detergent and
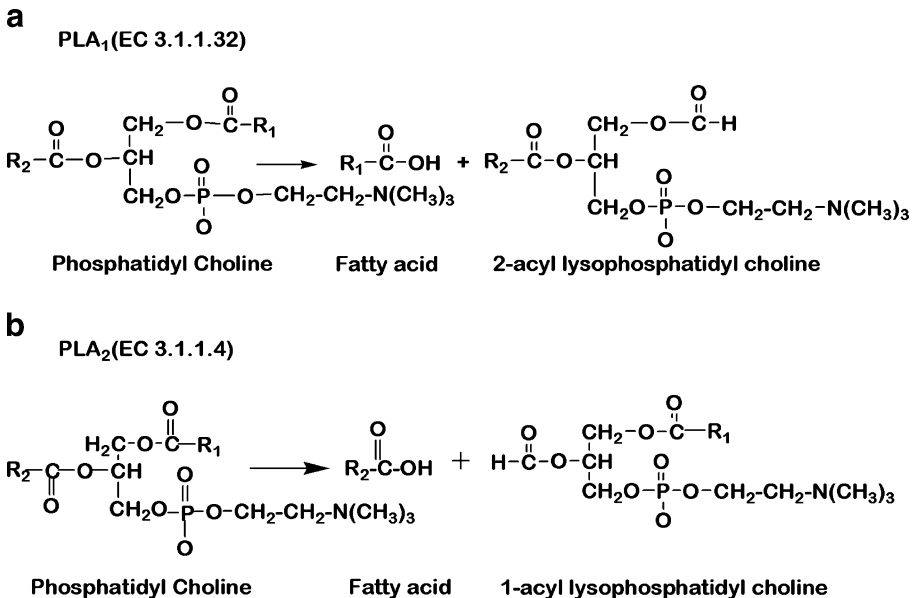
Fig. 2 **a** Phospholipase A_1 enzyme hydrolysis reaction at *sn*-1 fatty acid ester bond. **b** Phospholipase A_2 enzyme hydrolysis reaction at *sn*-2 fatty acid ester bond

can also act as a transacylase. OMPLA is one of the few enzymes present in the outer membrane of gram-negative bacteria [105]. OMPLA is not required for the bacterial cell under laboratory conditions, but it might be essential for growth in the natural environment. This enzyme is unusually resistant to inactivation by heat and ionic detergent and requires calcium for maximal activity. Another selective function of OMPLA is related to organic solvent tolerance in the bacterium; however, the role of OMPLA in this process needs to be clarified [110].

Recently, it has been reported that PLA_1 plays important biological role in both phospholipidosis—a pathological condition in which phospholipids accumulate in lysosomes and virulence factors for bacterial and fungal pathogenesis [58]. In addition to its physiological roles, PLA_1 is of particular interest for industrial applications as it yields 2-acyl-lysophospholipids. Lysophospholipids are excellent emulsifiers and are particularly suitable for use in many industrial applications, such as food technology, cosmetics and pharmaceutical industries. Lysophospholipids are able to enhance emulsification in oil–water emulsions due to increased solubility in water, form emulsions which are more stable to changing pH and temperature conditions and keep emulsions stable in the presence of magnesium or calcium ions. Lysophospholipids also have several physiological functions, including a role in platelet aggregation and a role as a signalling molecule [111]. They also affect ripening and storage characteristics of fruits, leaves and green plant tissue [112]. Immobilized PLA_1 was used for preparation of 1-ricinoleoyl-2-acyl-sn-glycero-3-phosphocholine from soya to egg phosphatidylcholine [7, 8]. The ricinoleic acid was incorporated at sn-1 position of egg and soybean lecithin. PLA_1 is not yet widely available and provides the scope in search for superior PLA_1 sources.

PLA_2 has received extensive characterization and is more frequently reported enzyme in bacteria, protozoa, invertebrates and mammals (Table 3). PLA_2 participates in a wide variety of physiological processes, including phospholipids digestion, signal transduction, remodelling of cell membrane and host defence. It also takes part in pathophysiological processes by producing precursors of various types of biologically active lipid mediators, such as prostaglandins, leukotrienes, thromboxanes and platelet-activating factor (PAF) [10]. Advances in molecular biology, numerous eukaryotic PLA_2s have been identified and characterized. According to their biochemical features, such as cellular localization, requirement of Ca^{2+}, substrate specificity and the primary structure, these PLA_2s are classified in to three main categories, including (a) low MW (13–18 kDa) secretary PLA_2, (b) Ca^{2+}-sensitive arachidonoyl-specific (~85 kDa) cytosolic PLA_2, (c) Ca^{2+}-independent PLA_2 (85–88 kDa) and (d) several subdivision, e.g. PLA_2-IB, PLA_2-IIA, PAF-acetylhydrolase etc. [113]. Secreted and membrane-bound PLA_2 activity has been described in bacteria, fungi and protozoa [4, 114]. This activity is reported to be calcium-dependent and related to pathogenesis in most microorganisms. The crystal structure and the tertiary structure of the secreted PLA_2 (EC 3.1.1.4) from *Streptomyces violaceoruber* A-2688 have been determined by nuclear magnetic resonance (NMR) and X-ray analyses. Interestingly, the X-ray diffraction data of this PLA_2 indicated that although the tertiary structure of the microbial PLA_2 is strikingly different from that of the bovine pancreatic phospholipase, the internal motion which is associated with the calcium binding, phospholipids binding and allosteric interfacial activation is common to both [114].

Other major functions of PLA_2 in the toxicity of venomous organisms [97], the virulence factor in bacterial infections [40, 105], biocatalytic uses for the phospholipid modification [6, 8] and industrially used as degumming process for refining vegetable oils [7, 115] were also discussed. Therefore, a screening program for new microbial PLA_1 or PLA_2 would be always required to develop as more suitable for commercial production.

Phospholipase B

Phospholipase B enzymes catalyse three distinct activities: a *sn*-1 and *sn*-2 fatty acid ester hydrolase, a lysophospholipase and a transacylase activity (Fig. 3a). Thus, it created confusion in nomenclature [84]; therefore, synonyms lysophospholipase and lysophospholipase–transacylase are used for PLB. The hydrolase activity allows the enzyme to cleave fatty acids from both phospholipids (PLB activity) and lysophospholipid (lysophospholipase activity; Fig. 3b), while the transacylase activity allows the enzyme to produce phospholipids by transferring a fatty acid to a lysophospholipid (Fig. 3a). Many fungal species appear to produce PLB with both hydrolyzing and acyltransferase activity [4, 59, 116]. These activities have also been described in bacteria [2], *Dictyostelium discoideum* (protozoa) [84] and in mammalian cells [87, 94, 96]. The fungal PLBs range 600–700 amino acids in length and have a potential role in virulence and in fungal pathogenesis [4]. PLB has also been cloned from mammalian cells but is not sequenced related to the fungal PLBs or bacterial PLB. The focus on potential role of PLB in pathogenesis of fungal infections is reviewed later.
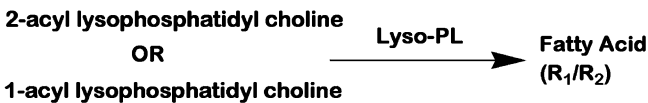
Phospholipase C

C-type phospholipases catalyse the cleavage of membrane phospholipids to 1,2-di-acyl glycerol and the respective organic phosphate (Fig. 4). According to their substrate

Fig. 3 **a** Types of reaction of phospholipase B enzyme. **b** Phospholipase B enzyme hydrolysis reaction

