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Purification and Characterization of *Listeria monocytogenes* Phosphatidylinositol-Specific Phospholipase C

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We have purified to homogeneity the 33-kDa phosphatidylinositol-specific phospholipase C (PI-PLC) from the culture fluid of *Listeria monocytogenes*, a facultative intracellular pathogen. The protein was overexpressed, and secretion of PI-PLC was further enhanced by the addition of divalent cations to the culture medium. The basic protein (pI, ~9.4) was complexed with anionic proteins in the crude culture fluid. It bound to DEAE-Sepharose and was eluted from Sephacryl S-200 near the void volume in low-ionic-strength buffer, suggesting aggregates of ≥150 kDa. Gel filtration chromatography on Sephacryl S-200 in the presence of 1 M ammonium sulfate resulted in disaggregation and complete separation of PI-PLC, which interacted with the column matrix. Amino-terminal sequencing of the pure protein gave results consistent with the previously deduced sequence and showed that the signal cleavage site was between alanine 29 and tyrosine 30. The enzyme was specific for PI and showed no activity with phosphatidylethanolamine, phosphatidylcholine, or phosphatidylserine. It did not cleave PI-4-phosphate or PI-4,5-bisphosphate, but it was active on the membrane form of the variable surface glycoprotein from *Trypanosoma brucei*, a PI-glycan-anchored protein. When assayed with deoxycholate-mixed micelles of PI, activity was highly dependent on added salt. Activation by salt was also observed with Triton X-100-mixed micelles. The optimal concentration of $CaCl_2$ or $MgCl_2$ was lower than that of KCl or $(NH_4)_2SO_4$, but activity was not specifically dependent on divalent cations and was not inhibited by addition of EDTA. With deoxycholate, the optimum pH was 7.0. A broader pH optimum ranging from 5.5 to 6.5 was observed with Triton X-100-mixed micelles. These results are consistent with a postulated role for secreted PI-PLC in the acidified primary phagocytic vesicle of infected cells.

Listeria monocytogenes, a gram-positive, nonsporeforming, facultatively anaerobic rod, is the cause of serious infections, including septicemia in pregnant women and meningoencephalitis in neonates and in immunocompromised adults (7). It has been studied as a model facultatively intracellular bacterial pathogen, chiefly in terms of its interactions with the cell-mediated immune system of the host (31, 37). Recently, the processes of host cell invasion, escape from the phagosome, growth in the cytoplasm, and cell-to-cell spread have been morphologically delineated (6, 10, 11, 36, 48). Several genes have been shown to be significant for intracellular growth and cell-to-cell spread, and these have been the subject of a recent review (39). Three of these genes encode proteins that interact with host cell membranes, including *hly*, the gene for listeriolysin O, a pore-forming cytolysin; *plcB*, the gene for a broad-specificity phospholipase C (PC-PLC); and *plcA*, the gene for a phosphatidylinositol (PI)-specific phospholipase C (PI-PLC), the enzyme which is the topic of this report. These three genes are coregulated by the product of *prfA*, a positive regulatory protein (9, 26, 34). Both lysteriolysin O (13) and the PC-PLC (14) have been purified, but the recently identified PI-PLC (4, 25, 33) had not been isolated.

Phosphoinositide-specific PLCs have been identified in both eukaryotic and prokaryotic organisms. The eukaryotic enzymes are involved in signal transduction processes, in which polyphosphoinositides are cleaved to yield water-soluble inositol phosphates and diacylglycerol. These products play distinct roles in mobilizing intracellular calcium ions and in the activation of protein kinase C, respectively (2). The enzymes isolated from prokaryotic sources catalyze the hydrolysis of PI and proteins that are anchored to eukaryotic cells by means of a PI-glycan (28). Although PI-PLC activity has been found in the culture filtrates of several human pathogens, including *Bacillus anthracis* (29), *Staphylococcus aureus* (27), and *Clostridium novyi* (47), and is known to be made in vivo during *B. anthracis* infections of laboratory animals (29), a role in pathogenesis, if any, has yet to be documented. PI-PLC was recently shown to be an extracellular product of *L. monocytogenes* (4, 25, 33). Strains defective in *plcA* were originally isolated during a screen of transposon insertion mutants that formed small plaques in monolayers of mouse fibroblasts (44). However, recent studies indicate that the small-plaque phenotype resulted from a polar effect of these mutations on the downstream gene *prfA* (5, 33).

Although PI-PLC from *L. monocytogenes* has homology with the enzymes isolated from *Bacillus thuringiensis* (16, 24) and *Bacillus cereus* (22, 51), there are a number of significant differences in its predicted amino acid composition and sequence (4, 25, 33). The protein from *L. monocytogenes* has an excess of basic amino acids and would be expected to have an isoelectric point (pI) of >8.5, unlike the enzymes from the *Bacillus* species, which have a net negative charge. In view of its postulated role in the intracellular growth of *L. monocytogenes* (5), we have undertaken the isolation and characterization of this protein.

