Novel Lysophospholipase A Secreted by *Legionella pneumophila*

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**We show that *Legionella pneumophila* possesses lysophospholipase A activity, which releases fatty acids from lysophosphatidylcholine. The NH$_2$-terminal sequence of the enzyme contained FGDSLS, corresponding to a catalytic domain in a recently described group of lipolytic enzymes. Culture supernatants of a *L. pneumophila* pilD mutant lost the ability to cleave lysophosphatidylcholine.**

*Legionella pneumophila* is a gram-negative bacterial pathogen, which secretes several enzyme activities, including phospholipase A (PLA) activity (2, 11). PLAs, a heterogeneous group of enzymes produced by bacteria as well as eukaryotic cells catalyze the removal of a fatty acid from phospholipids generating cytoxic lysophospholipids (27). When fatty acids are then liberated from lysophospholipids, the responsible enzyme is named lysophospholipase A (LPLA). Although more frequently described for phospholipase C enzymes, PLAs are suspected to be virulence factors of bacteria (9, 13, 26). Phospholipases contribute to bacterial escape from phagosomes and host cells after intracellular multiplication (7), the destruction of macrophages and epithelial cells (3, 21, 23, 30), the generation of signal transducers such as lysophosphatidylcholine and derivatives of both arachidonic and linoleic acid (18, 24), the destruction of lung surfactant (12, 15), and the induction of inflammation (16).

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Since *L. pneumophila* destroys phospholipids such as phosphatidylcholine and phosphatidylglycerol, as well as lysophosphatidylcholine and monoacylglycerol (2, 11, 12), we investigated whether distinct lipolytic enzymes were responsible for the cleavage of the different lipid substrates. A 10-fold-concentrated supernatant was prepared as described before from 5 liters of *L. pneumophila* strain 130b (Wadsworth, ATCC BAA-74) culture material using BYE broth containing 0.25% yeast (10, 11, 12). Anion-exchange chromatography (AEC) was performed as before, except 5 mM Na$_2$EDTA was added to both equilibration and elution buffer (11). Eluted fractions were tested for PLA, LPLA, and lipase. For PLA, we identified activities that preferentially hydrolyzed phospholipids containing both fatty acids, such as dipalmitylophosphatidylglycerol (DPPG) and dipalmitoylphosphatidylcholine (DPPC) at final concentrations of 5 mg/ml. LPLA activities were defined as those that released free fatty acids (FFA) from monopalmitylophosphatidylcholine (MPLPC; final concentration, 3.4 mg/ml). Finally, lipase activities were those that hydrolyzed 1-monopalmitoylglycerol (1-MPG; final concentration, 2.2 mg/ml). All substrates were obtained from Sigma-Aldrich (Munich, Germany), and methodologies were as described before (11). AEC revealed four major peaks (fractions 7, 10, 13, 15) capable of causing DPPG and DPPC hydrolysis, suggesting that *L. pneumophila* secretes different PLA activities (Fig. 1). All four fractions liberated more FFA from DPPG than from DPPC. Fraction 17, however, showed a distinct ability to release fatty acids from MPLPC, indicating that *L. pneumophila* possesses PLA activity. Since this fraction also cleaved 1-MPG (Fig. 1), it is possible that the *Legionella* PLA acts on nonphospholipids, a scenario with some precedent (25, 28).

Reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on AEC fractions containing the novel PLA activity (Fig. 1). A 28-kDa protein in fractions 16 to 18 paralleling the hydrolytic activities on MPLPC and 1-MPG was suggested to be responsible for release of FFA from both substrates (Fig. 1). For better resolution of PLA, fraction 17 was then applied onto a 1-ml Resource Q column (Amersham Pharmacia Biotech, Freiburg, Germany) using the same conditions as in the earlier AEC (Fig. 2). One peak (i.e., fractions 3 and 4) capable of hydrolyzing both MPLPC and 1-MPG was identified. Fraction 3 contained several proteins, with the predominant band being 28 kDa (Fig. 2). The intensity of the 28-kDa-protein band paralleled the hydrolytic activity of MPLPC, suggesting that this band corresponds to the PLA enzyme. To further purify PLA, fraction 3 was concentrated five-fold by speed vacuum evaporator (Savant, New York, N.Y.) and then subjected to gel filtration, using the same equilibration buffer as that described for AEC, except without the addition of Na$_2$EDTA. As shown in Fig. 3, the PLA activity elution profile consistently paralleled the fractionation of the 28-kDa band, supporting our notion that this protein represents PLA.

As a next step toward identifying the *L. pneumophila* PLA, NH$_2$-terminal amino acid sequence was determined from the major protein bands (28, 29, 41, and 50kDa) present in fraction...
3 (see Fig. 2) according to the following method. After fraction 3 was concentrated 20-fold using a MICROCON centrifugal filter device with a 10-kDa molecule cutoff (Millipore, Eschborn, Germany), it was subjected to SDS-PAGE. The separated proteins were then electrotransferred to an Immobilon-PSQ membrane (Millipore) according to the manufacturer’s specifications. After staining with Ponceau-S solution (Sigma-Aldrich), the protein bands were excised and placed upon a trifluoroacetic acid-treated glass fiber filter, coated with 0.75 mg of Polybrene (BioBrene Plus; PE Biosystems, Weiterstadt, Germany), within the standard reaction cartridge of the protein sequencer Procise (PE Biosystems model 494A). Sequencing was performed according to the manufacturer’s protocols.