PLC(EC 3.1.4.3)
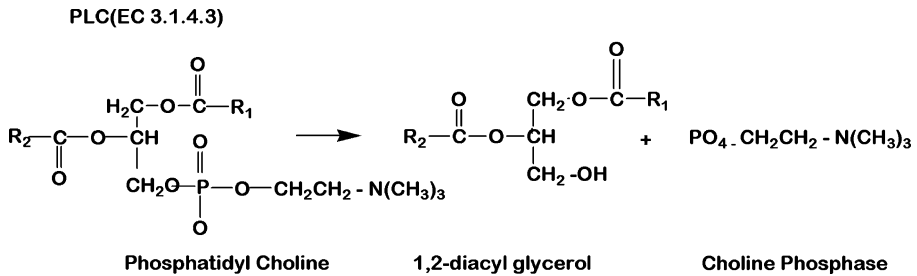
Fig. 4 Phospholipase C enzyme hydrolysis reaction at first phosphodiester bond

specificities, PLC can be classified as (a) PI-specific PLC (PI-PLC), (b) PC-preferring PLC (PC-PLC) and (c) non-specific PLC. PI-PLC enzyme in eukaryotes play key role in generating membrane-associated second messengers [117, 118], and in prokaryotes, they act as virulence factor in some pathogenic bacteria [13, 30, 31]. Bacterial PI-PLCs consist of a single domain of 30–35 kDa, while the much larger eukaryotic enzymes (80–150 kDa) are organized in several distinct domains. Both the eukaryotic and prokaryotic enzymes need cofactors calcium and zinc, respectively, for optimum catalytic activity [118]. PI-PLC [117] catalyses the hydrolysis of PI into two steps: (a) intra-molecular phosphotransferase reaction to inositol 1,2-cyclic phosphate (CIP) followed by (b) a cyclic phosphodiesterase activity that converts CIP to inositol-1-phosphate. Feng et al. [119] studied the PI-PLC from *Bacillus thuringiensis* which exhibits several types of kinetic interfacial activation by interfaces. Micellar PI is a better substrate than monomeric PI, and interface of PC is a non-substrate (does not bind at the active site) that activates the enzyme for both PI cleavage and CIP hydrolysis. PI-PLC has seven tryptophan residues at active site domain. Two of the seven tryptophan (Try-47 and Try 242) clearly have role in PC activation of PI-PLC hydrolysis of water soluble CIP because they also affect PI cleavage to CIP. They are important for orienting the enzyme at any interface, either activating interfaces such as PC or a substrate interface (PI). Removal of both rim tryptophan produces an enzyme that is less sensitive to both activating and a substrate interfaces. PC-PLC have relatively broad specificity towards the head group of phospholipid. They have been detected in a variety of bacteria: *Listeria monocytogenes* [15–19] showed PLC as virulence factor while the virulence of *Bacillus cereus* [27–29] has not been investigated in animals and non-specific PLC of this bacterium was considered to be nontoxic (Table 3).

PLC has been mainly studied for its significance in biochemistry to the eukaryotic cell metabolism [5]. Extracellular microbial production of PLC is still at relatively low level; thus, their industrial applications are limited [7].

Phospholipase D

Phospholipase D (PLD) activities are present in prokaryotic and eukaryotic organisms including mammalian tissues and cell lines. They are involved in phospholipids metabolism, nucleases, toxins and virus envelop protein of unknown function [120, 121]. Different PLDs can catalyse the hydrolysis of PC, PE, PI, phosphatidylserine (PS), lysophophatidylcholine, sphingomyelin, phosphatidylglycerol (PG) or *N*-acyl phosphatidylethanolamine (NAPE), generating phosphoric acid (PA) and the accompanying polar head group (Fig. 5a). PLs also exhibit a unique trans-phosphatidylation activity that

a

b

Phospholipase D
(Hydrolysis and Transphosphatidylation activity)
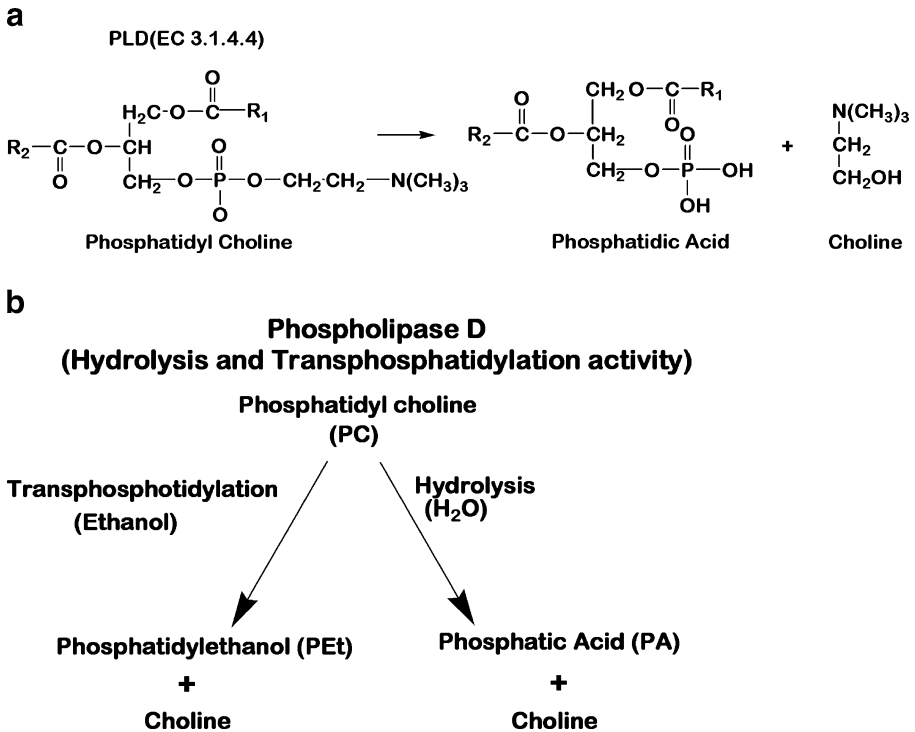
Fig. 5 **a** Phospholipase D enzyme hydrolysis reaction at second phosphodiester bond. **b** Types of reaction of phospholipase D enzyme

transfers the phosphatidyl moiety of the substrate of certain nucleophiles such as ethanol, thereby forming phosphatidylethanol [3, 120, 122, 123]. Thus, in trans-phosphatidyl transfer reaction, the primary alcohol acts as nucleophilic acceptor in place of water (Fig. 5b). The significant amount of PLD has been secreted by *Streptomyces antibioticus* [48, 124], *Streptomyces septatus* [125], *Streptomyces halstedii* [44, 45] and *Streptomyces chromofuscus* [46]. Their trans-phosphatidylation activity can be enzymatically used in modification of phospholipids and synthesis of phospholipid conjugates for the use of food, cosmetics and pharmaceutical industries. Although many *Streptomyces* strains have been isolated as high producer of extracellular PLD, the productivity has not been high enough to permit industrial application. Therefore, many researchers are still trying to isolate PLD hyper producing strains. PLD-catalysed trans-phosphatidylation reactions were performed by commercially available PLD of plant particularly cabbage and microbes mainly *Streptomyces* species [124], *Saccharomyces cerevisiae* (encoded by SPO14). PLD was described as essential for meiosis [3]. The role of PLD as virulence factor for *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans* and *Arcanobacterium haemolyticum* has been reported [36, 37] as a result of targeted mutagenesis of the phospholipase D gene with decrease in virulence of *C. pseudotuberculosis*. Mammalian PLD has one of the key enzymes in intracellular signalling, where phosphatidic acid is greatly involved in signal transduction. Interaction of extracellular signal molecules with cell surface receptors often activates a PLD which is believed to play an important role in the regulation of cell function and cell fate.

Multiple PLD activities were characterized in eukaryotic cells and several PLD genes have been cloned [2]. Mammalian cell contain two PLD genes, PLD_1 and PLD_2, both of which have been cloned and overexpressed in variety of cell lines. PLD_1 was found primarily on intracellular membranes (e.g. Golgi vesicles, endoplasmic reticulum, nuclear membrane etc.) and became active only when stimulated with variety of activators. It was active in vitro but inactive in vivo. PLD_2 was associated mainly with plasma membrane and modestly stimulated by the known PLD activators. It showed low activity in vivo but high activity in vitro.

Signal-activated phospholipases and lipid kinases together represent the molecular basis for the rapidly growing field of lipid signalling. The activity of PLD was stimulated upon receptor ligation by agonists resulting in modification of various lipid constituents of the membrane. This was done either by degradation or by phosphorylation and generation of one or more products (messengers) through recruit or modulates specific target proteins. PC-specific PLD generated PA was considered as the major messenger molecule and choline does not have a signalling function, but it serves as a substrate for acetylcholine synthesis in neurons. Physiological function and molecular biological approaches clears its biological role by several mechanisms [120, 121]. The PA can then be dephosphorylated by PA phosphohydrolase to produce diglycerides (DG) [121]. The DG can activate protein kinase C to regulate a host of cellular responses [126] such as induction of Ca^{2+} influx and inhibition of adenylate cyclase in signal transduction (central component in signal transduction). PLD was also thought to be an integral part of the signalling network involving various phospholipases [120]. The various physiological processes linked to lipid second messengers (PA and DG) are secretion, vesicle trafficking mitosis and meiosis [3]. In phagocytic leukocytes (monocytes, macrophages and neutrophils), activation of PLD regulates cytoskeletal-dependent anti-microbial responses, generation of reactive oxidants and granule secretion [127]. Liscovitch et al. [120] tabulated PLD from different cell types, which were activated by various stimuli, and the main PLD activators were various hormones (e.g. vasopressin, gonadotrophin-releasing hormone), neurotransmitters (cholinergic agonists, histamine and nor adrenaline), growth factor (platelet-derived growth factor, epidermal growth factor), cytokines (tumour necrosis factor-α, fas ligand, Rantes), protein kinase C, ADP-ribosylation factor (Arf) family member, Rho family members, cell surface receptors (e.g. antigen, IgG receptor), extracellular matrix (collagen, fibronectin), drugs and physical stimuli.