MATERIALS AND METHODS

Materials. L-3-Phosphatidyl[2-^{3}H]inositol ([^{3}H]PI, 17.5 Ci/mmol), L-3-phosphatidylethanolamine 1,2-di[^{14}C]palmitoyl (117 mCi/mmol), and L-3-phosphatidyl-L-[U-^{14}C]serine, 1,2

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dioleoyl (60 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. *sn*-1,2-Dipalmitoyl phosphatidyl[*methyl*-^{3}H]choline (37 Ci/mmol), phosphatidyl[2-^{3}H] inositol-4-phosphate ([^{3}H]PIP, 4.4 Ci/mmol), and phosphatidyl[2-^{3}H]inositol-4,5-bisphosphate ([^{3}H]PIP_2, 5.4 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. The membrane-bound form of [^{3}H]myristate-labeled variable surface glycoprotein (mfVSG) (11,000 dpm/μg) from *Trypanosoma brucei* was the kind gift of Paul T. Englund. PI-PLC isolated from *B. thuringiensis* was kindly provided by Martin G. Low. Phospholipids, bovine serum albumin, and protein molecular weight markers were obtained from Sigma Chemical Co., St. Louis, Mo. Molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were also obtained from Bio-Rad, Richmond, Calif. The sources of chromatographic materials are indicated in the text. Other reagents were obtained from a variety of sources and were of reagent grade or better.

Bacterial strains and culture conditions. *L. monocytogenes* 10403S (40) was the wild-type strain initially used for studies on the secretion of PI-PLC (4). *L. monocytogenes* DP-L1470 was constructed by transforming strain 10403S with plasmid pAM401 (53), containing the *L. monocytogenes* genes *plcA* and *prfA* (5). For isolation of PI-PLC, strain DP-L1470 was grown in Luria-Bertani (LB) (32) broth supplemented with 40 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 μg of chloramphenicol per ml. Cultures containing 300 ml of this medium in 2-liter flasks were inoculated with a 10% inoculum of cells grown overnight in the same medium without $CaCl_2$ and $MgSO_4$. Cultures were incubated at 37°C on a rotary shaker at 180 rpm for 7 h. Cells were harvested in a refrigerated centrifuge at 3,000 × *g* for 30 min. The supernatant fluid from several flasks was pooled and stored at 4°C after addition of 0.05% sodium azide.

Assays. PI-PLC was assayed essentially as described (27) except that [^{3}H]PI (0.025 μCi, 17.4 Ci/mmol) and 13.5 μg of PI were sonicated in 100 μl of 40 mM Tris-HCl (pH 7.2)–0.2% deoxycholate per assay. The standard assay mix also contained 1 mM $CaCl_2$ and 114 mM $(NH_4)_2SO_4$. The PI sonicate was incubated with enzyme diluted in brain heart infusion (BHI) broth unless otherwise indicated, in a final volume of 200 μl. Incubations were from 1 to 5 min, depending on the state of purification of the enzyme. One unit of activity represents the release of 1.0 μmol of water-soluble radioactive [^{3}H]PI per min. For assay of hydrolysis of ^{14}C-labeled acyl chain phosphatidylethanolamine, the chloroform-soluble products were chromatographed with carrier diacylglycerol on Silica Gel 60 thin-layer chromatography (TLC) plates (E. Merck, Darmstadt, Germany) in petroleum ether-ethyl ether-acetic acid (50:50:1, vol/vol/vol), and the diacylglycerol and unhydrolyzed phospholipid were scraped off and assayed for radioactivity. For assay of hydrolysis of PIP and PIP_2, the water-soluble hydrolysis products were assayed for radioactivity as described above, and the lipid-soluble material was chromatographed with carrier PI, PIP, and PIP_2 in chloroform–methanol–4 N NH_4OH (9:7:2, vol/vol/vol) on Silica Gel 60 TLC plates which had been treated with potassium oxalate (49). Protein concentration was determined by the bicinchoninic acid method (43) with bovine serum albumin as a standard and the reagents supplied by Pierce (Rockford, Ill.).

mfVSG phospholipase activity. The release of [^{3}H]myristate from *T. brucei* mfVSG was measured as described by Hereld et al. (17) with the following changes. Tris buffer at pH 7.2 rather than pH 8.0 was used, and the reaction was stopped with toluene-acetic acid (10:1, vol/vol) as described by Ferguson et al. (8), rather than with butanol, as preliminary experiments revealed rapid activation of phospholipase activity on mfVSG after the addition of butanol. The volumes of toluene-acetic acid and water were adjusted to the volume of the incubation, 25 μl. The lipid product was recovered in the toluene phase from 100-μl incubations and chromatographed on a Silica Gel 60 TLC plate in ethanol-ethyl ether-acetic acid (50:50:1, vol/vol/vol).

Purification of PI-PLC. (i) Ammonium sulfate precipitation. Protein in the culture supernatant was concentrated at 4°C by addition of solid ammonium sulfate to 80% saturation followed by 15 to 30 min of stirring. The insoluble proteins were sedimented at 10,000 × *g* for 10 min at 4°C. The precipitates were dissolved in 20 mM MOPS (morpholinepropanesulfonic acid) buffer, pH 7.2, and any undissolved material was removed by centrifugation as described above. The protein solution was then dialyzed twice against MOPS buffer at 4°C. Enzyme preparations were stored at 4°C; however, all subsequent purifications were performed at room temperature.