Both the 28-kDa and the minor 29-kDa band yielded an identical NH₂-terminal amino acid sequence, TPLNNIVVFG DSLSDNG, indicating that they derive from one parental protein. Upon using the BLAST program of the National Center for Biotechnological Information, the 17 NH₂-terminal amino acids revealed its closest homology to a portion of the lecithinase from Vibrio mimicus (accession number AF035162) (1). It also showed relatedness to the lipase/acyltransferase of Aeromonas hydrophila (P10480), which belongs to a new family of lipolytic enzymes containing the sequence FGDSLS in their active sites (29). The serine centremost in the consensus motif is proposed to be the nucleophilic agent that attacks the lipid substrate (29). In contrast to its location in other lipases, the
active site of this family is closer to the NH$_2$ terminus (29). Since the 28- and 29-kDa proteins of \textit{L. pneumophila} contain the FGDSLS sequence in their NH$_2$ terminus, we hypothesize that they are members of this new enzyme family.

The FGDSLS enzymes often reveal higher molecular masses than the 28-kDa LPLA detected in \textit{L. pneumophila} (29). For example, the lipase/acyltransferase from \textit{A. hydrophila} possesses an unprocessed molecular mass of 37.1 kDa. However, proteolytic cleavage by trypsin results in a 27-kDa protein connected to a 4.7-kDa C-terminal peptide by a disulfide bridge which is lost under reducing SDS-PAGE (14). Therefore, we believe that the unprocessed molecular mass of LPLA is higher than 28 kDa. Indeed, the observation of 28- and 29-kDa \textit{Legionella} proteins with identical NH$_2$-terminal sequences suggests an incomplete activation of LPLA by a serine protease. In addition to being subject to proteolytic processing, the FGDSL enzymes appear to exist in multimeric complexes, e.g., the well-characterized lipase/acyltransferase of \textit{A. hydrophila} forms dimers (4). When the native molecular size of LPLA was estimated by gel filtration, it revealed a size of about 90 kDa (data not shown), implying that the \textit{Legionella} molecule may also combine in a multimer or protein complex. A higher native molecular size of LPLA was also suggested by the fact that LPLA activity was not found in the filtrate (<30 kDa) but in the concentrated fraction (>30kDa) after ultrafiltration of the culture supernatant (data not shown).

Since fractions which liberated high amounts of fatty acids from MPLPC exhibited hydrolytic activity toward 1-MPG (Fig. 1, 2, and 3), we believe that the \textit{L. pneumophila} LPLA is indeed able to hydrolyze nonphospholipid substrates. The hydrolysis of both MPLPC and 1-MPG by LPLA corresponds to the substrate specificities of the FGDSL enzyme family noted above (29). However, it should be acknowledged that \textit{L. pneumophila} may produce other lipases able to act on more apolar lipids.

The 50- and 41-kDa proteins, which fractionated with LPLA (Fig. 1, 2, and 3), revealed the NH$_2$-terminal sequence XKNV VLDAIKEHDAKFV and EKVQAKGMGFGGNRK, respectively. The 50-kDa protein sequence showed closest homology to a portion of the glutamine synthetase from \textit{Azospirillum brasilense} (P10583) (6). The second sequence identified the 41-kDa protein as the well-characterized zinc metalloprotease of \textit{L. pneumophila} (M31884) (5). It is possible that the zinc metalloprotease cleaves LPLA under natural and/or experimental conditions.

Many exoenzyme activities of \textit{L. pneumophila} are dependent upon the \textit{Legionella} prepilin peptidase (PilD) and type II protein secretion apparatus (2, 24a). This is the case for PLA and lipase activities (2). Now, we wanted to clarify whether LPLA belongs to the \textit{pilD}-dependent secreted enzyme activities. Thus, culture supernatants of a \textit{pilD}-deficient mutant (NU243) of strain 130b were prepared as described before and then tested for their relative abilities to release FFA from MPLPC
(2, 11, 20). In four trials, the ability of the pilD mutant to hydrolyze MPLPC was considerably reduced, i.e., NU243 supernatants had 7.47% ± 4.44% of the activity of wild-type 130b. Thus, the LPLA activity is PilD dependent and therefore its loss could, at least in part, account for the reduced cytotoxicity, intracellular multiplication, and virulence of the L. pneumophila pilD mutant (20).

There are a number of ways in which LPLA of L. pneumophila might contribute to the development of Legionnaires’ disease. For example, since cell swelling may be essential for bacterial multiplication in host cells and since glycerophospho-

rycholine, a reaction product of LPLA, causes cell swelling, LPLA is suspected to contribute to Legionella intracellular growth (11, 19, 22). Furthermore, LPLA might protect L. pneumophila from toxic lysophosphatidylcholine that is generated by action of PLA on surfactant (12, 30).

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