Molecular biological approaches concluded that PLD is a membrane-associated enzyme, and its activities have been reported for diverse subcellular organelles including plasma membrane, Golgi vesicles, endoplasmic reticulum, secretary vesicles and nuclear membranes. The major question remains—learning more about its localization, identification, structure and functions of different isoenzymes and its regulatory properties, detail of molecular mechanism of regulation and role of protein kinase C, Rho proteins, ADP-ribosylation factor, G-proteins and Ca^{2+} in each subcellular locations and finally the physiological functions of PLD need to be clarified.

Assay Methods of Phospholipases

Several accurate phospholipase assay methods are known, but a rapid and reliable method for high-throughput screening is still a challenge for efficient detection of superior phospholipases and their practical applications. Some remarkable methods for high-throughput screening of enantioselective biocatalysts particularly lipase have recently been developed using GC and HPLC chromatography, mass spectrometry, IR thermography and

circular dichroism analysis [128]. A proper instrumental configuration for high-throughput methodology will also be required for effective detection and screening of lipase/phospholipase-catalysed reactions. In practice, assay methods (Table 4) for screening and measuring different class of phospholipase activities in a reliable manner are discussed in the paragraphs below.

Plate assay methods are most commonly used for microbial cell-based primary screening of phospholipase activity. Estimation of total phospholipase activity in less quantitative manner can be made using an agar diffusion assay based on the plate assay [129]. Eight-millimetre-diameter wells were cut into 20 ml diffusion plates containing 0.5% (w/v) soybean phospholipids, 50 mM/l CaCl$_2$ and 1% (w/v) agar. Then 150-μl filter-sterilized culture supernatant was added into wells. Plates were incubated at 37 °C for 4 days, and diameter of white zone of precipitation was measured [130]. Ghannoun [4] described a plate method for the detection of phospholipase activity in 30 species of *Candida*, where egg yolk incorporated in dextrose agar-based medium. When grown on this medium, phospholipase-positive *Candida* isolates produce a distinct dense white zone of precipitation around the colony. The white zone was due the formation of calcium complex with the fatty acids released by the action of phospholipase on the phospholipids present in soybean and egg yolk. These assay plate methods are limited to screen only the extracellular phospholipase activity of any microbe. Soybean and egg yolk contain substrates for both phospholipase (phospholipids) and lipases (triglycerides) which makes the assay non-specific. Furthermore, the assay is not suitable for the screening of microbial isolates that produce low levels of phospholipids. Conformation of phospholipase activity necessitates the use of a specific radiometric or colorimetric assay (Table 4) or fast atom bombardment–mass spectrophotometer (FAB-MS) and the use of concentrated cultrate filtrate, particularly in poorly phospholipase-producing strains.

The PLA$_1$, PLA$_2$ and PLC producing microorganisms can be screened, but PLD activity on CaCl$_2$-agar medium cannot be detected. A colorimetric method was developed for detecting PLD activity on solid media using phosphatidyl-2-naphthol as substrate [131]. In this method, the synthetic substrate phosphatidyl-2-naphthol was hydrolyzed by the action of PLD and released 2-naphthol and phosphoric acid. The liberated 2-naphthol was spontaneously coupled with a coexisting diazonium salt (fast blue B salt) and generated coloured azo dye. The generated azo dye was water insoluble; thus, the positions of the enzyme on solid materials such as nitrocellulose membrane, polyacrylamide gel and agar media can be visualized as coloured bands or spots The water-insoluble azo dye was generated from the 2-napthol liberated by PLD, and the colour of azo dye was stable on a nitrocellulose membrane, polyacrylamide gel and agar. Determination of phospholipase activity by a colorimetric assay using pH indicator is well-known screening method. The pH-stat assay using egg yolk lecithin can measure PLA$_1$ and PLA$_2$ activities quantitatively by titration of the acid products released with time by adding NaOH to maintain a constant pH value [132]. Although it was less quantitative method, it can be more suitable for high-throughput screening by adding a proper instrumental configuration such as autotitrator, autoburette, data recorder etc.

The enzyme activities of PLB, lysophospholipase (LPL) and lysophospholipase–transacylase (LPTA) by applying radiometric analysis using thin-layer chromatography (TLC) and NMR spectroscopy were carried out by Cox et al. [133]. The determination of PLB activity, carriers dipalmitoyl phosphatidylcholine (DPPC; 102 nmol) and 1, 2-di-[1-^{14}C] palmitoyl-phosphatidylcholine (20,000 dpm) was dried under nitrogen and resuspended in 125 mM imidazole-acetate buffer, pH 4.0(assay buffer), by sonication. After addition of culture filtrate (1 to 2 μg of total protein) and incubated for 22 min, the

Table 4 Assay methods of different class of phospholipases

PL-type	Substrate	Product	Assay type and principle involved	Experimental procedure	References
PLA$_1$, PLA$_2$	Natural phospholipid, e.g. egg yolk/ soybean	FFA and lysophospholipids	Plate assay: dense white zone of precipitation of calcium complex with FFA around the colony, non-specific, less quantitative, useful for primary screening	Diffusion plates of 0.5% soybean/egg yolk +50 mM CaCl$_2$ + 1% agar were inoculated and allowed for growth at suitable temperature and time	[4, 129, 130]
PLC	Natural phospholipid, e.g. egg yolk/ soybean	Free organic phosphates and DAG	Same as PLA$_1$ and PLA$_2$	Measure area of precipitation	[130]
PLD	Phosphatidyl-2-naphthol	2-Naphthol and phosphoric acid	Solid assay: specific for PLD, less quantitative, useful for primary screening. The liberated 2-naphthol coupled with diazonium salt and generated coloured azo dye on solid material	0.1% emulsified phosphatidyl-2-naphthol, 0.05% fast blue B salt, 0.5% Triton X-100 and 50 mM sodium acetate-acetic acid buffer (pH 5.6) PLD was spotted on nitrocellulose membrane and dipped in substrate mixture and incubated at 37 °C for 1 h. PLD spots become purple colour	[131]
PLA$_1$, PLA$_2$	Egg yolk, lecithin (+ pH indicator)	FFA/phosphoric acid	pH state colorimetric assay: colour change of pH indicator supplemented in agar plates/solution base. Less quantitative can be suitable for high-throughput screening	At a particular time released products were titrated with NaOH and measure change in colour	[132]
PLD	1,2-Dipalmitoyl glycerol [3H] phosphocholine	[3H] Choline	Radioactive TLC assay: enzyme incubated with radiolabelled substrate and phospholipids extraction followed by thin-layer chromatography	250 nCi of L-dipalmitoyl-[choline-methyl-3H] phosphatidylcholine per ml + 14 mM PC (in 20 mM Tris–HCl, pH 7.2) + % Triton X-100 and 6 mM sodium azide. The resulting mixture was incubated, yielding a final volume of 200 μl enzyme for 5 h at 37 °C with continuous agitation	[133]
PLA	Phosphatidylcholine	Free fatty acid	Spectrophotometry assay: coloured product measured at 500 nm	5 mg/ml PC + 3 mm NaNH$_3$ + 0.5% Triton X-100 and 20 mm Tris–HCl (pH 7.2) and PL were incubated for 16 h at 37 °C. The re action stopped by 1 ml chloroform/methanol/ conc. HCl. FFA precipitated with CaCl$_2$ and measured increase in absorbance	[39, 134]
PLA and PLB	4-Nitro-3-octanoyloxybenzoic acid	3-Hydroxy-4-nitrobenzoic acid	Absorbance measured at 410 nm	NOBA (1 mg/ml in assay buffer, i.e. 10 mm Tris, 10 mM/l CaCl$_2$, 0.1 M/	[71, 135]

Table 4 (continued)