(ii) DEAE-Sepharose chromatography. The conductivity of the dialyzed enzyme was measured, and the enzyme was either diluted with MOPS buffer or dialyzed again to give a conductivity below 1.5 mMho, if necessary. The solution was then applied to a column of DEAE-Sepharose (1.6 by 10 cm; Sigma Chemical Co.) which had been equilibrated with MOPS buffer. The column was washed with 10 to 20 ml of MOPS buffer and eluted with a linear gradient from 0 to 0.8 M KCl in MOPS buffer; the total volume was 110 ml. Elution was completed with a further addition of 20 ml of 1.0 M KCl in MOPS buffer.

(iii) Sephacryl S-200 chromatography. The active fractions from the DEAE-Sepharose column were pooled and concentrated with a CX-30 filter (Millipore, Bedford, Mass.) to approximately 8.0 ml. This was then loaded onto a Sephacryl S-200 (Pharmacia, Piscataway, N.J.) column (1.7 by 78 cm) which had been equilibrated with MOPS buffer, and elution was done with the same buffer.

(iv) Sephacryl S-200 chromatography in 1 M $(NH_4)_2SO_4$. The active fractions from the first Sephacryl column were concentrated as described above and brought to 1.0 M $(NH_4)_2SO_4$ by addition of solid reagent. This solution was applied to the same Sephacryl S-200 column, which had been equilibrated with 1 M $(NH_4)_2SO_4$ in MOPS buffer. The column was eluted with 1 M $(NH_4)_2SO_4$ in MOPS buffer.

SDS-PAGE. Protein solutions were boiled for 3 min with an equal volume of treatment buffer (0.125 M Tris-Cl [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol, 4% SDS. Trichloroacetic acid (10%) precipitates were boiled in 2× treatment buffer. Samples were subjected to SDS-PAGE in a discontinuous buffer system (23) with slab gels of 10% acrylamide. Gels were stained with either 0.125% Coomassie brilliant blue R or a silver staining kit (Bio-Rad, Richmond, Calif.), according to the instructions provided by the manufacturer.

Isoelectric focusing. Gels (0.5 mm thick) were prepared with 4% acrylamide and 2% BioLyte Ampholines (Bio-Rad). The gradient was from pH 3.5 to 10. The gel was run at 4°C for 3 h at 200 V, fixed in 12.5% trichloroacetic acid, and stained with the Bio-Rad silver staining kit. The pH was read directly from gel slices in a separate lane, which were placed in distilled water.

N-terminal amino acid sequence analysis. Sequence analysis was performed by the Laboratory of Protein Chemistry, University of Pennsylvania School of Medicine, with a model 473A (Applied Biosystems, Inc., Foster City, Calif.)

TABLE 1. Effect of addition of salts to LB broth on the release of PI-PLC by *L. monocytogenes*

Addition(s) (mM)	Enzyme activity[a] (nmol/min/ml)			
	10403S		DP-L1470	
	5–6 h	7–8 h	5 h	6 h
None	1.2, 3.4	3.9, 3.7	46	86
$CaCl_2$ (2.5) + $MgSO_4$ (40)	40 ± 6	33 ± 3	1,680	1,950
$CaCl_2$ (2.5) + $(NH_4)_2SO_4$ (40)	38, 30	12, 34		
$MgCl_2$ (40)	ND[b], 23	74, 26		

[a] Cultures (10 ml) were grown in 250-ml flasks with shaking at 37°C, and activities were measured in culture supernatant fluids. The activities are expressed as nanomoles of PI hydrolyzed per minute per milliliter of culture supernatant. Individual values or means ± standard deviation (n = 4) are shown.

[b] ND, not detectable.

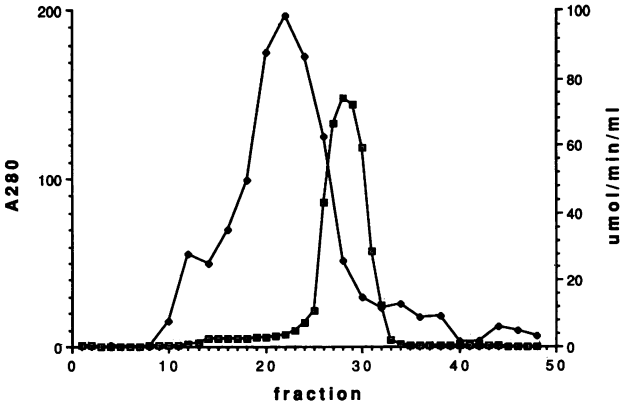

FIG. 1. Chromatography of the PI-PLC of *L. monocytogenes* DP-L1470 on DEAE-Sepharose. The enzyme solution, containing 230 mg of protein, was applied to a column (1.6 by 10 cm) in 20 mM MOPS buffer, pH 7.2. Fractions (3 ml) were collected at a flow rate of 1.4 ml/min. □, A_{280}; ◆, enzyme activity.

gas phase sequencer according to the instructions of the manufacturer.