PL-type	Substrate	Product	Assay type and principle involved	Experimental procedure	References
				l NaCl, pH 8.0) 1:60 samples, substrate ratio was mixed and incubated at 37 °C and monitored periodically over time	
PLC and PLD	P-Nitrophenyl phosphorylcholine	P-Nitrophenol	Absorbance measured at 410 nm	1 ml reaction mixture containing 1 mm P-NPPC + 3 mm NaN$_3$ + 20 mm CaCl$_2$ + 20 mm MnCl$_2$ + 0.5% Trintox 100 + 20 mm Tris–HCl (pH 7.2), the reaction was started by adding PLC and incubated at 37 °C for 20 h with continuous agitation and stopped by adding 1 ml conc. HCl/chloroform/n-butanol	[39]
PLA$_1$, PLA$_2$	Dilinoleoyl phosphatidylcholine	Linoleic acid	Continuous spectrometric assay (coupled lipoxygenase enzymatic assay): The released linoleic acid was oxidized by lipoxygenase and increase in absorbance measured at 234 nm due to formation of hydroperoxide	1 ml reaction mixture containing 65 μM DLPC, 0.23 μg/ml lipoxygenase and 0.25 μg/ml PLA$_2$ in 50 mM Tris buffer pH 8.5 + 3 mM deoxycholate at 25 °C	[136]
PLC	1,2-sn-Dimyristoyl phosphatidylcholine	1,2-Di-acyl glycerol and phosphate head group	Improved spectrophotometric assay (alkaline phosphatase coupled enzymatic assay): Released phosphate head group was cleaved with alkaline phosphatase and extracted with n-butanol and quantified as phosphomolybdate complex at 700 nm	500 μl of organic solvent DMPC (conc. 1 mM to 10 mM) + 500 μl PLC in borax buffer (100 mM, pH 7.5), 25 μl alkaline phosphatase (60 U/ml) + 200 μl borax buffer incubated for 12–18 h at 37 °C at 300 rpm shaking. Add 150 μl aqueous phase solution (containing 75 μl HCl + 450 μl distilled water+150 μl 2% ammonium molybdate), shake and incubate for 30 min. The formed phosphomolybdate complex was extracted with 600 μl butanol and add saturated SnCl$_2$ in 10% HCl. Organic phase washed with distilled water and blue colour was measured	[137]
PLA$_1$ and PLA$_2$	Phospholipid with alkyl group with a fluorescence	Pyrene-labelled lysophospholipid	Fluorescence-based assay: applicable to all types of PL. Assay based on change in	Shifted in fluorescence emission after phospholipid hydrolysis.	[138, 139]

Table 4 (continued)

PL-type	Substrate	Product	Assay type and principle involved	Experimental procedure	References
	pyrenyl group. PC-CPE (conjugated PC polyelectrolyte)		properties of probe from substrate converted to product	Addition of PLC caused decreased in the fluorescence intensity	
PLC, PLD		CPE-labelled complex	PLC-catalysed hydrolysis of PC and disrupts PC–CPE complex		[140]
PLC	*sn*-1,2-Dimyristoyl phosphatidylcholine	*sn*-1,2-Dimyristoyl glycerol	Bioluminescent assay (using bioluminescent marine bacteria). The substrate *sn*-1,2-dimyristoyl phosphatidylcholine and PLC reaction product *sn*-1,2-dimyristoyl glycerol was further hydrolyzed with lipase. The released myristic acid was quantified directly using the dark mutant of *Vibrio harveyi*	10 μl DMPC (4 mM in ethanol) + 0.5 ml 50 mM Tris–HCl buffer (pH 8.0)+30 mM $CaCl_2$ + 50 mM sodium deoxycholate + PLC, incubated at 37 °C; 0.1 ml of reaction mixture + luminous M-17 bacterium suspension (1 ml), vortex and measured maximum light intensity (I_i). Another 0.1 ml reaction mixture + cold (−70 °C) acetone (0.2 ml) for precipitation of remaining DMPC. Centrifuged and transfer the supernatant, add 0.1 ml lipase, incubate for 10 min, then add luminous M-17 bacterium suspension (1 ml), vortex and measured maximum light intensity (I_f). The peak intensity difference ($\Delta I = I_f − I_i$) was directly related to the amount of myristic acid in produced from DG by lipase or the amount of DG produced from DMPC by PLC	[141]

PC phosphatidylcholine, *P-NPPC* P-nitrophenyl phosphorylcholine, *P-NP* P-nitrophenol, *DLPC* dilinoleoyl phosphatidylcholine, *NOBA* 4-nitro-3-octanoyloxy-benzoic acid, *DMPC* 1,2-*sn*-dimyristoyl phosphatidyl-choline

rate of loss of radiolabelled PC was measured. Both LPL and LPTA activities were measured simultaneously with carrier lyso-PC and radiolabelled 1-[1-^{14}C] palmitoyl-lyso-PC (25,000 dpm) suspended in assay buffer. The reaction was carried out at 37 °C for 15 s by adding enzyme solution give a final volume of 0.125 ml. All the reactions were terminated by adding 1.0 ml of chloroform/methanol (2:1, *v/v*). Reaction products were extracted, separated by TLC and quantitated. LPL activity was measured by the rate of loss of 1-[1-^{14}C] palmitoyl-lyso-PC with release of radiolabelled fatty acids. LPTA activity was estimated by the rate of formation radiolabelled DPPC from radiolabelled 1-[1-^{14}C] palmitoyl-lyso-PC. Phospholipids with [3]H-, [14]C- and ^{32}P-labelled fatty acids are commercially available for radioisotope assay procedures. Although the assay has routinely been used as highly sensitive and reliable method, it has some drawbacks such as

discontinuous, expensive, time and labour-consuming extraction and separation procedures as well as regulations regarding the use of hazardous radio isotopes chemicals.

The radioactive TLC assay [142] using phosphatidylcholine as substrate was commonly used for determination of PLD activity in vitro. The assay involved incubation with a radiolabelled substrate and lipid extraction followed by thin-layer chromatography in order to separate and quantify substrate and product(s). However, the separation principle was not applicable in evaluating anionic substrate phospholipids (like PE, PG, PS and NAPE), NAPE-hydrolyzing PLD activity [143] which produced two lipophilic products, N-acyl ethanolamine (NAE) and PA. Therefore, they developed a rapid assay for the routine detection of NAPE-hydrolyzing PLD activity based on precipitation of radiolabelled substrate (NAPE) in the presence of $ZrOCl_2$, followed by quantification of radiolabelled NAE released into a methanolic supernatant. These assays were discontinuous, expensive and not appropriate when careful kinetics was required.

The spectrophotometric thiol assay was often used as more sensitive and convenient assay for PLA_1, PLA_2 and PLC [144]. The method of Cho and Kezdy [135] was originally used to determine PLA_2 activity with synthetic substrate 4-nitro-3-octanoyloxybenzoic acid. However, Birch et al. [70, 71] showed that it also acts as a substrate for phospholipase B (phospholipid acyl hydrolase activity) and measured phospholipid acyl hydrolase activity, i.e. combination of both phospholipase A and B (Table 4). Jiménez et al. [136] developed a continuous spectrophotometric method for assaying PLA_2 activity. The assay was based on a coupled enzymatic assay, using dilinoleoyl phosphatidylcholine as substrate and lipoxygenase as coupling enzyme. The linoleic acid released by PLA_2 was oxidized by lipoxygenase, and then, PLA_2 activity was measured spectrophotometrically at 234 nm. The increase in absorbance is due to formation of the corresponding hydroperoxide from the linoleic acid. The preparation of phosphatidyl-p-nitrophenol was used as a substrate in assay for the quantitative evaluation of phospholipase D catalysed hydrolytic activity [145]. The method is rapid, accurate and inexpensive. The trans-phosphatidylation activity of PLD enzyme was assayed as specific spectrometric method, in which p-nitrophenol liberated by PLD-catalysed reaction of phosphatidyl-p-nitrophenol and ethanol in an aqueous-organic emulsion system [146]. The most frequently reported spectrophotometric assay for PLC activity is using p-nitrophenyl phosphorylcholine as substrate liberated p-nitrophenol was measured at 410 nm [39, 134]. An improved assay for PLC activity was described by Durban and Bornscheuer [137] in which the liberated phosphate compound was cleaved with an alkaline phosphatase. The phosphate formed was then extracted with n-butanol and reduced by an acidic $SnCl_2$ solution. The phosphate was converted to phosphomolybdate complex and quantified by spectrophotometric measurement at 700 nm. The method was demonstrated for concentration range from 10 nM to 10 mM with different head group phospholipids in an aqueous and a two-phase system. This method has additional advantage that crude enzyme can be used without the purification.