RESULTS

Expression of PI-PLC in LB broth. We tested for the release of PI-PLC in various media. In LB broth, growth of the wild-type strain 10403S was essentially complete after 3 h, resulting in low culture densities (optical density at 600 nm, ~0.5). PI-PLC activity was generally highest at 6 to 8 h after inoculation, but the final yield of PI-PLC in LB broth was low (Table 1). Addition of $CaCl_2$ (2.5 mM) and $MgSO_4$ (40 mM), which were found to stimulate expression of *plcA* transcription (38a), resulted in a ninefold increase in activity. No other combination of salts tested resulted in consistently greater activity at both 5 and 7 h after the culture was initiated. Addition of 40 mM $MgCl_2$ resulted in PI-PLC activity close to that observed when $CaCl_2$ and $MgSO_4$ were added (Table 1). With strain DP-L1470, there was a 20-fold increase in PI-PLC activity in LB medium, and stimulation with added $CaCl_2$ and $MgSO_4$ was still seen. In other experiments, it appeared that similarly high activities could also be obtained by addition of $MgSO_4$ alone at 40 mM (data not shown).

Purification. The DNA sequence predicted a mature protein with 25 acidic amino acids and 38 basic amino acids and a pI of >8.5. However, preliminary trials with the cation-exchange medium carboxymethyl-Sepharose revealed no binding of the enzyme in 20 mM MOPS buffer (pH 7.2). Surprisingly, 80 to 90% of the enzyme bound to an anion-exchange column of DEAE-Sepharose in the same buffer and could be eluted with a salt gradient. Most of the activity emerged near the beginning of the gradient with a small peak of protein, with the bulk of the protein emerging after the PI-PLC activity peak (Fig. 1). After concentration, the material was loaded onto a Sephacryl S-200 column and eluted with MOPS buffer (Fig. 2A). Most of the activity emerged close to the void volume, suggesting that the protein was complexed with other proteins in aggregates of ≥150 kDa. This was also consistent with the anomalous behavior of the enzyme on ion-exchange resins.

In order to test this hypothesis, a means of gently breaking these complexes was sought. Trials with either 6 M urea or guanidine at concentrations up to 4 M revealed substantial loss of activity (data not shown). Since experiments had shown stimulation of the PI-PLC activity of crude enzyme by the presence of up to 0.7 M $(NH_4)_2SO_4$, we tested for the dissociation of enzyme aggregates by 0.5 M $(NH_4)_2SO_4$ on Sephadex G-100 columns and found that PI-PLC no longer emerged near the void volume. Disaggregation and separation were achieved on a column of Sephacryl-S200 in the presence of 1 M $(NH_4)_2SO_4$ (Fig. 2B). PI-PLC activity emerged beyond the expected volume for a 34-kDa protein and the activity peak was broad, suggesting either protein heterogeneity or interaction with the column. The protein that emerged, however, gave a single band on SDS-PAGE when stained with either Coomassie blue (Fig. 3) or silver stain (not shown), indicating interaction with the column as the more likely possibility. A summary of the purification scheme is presented in Table 2, and the results of SDS-PAGE of the active fractions obtained during this purification from a culture in which the protein was highly expressed by *L. monocytogenes* DP-L1470 are shown in Fig. 3.

N-terminal sequence and pI. The N-terminal sequence of the pure protein, determined by the solid-phase phenylthiohydantoin amino acid derivative method, was found to be YSLNNWNKPI, corresponding to amino acids 30 to 39 after a putative translational start site (4, 25, 33) at base number 1247 in the DNA sequence reported by Mengaud et al. (35). The pI was found to be between 9.2 and 9.6 by isoelectric focusing followed by silver staining. The protein appeared to be homogeneous after isoelectric focusing.

Properties of the purified enzyme. (i) Activation by detergents and substrate dependence. The PI-PLC activity of both the crude and purified enzyme was almost completely dependent on the presence of detergents. As shown in Fig. 4, at pH 7.2, 0.1% deoxycholate- and 0.05% Triton X-100–PI mixed micelles were equally effective substrates when assays were performed with $(NH_4)_2SO_4$ at optimal concentrations for each detergent (see below). Higher concentrations of detergent resulted in greatly decreased activity. In 0.1% deoxycholate, the reaction is essentially saturated with PI above 0.15 mM (data not shown). The standard assay condition, 0.08 mM PI, gave optimal activity with a detergent-to-substrate molar ratio of 29:1.