The sensitive and continuous fluorescence-based assays are applicable to all types of phospholipases with availability of suitable substrates and instrumentation. This can be readily applied to high-throughput screening for kinetic analysis and inhibitor discovery [138]. The assays based on change in the properties of the probe as the substrate was converted to product. Pyrene, Bis-BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-S-indacene), coumarin, dansyl, NBD-hexanonyl and naphthyl-labelled phospholipids were used for phospholipases continuous fluorescence assays [139]. A convenient and specific fluorescent turnoff assay for PLC was developed and applied with additional advantages of using natural lipid substrates [140]. The assay principle is based on the reversible interaction between the natural substrate (PC) and a fluorescent (water soluble conjugated polyelectrolyte, CPE). The fluorescent

intensity of the CPE in water was increased substantially by the addition of the phospholipid due to the formation of a CPE–phospholipid complex. Addition of the enzyme PLC caused decrease in the fluorescence intensity (turnoff sensor) due to PLC-catalysed hydrolysis of the PC, which effectively disrupts the CPE–phospholipid complex. The assay has micromolar to 1 nM analytical detection range for PLC.

Spectrophotometric endpoint assay do not permit continuous monitoring of phospholipase activation in live cells. Feng et al. [138] proposed the synthesis as well as the in vitro and in situ evaluation of a novel PC analogue that allowed monitoring of PLA$_2$ activity via sensitive fluorogenic assay. The fluorogenic analogue of PLA$_2$ substrate named Dabcyl-BODIPY-PC or simply DBPC (Dabcyl, 4-4-N,N-dimethylamino phenylazo benzoic acid) was synthesized with a fluorescence quencher, in the sn-1 acyl chain and a BODIPY fluor in the sn-2 acyl chain. PLA$_2$ recognized DBPC and quantifiable fluorescence enhancement of DBPC occurred upon PLA$_2$ hydrolysis. Thus, DBPC can be used as a sensitive fluorogenic probe for in vitro screening for drug discovery and subcellular localization of enzyme activity and would expedite studies of PLA$_2$ in cellular signalling.

FAB-MS had been applied to the study of bacterial phospholipids for chemotaxonomic purposes, and the technique provides useful information about phospholipids and fatty acids and can be applied to complex mixture of these compounds [70]. The negative-ion FAB-MS was used to monitor the breakdown of soybean phospholipids by extracellular phospholipase activities of *Aspergillus fumigatus.* Assay of PLA$_2$ using scattering mode of a spectrofluorimeter avoids time-consuming and expensive fluorescence-labelled reagents. The hydrolysis of dimyristoyl phosphatidylcholine by snake venom PLA$_2$ was monitored by spectrofluorimeter at 650 nm [147]. The detection sensitivity by the scattering mode was double when compared to the titrimetric assay.

A bioluminescent assay for detection of PLC activity using bioluminescent marine bacteria was developed by Cho et al. [141]. The substrate sn-1,2-dimyristoyl phosphatidylcholine and PLC reaction product sn-1,2-dimyristoyl glycerol was further hydrolyzed with lipase. The released myristic acid was quantified directly using the dark mutant of *Vibrio harveyi.*

Industrial Applications of Phospholipases

Phospholipase of the invention could be used in various industrial applications of phospholipases, e.g. use in oil refining, dairy, baking, health food industries etc. PLC and PLD were chiefly used for pharmaceutical, medical and analytical purposes. The microbially produced PLD was mainly discussed for the synthesis of chemicals by trans-phosphatidylation, i.e. exchange of the alcohol moiety attached to the phosphatidic acid residue. PLA$_2$ were reported to be suitable for enzymatic degumming of edible oils and synthesis of triglycerides enriched in polyunsaturated fatty acids (PUFAs). Mammal, plant or microbially produced phospholipases are being sold for these purposes, but the uses of microbial enzymes are cost-effective and environmental friendly.

Use in Health Food Industries

Preparation of Highly Unsaturated Fatty Acids

There has been an increasing awareness in recent years of the nutritional importance of certain fatty acids such as γ-linolenic acid, arachidonic acid and docosahexaenoic acid

(DHA). These occur naturally but often at low levels along with other acids. There is a demand of higher levels of these acids in industries and preparation of pure material for research purpose. While such material can be produced by conventional physical and chemical methods of separation, these are not always appropriate if the material is subsequently to be used for nutritional studies. The problem has been successfully overcome by exploiting the specificity of enzymes. Phospholipase A_2 was used to prepare phosphatidylcholine enriched with n3PUFA [6]. Phosphatidylcholine from egg yolk was hydrolyzed to lysophophatidylcholine (30 °C, 16 h) and re-acylated with a PUFA-enriched mixture of acids containing eicosapentaenoic acid (EPA) 41% and DHA 30%. The phosphatidylcholine finally contained 16% and 11%, respectively, of these two acids. Using a 90% concentrate of DHA, phosphatidylcholine with 35% of DHA was prepared [148].

Pig pancreas phospholipase A_2 was used to mediate trans-esterification of soy lecithin with highly unsaturated fatty acids (at sn2 position) to produce a lecithin. The lecithin could be used for pharmaceutical, nutritional and industrial applications [149–152]. For the trans-esterification, 14 μmol soy PC was combined with 60 μmol of acyl donors (EPA, DHA, EPA ethyl ester or DHA ethyl ester) and 0.55 g glycerol in a screw-cap vial at 600–800 rpm and 25 °C under Argon gas atmosphere. The products of the reaction were treated with chloroform/methanol/water (10:5:3$v/v/v$) and separated in a separator funnel. The chloroform layer with the reaction products was concentrated and applied to silica gel TLC plate and developed with chloroform/methanol/water (65:25:4 $v/v/v$). The lecithin fraction was scraped off and eluted with methanol. As a result, 70% of the total fatty acid at sn2 was incorporated as highly unsaturated fatty acid into soy lecithin. Maruha [152] has developed a method for production of glyceride containing arachidonic acid in high concentration (e.g. 16.5%) by phospholipase A_2. It involved yolk phospholipase hydrolysis, fatty acid extraction and glycerol addition to the fatty acid.

Functional Food Manufacturing

Functional foods are food products that provide a health benefit over and above their basic nutritional value. Degussa food ingredients has a range of phosphatidylserine that was derived from soy in process using trans-esterification (trans-phosphatidylation) reaction of PLD [153]. It is also possible to synthesize naturally occurring low abundance phospholipid such as phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol from phosphatidylcholine a high abundant phospholipid by PLD [154].

Cholesterol Reduction in Food

A method of reducing the cholesterol content of a food involves subjecting the food to either an enzymatic treatment (especially with cholesterol oxidase (EC 1.1 3.6) or a cholesterol oxidase producing microorganism) or to biochemical treatment (degradation of cholesterol) [155]. The treatment with hydrolytic enzyme and the biochemical treatment may be performed simultaneously. The food usually is egg yolk, meat, fish or dairy products. The biochemical, chemical and/or physical treatment of the food to obtain the cholesterol-reduced product was improved by treating it with a hydrolytic enzyme, e.g. phospholipase A1, phospholipase A_2, lysophospholipase phospholipase B or phospholipase D (but not phospholipase C) as an alternative to cholesterol oxidase treatment.

Removal of Mucilage and Content of Phosphorus Component or Degumming of Plant Oil

Vegetable oil contains phospholipids (commonly known as gums), which have a negative impact on the storage stability and downstream processing of the oil. In oil refining industries, PLs have been used for removal of mucilage and content of phosphorus component in crude vegetable oil. It is carried out by PLA_1, PLA_2 or PLB but not with PLC or PLD [156–161].

The phospholipase enzymatic treatment process is applicable to purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil [157–159]. The process for reducing the content of phospholipid in edible oil comprises of two steps, treating the oil with phospholipase (A_1, A_2 or B) to hydrolyze the phospholipids such as lecithin and other accompanying hydrophilic components and separating an aqueous phase containing the hydrolyzed phospholipids from the oil. The process was named 'wet refining to remove mucilage' as it was carried out by extraction with water. A part of phosphotides may be left in the oil through above treatment and are termed as 'non-hydrolic phosphotides' (NHP). NHP mainly consist of lysophospholipids and phosphatidic acids and/ or calcium and magnesium salts. The NHP content should be removed for production of edible oils, and the remaining phosphorus content should not exceed to 5 ppm [157]. One litre of soybean oil was processed for 'wet refining to remove mucilage'. The oil containing 130 ppm of residual phosphorus was heated to 50 °C in a Florence flask; 0.1 g of a pure phospholipase A_2 (an activity of 10,000 U/g/l of phospholipase A_2 unit liberates 1 μmol of fatty acid per minute from egg yolk at 40 °C and pH 8), 1 g of sodium citrate and 20 g of sodium dodecyl sulphate were dissolved in 33.3 g of water, and the solution was emulsified in the oil to form droplets 0.1 μm in diameter using an external centrifugal pump. After treatment for 3 h, a sample removed by centrifugation was found to have an NHP content of 34 ppm of phosphorus. After increasing the temperature to 75 °C and continuing the treatment for one further hour, the NHP content has decreased to 3 ppm of phosphorus. The oil which had thus been treated can be now subjected to physical refining. The microbial origin (e.g. *Aspergillus* sp.) phospholipase A_1, A_2 and B were used for reducing content of phosphorus in plant oils [115, 162, 163].

Use in Mayonnaise Preparations

PLA_2 was used for enzymatic hydrolysis of egg yolk to give better emulsion in mayonnaise preparations [164]. Enhancement of interaction between proteins and phospholipids may be the reason why PLA_2 increases emulsion stability. Structural and functional changes of low density lipoprotein were observed in PLA_2-treated hen egg yolk [165].

Use in Bread Making

Lipases and phospholipases are used in baking industry since 1990. A phospholipase from *Fusarium oxysporum* with both lipase and phospholipase activity was introduced to the market by novozymes A/S for baking application under the trade name Lipopan F™. The value of lipases in bread making lies in their ability to increase loft volume and improved softness. Phospholipase and galactolipase activities were shown to provide even better emulsification in the dough [158, 166, 167]. Lipase/phospholipase baking has optimal activity on the different substrate available in flour such as triglycerides/phospholipids/

galactolipids and giving the optimal gas bubble stability. The phospholipases are able to replace emulsifiers such as diacetyl tartaric acid ester of monoglycerides and sodium steroyl lactylate [7].

Use as Animal Feed Additives

A process for the production of animal feed comprises mixing the phospholipase and at least one phospholipid with feed substances. The composition contains a phospholipid, especially lecithin and PLA_2 [168]. Phospholipase A_2 can be obtained from mammals, plants, microorganisms and/or derived from cattle, pig, mouse, rat or humans. It can also be produced by expression of recombinant DNA in a host cell, especially *Escherichia coli*, *S. cerevisiae* and *Aspergillus niger*. The composition improves lipid digestibility and promotes feed utilization efficiency for feed of non-ruminating animals especially calves.

Use in Synthesis of Surfactants

A surfactant composition contains at least one lysophospholipid, where one of the aliphatic acyl group (R' and R'') and the H was other an aliphatic acyl group, and X was a residue formed by removal of H from a hydroxyl group of polyhydric alcohol (e.g. ethylene glycol, glycerol, sorbitol, mannitol, glucose, galactose, fructose, sucrose or lactose). Partial hydrolysis of lysophospholipid, was performed using a lipase (EC 3.1 1.3) or preferably PLA_2 (EC 3.1.1.4) at pH 7.0–8.5 in the presence of calcium ions. The composition was a water soluble and efficient surfactant [169].

Preparation of Pork Meat Biosensor

A novel time–temperature indicator based on a pig pancreas PLA_2–phospholipid system was developed to monitor quality change of frozen food during storage [170].

Use in Detergent

The detergent composition using phospholipase variant was formulated for hand or machine dishwashing operations, e.g. as described in GB 2,247,025 (Unilever), WO1998/26057 and WO 99/01531 (Procter & Gamble) [158]. In a dishwashing composition, the variant might be effective for removal of greasy/oily stains, for prevention of the staining/discoloration of the dishware and/or plastic components of the dishwasher by highly coloured components and the avoidance of lime soap deposits on the dishware. It was also used as hand or machine laundry detergent compositions, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup [166].

Use in Dairy Industries

Maximizing cheese yield without compromising product quality is a key concern for industrial cheese manufacturers. Normally 85–95% milk fat and 75% of milk protein are

entrapped in the cheese curd. The rest is lost in whey and brine [171, 172]. An enzymatic method for increasing cheese yield using phospholipase was patented by Nielsen [173].

The hydrolysis of milk phospholipids with PLA_1 from *Fusarium venenatum* significantly increased the cheese yield through better moisture and fat retention during whey drainage and stretching. The fungal PLA_1 from *F. venenatum* hydrolyzed the major milk phospholipids PE and PC. The phospholipase A_1 was used to decrease the oiling-off effect in cheese and to increase cheese yield. The oiling-off effect is the tendency of the cheese to form free oil upon storage and/or melting. A suitable dosage of phospholipase will usually be in the range 0.001–0.5 mg enzyme protein per gram milk fat. They manufactured low-moisture mozzarella cheese as an ingredient in food, for instance in pizza, where it is most important functionality was the melting behaviour. They evaluated the flow ability, stretch ability and browning of the phospholipase cheeses [174].

Lipid Second Messengers and Phospholipases

Genetic and pharmacological approaches implicated PLD for control of intracellular membrane transport and recognition of the actin cytoskeleton [121]. The PLD activity leads to formation of phosphatidic acid included in phosphatidylinositol-4 phosphate-5-kinases and the RAF protein kinase. Phosphatidic acid can also be converted to two other lipid mediators, diacylglycerol and lysophosphatidic acid. Regulation of PLD activity is complex, and studies in this area have been further complicated by the lack of good antibodies, purified enzymes and the existence of several isoforms. Mammalian PLD activity was highly regulated by the large number of factors like fatty acids, phosphoinositol, small GTP binding proteins, protein kinase C, Ca^{2+}, phosphorylation and other negative regulators. Many of these factors regulate PLD activation in a synergistic or antagonistic manner. Phospholipase D generates PA which subsequently can be either metabolized by PLA_2 generating lysophosphatidic acid, a potent cellular mitogen, or phosphatidate phosphohydrolase yielding DAG. Sphingomyelinase, a phospholipase C-type enzyme, and related enzymes of sphingolipid metabolism were implicated in apoptosis and other signalling processes.

Other important phospholipases include the phosphatidylinositol-specific PLC, which controls the production of inositol-1,4,5-triphosphate (second messenger) and initiates the signal transduction cascade in response to cell surface receptor activation [117, 118]. C-type phospholipases catalyses the cleavage of membrane phospholipids to 1,2-diacylglycerol and the respective phosphoryl compounds. In eukaryotic cells, activation of PLC frequently occurs through surface receptor stimulation. PLC specific for phosphatidylinositol and its phosphorylated derivatives have been studied extensively [121]. 1,2-Diacylglycerol acts as second messenger in cellular signalling through activation of protein kinase C.

Numerous signal transduction processes involved lipids as signalling molecules. Many of those molecules generated by phospholipases such as PLA_2 released fatty acids (arachidonic acid and lysophospholipids). Each of those products was implicated in signal transduction processes and also served as a precursor for eicosanoids including prostaglandins, leukotrienes and lipoxins or PAF [175]. These compounds were implicated in numerous inflammatory diseases such as rheumatoid arthritis, sepsis, intestinal bowel disease, asthma as well as playing a role in cancer, atherosclerosis and premature parturition. In summary, the phospholipases generate numerous lipid products which control much of cellular signalling. Although the importance of these enzymes in

eicosanoid metabolism signal translation is not questioned, much remains to be studied regarding the regulation of these enzymes and their pathophysiological roles.

Potential Role of Phospholipase as Virulence and Pathogenicity Factor

Microbial pathogens use a number of genetic strategies to invade the host cell and cause infection. Invasion of host cells by microbes entails penetration and damage of the outer cell wall. The transmigration process is mediated by either physical or enzymatic means; phospholipases and proteinases are likely to be involved in the membrane disruption processes that occur during host cell invasion. Destruction of phospholipids, by microbial extracellular PLs and subsequent changes of membrane constituents which lead to cell damage is regarded as major virulence mechanism in infection. Table 2 summarizes the major sources of phospholipases: its involvement in pathogenesis and the substrate specificity for each class of PL.

The phospholipases have been included among the virulence and pathogenicity factor that damage host cells [105, 106]. Evidence implicating phospholipases in host cell penetration, cell lysis and injury by bacteria and fungus have been reported for *Rickettsia rickettsii* [94], *Clostridium perfringens* [12], *Legionella pneumophila* [38–40], *Helicobacter pylori* [55], *Yersinia enterocolitica* [58], *Candida albicans* [4], *Cryptococcus neoformans* [67, 68] and *A. fumigatus* [70].