(ii) Requirement for added protein. During purification, it was found that dilution of the enzyme into buffer resulted in low activities. Therefore, enzyme was usually diluted in BHI broth (4). With purified enzyme, however, addition of a

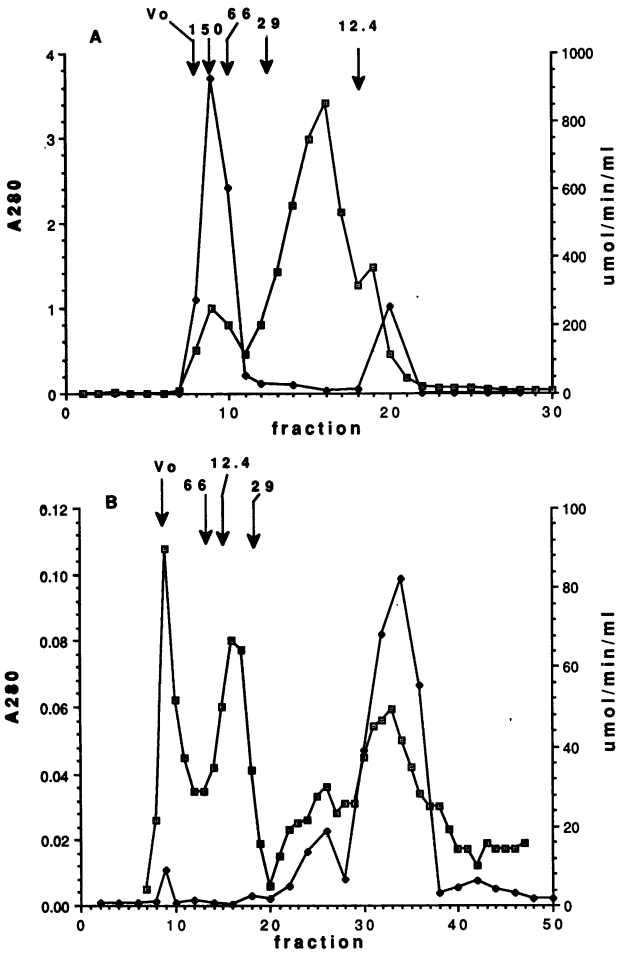

FIG. 2. (A) Chromatography of the PI-PLC obtained from a DEAE-Sepharose column on Sephacryl S-200 in 20 mM MOPS buffer, pH 7.2. The enzyme solution contained 58 mg of protein in 8.0 ml of MOPS buffer and was applied to a column (1.7 by 78 cm). Fractions (6 ml) were collected at a flow rate of 1.4 ml/min. □, A_{280}; ◆, enzyme activity. The positions of molecular mass markers (in kilodaltons) are shown: alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa. (B) Chromatography of the PI-PLC obtained from the first Sephacryl S-200 column on Sephacryl S-200 in 20 mM MOPS buffer, pH 7.2, containing 1 M $(NH_4)_2SO_4$. The enzyme solution, containing 5.5 mg of protein in approximately 8 ml of MOPS buffer containing 1 M $(NH_4)_2SO_4$, was applied to the same Sephacryl S-200 column, which had been equilibrated with MOPS buffer containing 1 M $(NH_4)_2SO_4$. Fractions (6 ml) were collected at a flow rate of 1.4 ml/min. □, A_{280}; ◆, enzyme activity. The positions of molecular mass markers chromatographed in 1 M $(NH_4)_2SO_4$ buffer are shown. Note the reversal of the positions of cytochrome *c* (12.4 kDa) and carbonic anhydrase (29 kDa) in high-salt buffer. V_0, void volume.

crude mixture of protein was not desirable. Titration with bovine serum albumin showed that maximal activity was obtained when the enzyme was diluted in a 0.5% solution in MOPS buffer before addition to the reaction mixture (data not shown).

(iii) Effect of pH. In the assay in the presence of 0.1% deoxycholate, there was a sharp pH optimum close to 7.0 with MOPS-MES (morpholineethanesulfonic acid) buffer mixtures (Fig. 5A). However, it was noted that at pHs below 6.5, the suspension of PI in deoxycholate was no longer

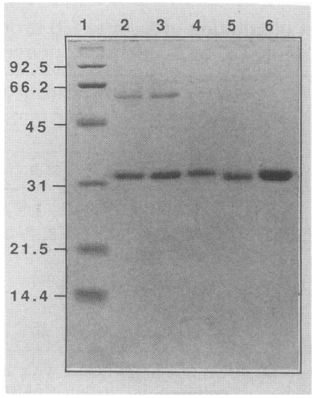

FIG. 3. SDS-PAGE done during fractionation of the PI-PLC of *L. monocytogenes*. Numbers indicate the molecular mass (in kilodaltons) of the standards. Lane 1, molecular mass standards; lane 2, concentrated culture supernatant (1 mg of protein); lane 3, 0 to 80% ammonium sulfate precipitate (1 mg of protein); lane 4, active fractions from DEAE-Sepharose (25 μg); lane 5, active fractions from the first Sephacryl S-200 column (10 μg); lane 6, active fractions from the second Sephacryl S-200 column (20 μg). The gel was stained with Coomassie blue.

clear. The effect of pH was therefore tested in the presence of a nonionic detergent. A broad pH optimum extending to pH 5.5 was observed (Fig. 5B).

(iv) Requirement for salt. During purification, it was observed that dilution of the enzyme in BHI after ammonium sulfate precipitation or DEAE-Sepharose chromatography resulted in decreased enzyme activity and that activity could be restored by addition of 0.1 M ammonium sulfate. With the pure enzyme assayed in the presence of 0.1% deoxycholate, addition of ammonium sulfate produced optimum activity at 100 mM. Activation by KCl was highest at 300 mM (Fig. 6A). When assayed in the presence of 0.05% Triton X-100, the dependence on added salt was not as strong. Activation by ammonium sulfate peaked at 20 mM, but considerable activity was seen at up to 300 mM (Fig. 6B). The addition of KCl resulted in optimal activation at 70 mM.