Bacterial Phospholipase and Pathogenesis

Extracellular phospholipase has been implicated as pathogenesis of disease for bacteria was the subject of some reviews (Istivan and Coloe [105], Schmiel et al. [57], Songer [176], Titball [177]). These reviews have focused on disease properties and the best characterized bacterial phospholipase virulence factors which were mostly PLC and later PLA. We wished to increase the scope of review in terms of the types of phospholipases covered. Some examples of bacteria phospholipase their potential role in virulence and pathogenicity are described here.

PLCs were the best studied bacterial phospholipase produced by the species of *Clostridia, Bacillus* or *Listeria* that were zinc-dependent metalloenzyme with molecular mass in the range 29–43 kDa. *C. perfringens* produces α toxin or lecithinase, i.e. a PLC that damages the membranes of erythrocytes and tissue cells. As in tetanus, gas gangrene results from contamination of wounds, by above bacteria. The bacteria *C. perfringens* is highly invasive and spreads rapidly through tissues. Awad et al. [178] studies the pathogenesis of *C. perfringens*-mediated gas gangrene. Their study provided definitive genetic evidence of the essential role of PLC in gas gangrene or clostridial myonecrosis. They used reverse genetics to construct a suicide plasmid in which the ple gene encoding PLC was inactive. Using this plasmid, they isolated mutant that had lost their ability to produce detectable PLC. Comparing the mutants and their parent in a mouse virulence model showed that the mutants were markedly lass pathogenic than the parent strain. Animals infected with the phospholipase-deficient mutants showed minimal swelling, less muscle destruction, inflammation or necrosis of the infected tissues and remained active with a healthy appearance. Animals challenged with the parent strain showed extensive swelling of the infected foot, leg and lips as well as demonstrable signs of haematuria and extensive muscle destruction. The precise mode of action of α toxin is not yet certain, but

the destructive effects were resulting from products of PLC activity. Incubation with purified α toxin was demonstrated to induce expression of cytokines TNF, αPAF and IL-8. In addition, α toxin activity was shown to trigger the arachidonic acid cascade which leads to cytokine release and inflammation [179]. Williamson and Titball produced genetically engineered vaccine against α-toxin of *C. perfringens*, which protects mice against experimental gas gangrene [180].

L. monocytogenes [16–18, 181] secretes two extracellular PLCs: (a) inosital-specific PLC (PI-PLC) and (b) PC-PLC. PC-PLC was broadly active PLC with the ability to hydrolyze macrophages, fibroblast and epithelial cell phospholipids. The zinc-metallo-PLC was active towards PC, but individual enzymes have different activities towards other phospholipids and sphingomyelin [177]. These two PLCs induced ceramide generation (product of PLC hydrolysis of sphingomyelin), nuclear factor-KB activation and *E*-selectin expression in human epithelial cells which contribute to immune modulation and the pathogenesis of *L. monocytogenes* [19].

Several PLCs including phosphatidylinositol-specific PLC, PC preferring, as well as sphingomyelinase were produced by *B. cereus* [27–29]. The contribution of PLs to the virulence of *B. cereus* has not been investigated in animals, and PLC of this bacterium was considered to be nontoxic. The PC-preferring PL and the sphingomyelinase were active in cancer to cause haemolysis [182]. Wazny et al. [183] reported that *B. cereus* strains producing PLC caused degranulation of human neutrophils with dose-dependent release tissue damage and suggested that *B. cereus* protects itself against phagocytosis by releasing PLs.

The phospholipase A activity has been identified in a number of pathogenic bacteria, but its role in virulence is yet to be fully elucidated. However, evidence suggests the PLA activity has a significant role in pathogenesis of *Rickettsia prowazekii* [34, 35]. The demonstration of host cells exposed to large number of *Rickettsia* species release considerable amounts of lysophospholipids and free fatty acids into the culture medium. Silverman et al. [33] confirmed these findings and provided suggestive evidence that phospholipase activity penetrates the host cells. *H. pylori* possess several different phospholipase activities such as PLA_1, PLA_2 and PLC. These were linked to the degradation of the phospholipids components of the mucosal barrier and were able to initiate production of leukotrienes and eicosanoids from arachidonic acid. This can also affect membrane permeability and mucous discharge [55]. Moreover, the pH-activated PLA_2 in *H. pylori* acted as an initial mucosal barrier breaker in pathogenesis of peptic ulcer [56]. The research indicated that the gastric juice of *H. pylori*-infected individuals contained significantly higher PLA_2 and PLC concentrations than those of healthy individuals.

The PLC activities in several *Legionella* were associated with pneumonia, destruction of lung surfactant and epithelial cells [38]. Flieger et al. [39] reported two PLA activities (PLA and lysophospholipase) in *L. pneumophila* and considered them as powerful agents in the mediation of pathogenicity due to destruction of lung surfactant and epithelial cells. PLA activity was secreted prior to lysophospholipase activity which led to accumulation of the cytotoxic agent [40].

The extracellular PLD of *C. pseudotuberculosis* [36] in caseous lymphadenitis of small ruminants played role in pathogenesis. *C. pseudotuberculosis* PLD predominantly found in the secreted fraction degraded sphingomyelin and lysophosphatidylcholine (product of PLA hydrolysis of PC). Purified PLD was dermonecrotic and lethal when injected into animals [184]. PLD-toxoid vaccines gave significant protection against caseous lymphadenitis in sheep, supporting the role of PLD in tissue destruction either directly or indirectly. The gene encoding PLD in *C. pseudotuberculosis* has been cloned and sequenced [37]. Targeted mutagenesis of PLD reduced the ability of the bacterium to establish a primary infection [36].

There are some reports of PLB produced by pathogenic bacteria such as *Moraxella bovis* [1]. However, there is no evidence that these enzymes are toxic or play role in virulence from bacteria.

Fungal Phospholipase and Their Potential Role in Virulence

Studies on fungal phospholipases have mainly focused on the characterization of PLB from non-pathogenic fungi, including *S. cerevisiae* [74, 75], *Torulaspora delbrueckii* [79], *Schizosaccharomyces pombe* [78] and *Penicillium notatum* [76, 77]. Of the medically important fungi, extracellular phospholipase activities have been described in *C. albicans* [4], *A. fumigatus* [70] and *C. neoformans* [67]. Fungi possess a broad range of hydrolytic enzymes that attack neutral lipid and phospholipids especially during infection of mammalian host. PLA_2 and PLB enzymes released by fungi can play important roles not only for nutrient acquisition and tissue invasion but also for intricate modulation of the host's immune response.

Extracellular phospholipases as virulence factor in *C. albicans* pathogenesis were proven [4]. *C. albicans* causes candidiasis of nail, skin and mucous membranes and are superficial mycoses or dermatomycoses type of fungal disease. The fungus invades subcutaneous tissues or the lungs from which they spread to other organs of the body, become established and produce disease. Infection with *C. albicans* in mouse also caused a mycotic endocarditis, pulmonary moniliasis and virginities. In the patients with impaired immunity due to cancer or AIDS, the incidence of fungal infections has risen dramatically. *C. albicans* was most commonly isolated pathogen from nosocomial blood stream infection in the USA, and oral candidiasis was one of the first indicators of HIV disease progression [60]. Samaranoyake et al. [61] screened 41 *Candida* isolates for PL activity by using plate assay method and found that 79% of the *C. albicans* strains tested produced extracellular PLs. Ibrahim et al. [62] carried out a comparative study in vitro on 22 isolates of *C. albicans* (11 isolates were obtained from patient with disseminated candidiasis and 11 were commensal isolates recovered from the oral cavities of healthy volunteers) by using egg yolk agar-based method. They found significant higher levels of PL production in the blood isolates then in the commensals. In the same study, they examined nine blood isolates for expression of virulence factors like phospholipase and proteinase production, adherence, germination, growth rate and ability to damage endothelial cell. The high phospholipase production was correlated with increased virulence in mouse model of disseminated candidiasis. To obtain further evidence of the contribution of PLs to candidal pathogenicity, the cloning of two genes encoding PLB (caPLB1 and caPLB2) and one gene (caPLD) coding for PLD was carried out [63, 64]. Gene disruption/deletion is frequently used to determine the functionality of a specific gene. A number of genes were disrupted to evaluate their contribution to the virulence of *C. albicans* [4, 155]. PLB-deficient mutants were constructed by targeted gene disruption using the URA3-blaster technique, which reduced the ability of *C. albicans* to secrete the enzyme [65]. Mouse models for haematogenous dissemination candidiasis as well as an infant mouse oral intragastric infection model were used to determine the role of caPLB1 in virulence of *C. albicans* [66]. In haematogenous dissemination model, 5×10^5 cells were intravenously injected in both parent type and the PLB-deficient mutant strain separately. Parent strain model caused infection in 9 days whereas no infection was found with PLB-deficient mutant till 15 days. In the experiments with oral intragastric infant mouse model, the infant mice were inoculated with 2×10^8 blastospore. Liver and kidney colonization for parental strain was

90% and 70%, respectively, whereas the liver and kidney colonization for caPLB1-deficient mutant was 45% and 27%, respectively.