(v) Requirements for divalent cations. Mengaud et al. found that PI-PLC activity in crude extracts was not inhibited by EDTA or EGTA (ethylene glycol tetraacetic acid) (33). We have performed similar experiments with the highly purified enzyme. Although 1 mM calcium chloride was included in the standard assay mix used during purification, we found that its omission did not decrease activity with the purified enzyme and that addition of EDTA at concentrations of up to 1.0 mM had no effect. Neither was an effect seen after addition of *o*-phenanthroline at concentrations up to 0.16 mM (data not shown). We also tested for inhibition by higher concentrations of divalent cations, as has been observed with PI-PLC from *B. cereus* (45) and *B. thuringiensis* (21, 46). When ammonium sulfate was omitted, both $CaCl_2$ and $MgCl_2$ stimulated PI-PLC activity in the presence of Triton X-100. The optimal concentration was determined for each salt (data not shown), and maximal activity was observed at 4 mM. Optimal $CaCl_2$ or $MgCl_2$ concentrations produced 28 and 9% greater activity than 20 mM $(NH_4)_2SO_4$, respectively. In a separate experiment, further addition of $CaCl_2$ up to 20 mM resulted in 40% less activity than was seen at 4 mM, whereas $MgCl_2$ at 20 mM resulted in 20% lower activity. These effects are similar to those seen with $(NH_4)_2SO_4$ or KCl at high concentrations in the presence of Triton X-100 (Fig. 6B).

TABLE 2. Purification of PI-PLC from *L. monocytogenes* DP-L1470

Purification step	Protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)	Recovery (%)
80% $(NH_4)_2SO_4$ precipitation	210	11,500	54.9		100
DEAE-Sepharose	55	12,400	220	4.0	108
Sephacryl S-200 in 20 mM MOPS	5.8[b]	4,300[b]	740	14	38[b]
Sephacryl S-200 in 1 M $(NH_4)_2SO_4$	1.06[b]	5,400[b]	5,100	93	47[b]

[a] Micromoles of PI hydrolyzed per minute.
[b] Values are corrected for samples taken for protein and activity determinations.

Activity on other phospholipids. The highly purified enzyme was tested on phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. Essentially no hydrolysis was observed with these substrates at concentrations varying from 0.03 to 0.45 mM in 0.1% deoxycholate. When the same preparations of these phospholipids were tested at 0.15 mM with the broad-specificity PLC from *B. cereus* (Sigma), the lipids were readily hydrolyzed. Activity was also tested on PIP and PIP_2 with the crude and pure enzyme. Although approximately 0.5% release of [^{3}H]inositol was observed in the water-soluble fraction, hydrolysis was not dependent on the time of incubation (1 to 8 min) or the amount of pure enzyme added (up to 6 ng). All of the water-soluble radioactivity released by either crude or pure enzyme could be accounted for by small amounts of contaminating [^{3}H]PI, which was rapidly hydrolyzed.

Phospholipase activity on mfVSG. The pure protein hydrolyzed *T. brucei* mfVSG ([^{3}H]myristate-labeled) to release labeled myristate in a toluene-soluble form. The activity of *L. monocytogenes* PI-PLC was compared with that of *B. thuringiensis* PI-PLC at pH 7.2. When 2 μg of mfVSG was treated in a 25-μl incubation, 1 mU (0.2 ng) of the enzyme from *L. monocytogenes* released 0.33% ± 0.05% (n = 3) of the radioactivity per min, compared with 4.2% ± 0.6% (n = 2) for the enzyme from *B. thuringiensis*. Incubation with 11.5 mU of the *L. monocytogenes* enzyme for 20 min resulted in release of 30% of [^{3}H]myristate. Ten milliunits of the *B. thuringiensis* enzyme produced the same degree of hydrolysis in 1 min and essentially complete hydrolysis in 5 min. The lipid product was identified by TLC. It migrated with carrier diacylglycerol and gave the same ratio of 1,3-diacylglycerol to 1,2-diacylglycerol as the product produced by treatment with PI-PLC from *B. thuringiensis*, 9.4 and 9.8% 1,3-diacylglycerol isomer, respectively.

DISCUSSION

We have purified and characterized the PI-PLC from a facultative intracellular human pathogen, *L. monocyto-*

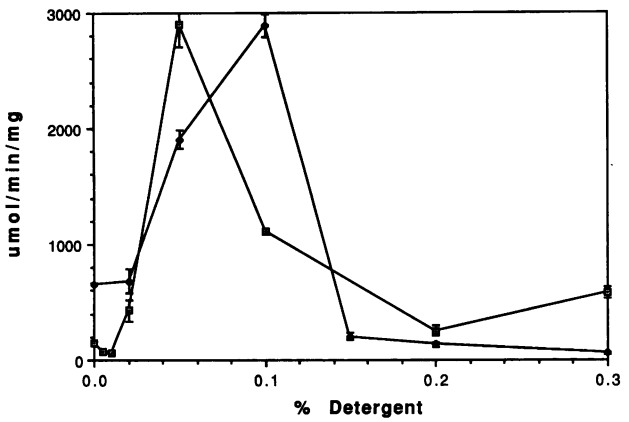

FIG. 4. Effects of detergents on enzyme activity. Enzyme purified through the second Sephacryl S-200 column (0.6 ng), diluted in 0.5% bovine serum albumin, was incubated under standard conditions with PI dispersed in 100 μl of various concentrations of Triton X-100 (□) or sodium deoxycholate (◆) to yield the final concentrations shown when diluted to a final volume of 200 μl. The ammonium sulfate concentration for the Triton X-100 curve was 20 mM. The bars represent the range of two measurements.

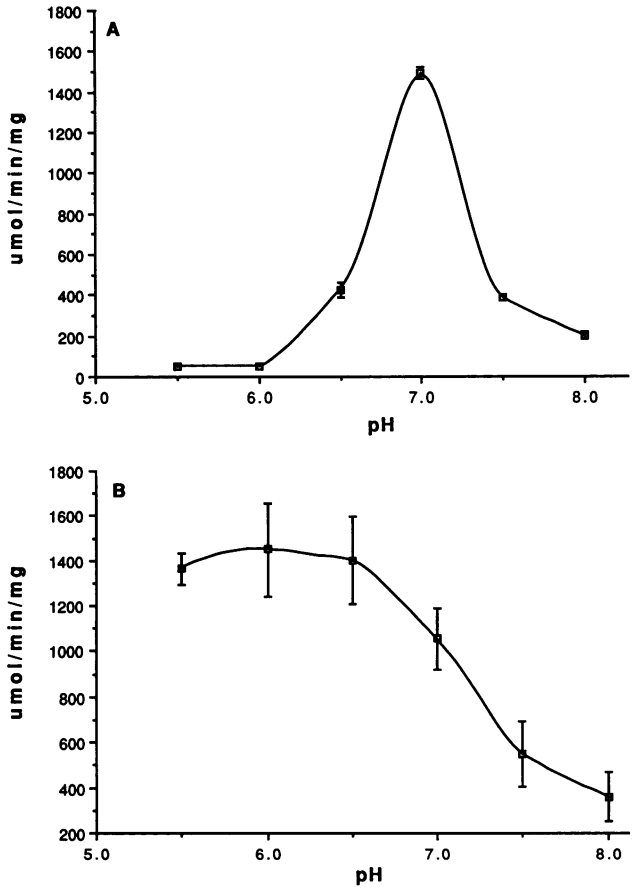

FIG. 5. Effects of pH on enzyme activity. Enzyme purified through the second Sephacryl S-200 column, diluted in 0.5% bovine serum albumin, was incubated under standard conditions with MOPS-MES buffers (50 mM) of the desired pH. (A) Substrate dispersed in 0.1% deoxycholate, 2 ng of protein per assay. The bars represent the range of two measurements. (B) Substrate dispersed in 0.05% Triton X-100, 1.2 or 2 ng of protein per assay. The data represent the mean ± standard deviation for four measurements.

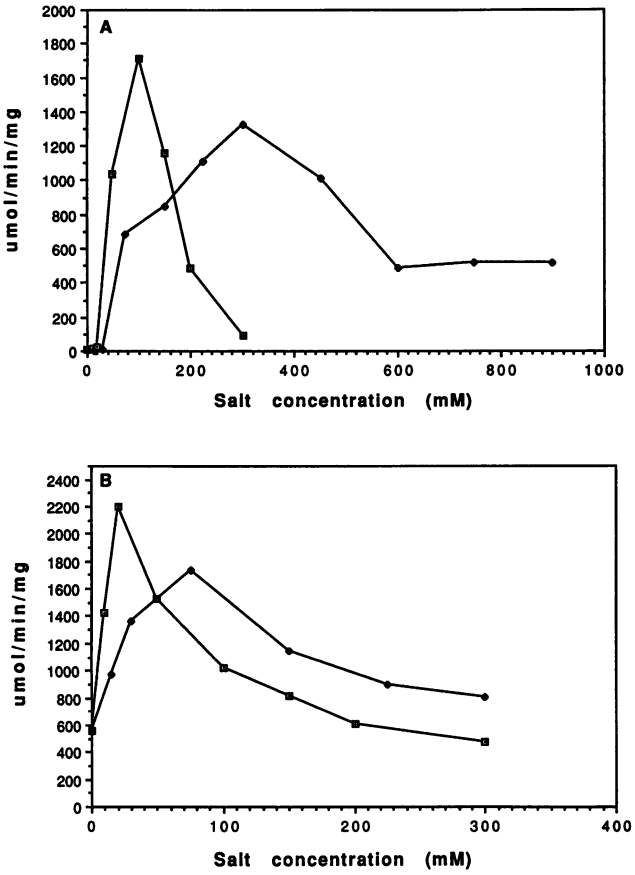

FIG. 6. Effects of salts on enzyme activity. Enzyme purified through the second Sephacryl S-200 column, diluted in 0.5% bovine serum albumin (2 ng per assay), was incubated under standard conditions. (A) PI was dispersed in 0.1% deoxycholate–$(NH_4)_2SO_4$ (□) or KCl (◆). (B) PI was dispersed in 0.05% Triton X-100–$(NH_4)_2SO_4$ (□) or KCl (◆). Each set of curves is from a representative experiment.