Two additional genes were isolated for PLB (caPLB3 and caPLB4) and another gene caPLB5 encodes PLA_2 [105]. caPLB5 null mutants were constructed in a wild-type strain of *C. albicans*. Phenotypic analysis of the caPLB5 null mutants revealed loss of cell-associated PLA_2 activity and significant decrease in liver colonization in an experimental mouse model for disseminated candidiasis. Thus, *C. albicans* has both PLA_2 and PLB activities involved in pathogenesis and may have high degree of regulation and functional diversity.

A. fumigatus is a human opportunistic fungal pathogen causing a wide range of diseases including allergic, bronchopulmonary aspergillosis and invasive aspergillosis mainly in immunosuppressed patients. Several pathogenicity determinants for virulence of *Aspergillus* species have been suggested. Some of these are products of elastase, ribotoxin and other extracellular toxins. There are no specific virulence factors which have been determined. Birch et al. [70] demonstrated the production of extracellular PLC activity in *A. fumigatus* by using FAB-MS. Further the same group compared clinical and environmental isolates of *A. fumigatus* for extracellular PLC and a phospholipids acyl hydrolase (PLA and/or PLB) activity. Clinical isolates of *A. fumigatus* produced the largest zone size in a diffusion assay and more extracellular PLC than environmental isolates. However, environmental isolates of *A. fumigatus* showed increased acyl hydrolase activity compared to clinical isolates. The study suggested that PLC activity were important in the pathogenicity *A. fumigatus*. Three PLB genes (afplb1, afplb2, afplb3) from *A. fumigatus* have been reported using degenerated primer in PCR amplification [72]. The influence of lecithin on the expression of these genes was investigated and found that gene transcripts of afplb1 and afplb3 were upregulated by lecithin (respectively 5-fold and 300-fold) whereas afplb2 expression was not affected. Lecithin (dipalmitoyl phosphatidylcholine) was major component of lung surfactant, and lungs were the main portal of entry for *A. fumigatus*, therefore indicating that the PLBs were potentially involved in the pathogenesis. The extracellular PLBs (Afplb1 and Afplb3) and PLC activities as virulence factor in cell biology of *A. fumigatus* remains to be tested. This may be important in the interaction of *A. fumigatus* with host cell membranes.

Chen et al. [67, 68, 116] examined 50 *C. neoformans* isolates for extracellular PL activity using an egg yolk-based agar assay. Forty-nine of these isolates produced a pericolonial precipitate indicative of PL activity. Further, they identified enzyme activities in one clinical isolate (strain BL-1 of *C. neoformans* var. *grubii*) as PLB, LPL (lysophospholipase) and LPTA (lysophospholipase/transacylase) activities by applying radiometric analysis using TLC and NMR spectroscopy. The enzyme was purified to homogeneity from *C. neoformans* using $(NH_4)_2SO_4$ fractionation and hydrophobic interaction, anion exchange and gel filtration chromatography. All three enzyme activities co-purified as a single protein that was an acidic glycoprotein containing *N*-linked carbohydrate moieties with p*I* values of 5.5 and 3.5. The enzyme was active only at acidic pH (pH optimum of 4.0 for PLB and 4.0–5.0 for LPL and LPTA). Wright et al. [69] isolated another phospholipase with only LPL and LPTA activities from *Cryptococcus gattii* and found it to be a 66-kDa protein (by SDS/PAGE). The PI was 6.3 and it was active at high temperatures (to 70 °C) as well as both acidic and neutral pH values.

The phospholipase-encoding gene (PLB1) was cloned from *C. neoformans* and constructed plb1 mutants using targeted gene disruption. The enzyme activities were markedly reduced in the mutant plb1 strain and were significantly less virulent than the control strains in both the mouse inhalational model and the rabbit meningitis model [67].

They were also able to restore virulence to the plb1 strain by reconstitution of extracellular phospholipase activities and concluded that the PLB1 gene product was virulence factor for *C. neoformans* in experimental cryptococcosis. The finding provided insight into the pathogenesis of fungal infections and allowed for the potential targeting of extracellular phospholipases in the development of anti-fungal drugs. The same group further showed that anti-cnplb1 antibodies were found in the sera of patients for up to 2 years after cryptococci infections. The potential of PL was demonstrated by Hanel et al. [185] who found increased survival of *C. albicans*-infected mice when phospholipase inhibitors were given in combination with the anti-fungal fluconazole.

Conclusion and Outlook

The phospholipases produced from mammals, plant or microbes are commercially available for industrial purposes. The use of microbial phospholipases is environmental friendly and cost-effective. The use of thermostable enzymes can improve the properties of industrial phospholipases and provide benefits like increased substrate solubility, decreased viscosity of the medium and a lower risk of microbial contamination. Thus, the search for microbial thermostable phospholipases is yet another area of study. A thermostable PLA_1 was created by evolutionary molecular engineering by using a combination of mutagenic PCR and filter-based screening. The thermal stability and catalytic activity of PLA_1 from *Serratia* sp. strain MK1 were successfully improved and had broadened the possibility of the application of this PLA_1 in various phospholipid-related industries [186].

The recent progress on virulence and pathogenic factor of phospholipases were derived from studies of phospholipases from pathogenic bacteria to fungi and their hydrolytic functions causing direct tissue damage. The other possible roles of phospholipases in pathogenesis include the liberation of lipid second messengers, such as inositol triphosphate, arachidonic acid, and the lysis of phagocytic cells and compartments [57, 187]. Prostaglandins are potent regulators of host immune response. Enhanced prostaglandin production during fungal infection could be an important factor in promoting fungal colonization and chronic infection. Host cells are one source of prostaglandins. Another potential source of prostaglandins is the fungal pathogen itself. The production of prostaglandins was determined in pathogenic yeasts *C. neoformans* and *C. albicans*. The role of prostaglandins in fungal biology and disease pathogenesis was also defined [188]. *C. neoformans* and *C. albicans* both secreted prostaglandins de novo or via conversion of exogenous arachidonic acid. Pathogenic fungi produce eicosanoids like prostaglanding or leukotrienes from arachidonic acid. The role of gene cnPLB in eicosanoid production from *C. neoformans* was investigated and demonstrated that PL was necessary for release of precursor arachidonic acid from arachidonoylphosphatidylcholine [188–191]. The discovery that pathogenic fungi produce and respond to immunomodulatory eicosanoids reveals a virulence mechanism that has potentially great implication for understanding the mechanisms of chronic fungal infection, immune deviation and fungi as disease cofactors. Moreover, the knowledge of phospholipase gene regulation in vivo would be helpful into further understanding of bacterial or fungal infection.

Once the importance phospholipase in bacterial or fungal virulence is proven, the next challenge would be drug discovery: to use these enzymes in vaccine development, to identify, improve and design phospholipase inhibitors and use enzyme as diagnostic marker of infections [192]. Approaches for evaluating the significance of phospholipases as virulence factor for the disease and identification of its inhibitors from different anti-fungal

or antibacterial herbs or by chemical inhibitor would be potentially an effective therapy for several inflammatory conditions. PLA_2 inhibitors are used as potential anti-inflammatory agents for inhibition of PLA_2 activity and are suggested for their therapeutic use [193, 194]. The starting point in seeking new and specific anti-inflammatory drug against such enzymes (PLs) are ellagic acid derivatives, koninginins E and F. Ellagic acid derivatives isolated from *Casearia sylvestris*. These derivatives are characterized as anti-PLA_2 activity [195]. *Trichoderma* fungi produce substances known as koninginins that have great structural similarity to compounds like flavonoids and vitamin E and are able to inhibit the PLA_2. These isolated compounds can be used for medicinal applications, particularly in inflammatory reactions. The isolated koninginins A, E and F from *Trichoderma koningii* (endophytic fungus) were analysed for capabilities of inhibiting oedema-inducing, myotoxic and enzymatic activities of the total venom of *Bothrops jararacussu* snake. Koninginins E and F are more efficient in the inhibition of PLA_2, and the structure and action mode of these molecules is similar to vitamin E.

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