genes. The enzyme has been overexpressed by taking advantage of strain DP-L1470 (5), which has a multicopy plasmid containing the structural gene for PI-PLC, *plcA*, and the gene for a protein that serves as a positive regulator of *plcA* expression, *prfA* (9, 26, 34). This strain showed an approximately 20-fold increase in PI-PLC activity over the wild type (Table 1). The addition of $CaCl_2$ and $MgSO_4$ to LB broth resulted in a further 30-fold enhancement of PI-PLC activity in the culture fluid. Purification of PI-PLC to homogeneity was achieved in four steps that can be carried out in 4 to 5 days and, with the exception of the ammonium sulfate precipitation, at room temperature. The enzyme is relatively stable and can be stored for 2 months in 1 M $(NH_4)_2SO_4$ at 4°C without loss of activity.

The first 10 amino acids have been sequenced, and cleavage of the signal sequence was shown to occur between alanine 29 and tyrosine 30 of the sequence proposed for this protein (4, 25, 33). The cleavage site obeys the "−3, −1" rule for predominantly uncharged, small residues at these positions, found in a variety of proteins secreted by bacteria (52). PI-PLC purified from the overexpressing strain 1470 gave a single band on SDS-PAGE after silver staining, with an apparent molecular mass of 32.5 ± 0.5 kDa. This can be compared with the predicted molecular weight of 32,935 determined from the sequence of the cloned *plcA* from strain 10403S. This sequence differs from that of *L. monocytogenes* LO28 (35) at amino acids 81 (Thr→Arg), 112 (Asn→Lys), and 119 (Phe→Tyr) in the mature protein and at amino acids 4 (Asn→Ile), 7 (Glu→Arg), and 13 (Leu→Val) in the signal sequence (5).

The PI-PLC secreted by *L. monocytogenes* is basic but appears to aggregate with anionic proteins. It bound to anion- rather than cation-exchange chromatography media, it migrated close to the void volume on Sephacryl S-200 columns at low ionic strength, and it was found to be dissociated on Sephacryl S-200 chromatography in the presence of 1 M $(NH_4)_2SO_4$. After this step, the enzyme bound to carboxymethyl-Sepharose, as expected (data not shown). If needed, further purification could be obtained by hydrophobic interaction chromatography on a propyl-agarose column. However, this step was not required when the enzyme was highly expressed, as shown in Fig. 3.

The properties of the PI-PLC varied markedly depending on the detergents used in the assay. In the presence of deoxycholate, the activity was highly sensitive to pH and to the presence of salts. It was not dependent on calcium ions, nor was it affected by the addition of EDTA, in agreement with the results obtained with the crude culture supernatant (33). It was also not affected by the addition of *o*-phenanthroline, which complexes other divalent cations, such as Zn^{2+} and Fe^{2+}. In the presence of Triton X-100, the pH optimum is shifted to lower pHs. Similar disparities have been found with PI-PLC from *B. thuringiensis* assayed in the presence of deoxycholate, which gave a pH optimum of 7.5 (20), or Triton X-100, which gave a broad pH optimum at 5.6 to 6.5 (21). The pH curves with both deoxycholate- and Triton X-100-mixed micelles (Fig. 5) were obtained in the presence of 114 mM ammonium sulfate. However, the optimum ammonium sulfate concentration with 0.05% Triton X-100 was 20 mM at pH 7.2 (Fig. 6B). As expected, when PI-PLC activity was measured with Triton X-100-mixed micelles in the presence of 20 mM ammonium sulfate, we observed 2.4 times higher activity at pH 7.5 and 1.5 times higher activity at pH 7.0 than in the presence of 114 mM ammonium sulfate (data not shown). At lower pHs, the assay was not linear during a 1-min incubation. It appeared that the enzyme was losing activity below pH 7.0 at the lower salt concentration and that high ionic strength may serve to preserve enzyme activity at low pHs. Although the enzyme is not as strongly dependent on added salt when PI is dispersed in Triton X-100 micelles, significant activation was seen with $(NH_4)_2SO_4$, KCl (Fig. 6B), $CaCl_2$, and $MgCl_2$, with lower optimal concentrations for the divalent cation salts. The enzyme is clearly not specifically dependent on added divalent cations. Stimulation by salts can result from interactions with either the substrate or the enzyme.

Salt dependence and lack of strong inhibition at high salt concentrations differentiate *L. monocytogenes* PI-PLC from the *Bacillus* enzymes (21, 45, 46), suggesting direct effects on the enzymes. However, it is also possible that differences in the structures and surface charges of these enzymes modify their interactions with PI-detergent micelles of various sizes and charges. Further detailed kinetic studies may provide insights into these complex interactions. We suggest that the purified enzyme be assayed in the presence of Triton X-100 in future work, in order to avoid the strong salt and pH effects observed in the presence of deoxycholate, as has been found for other phospholipases (41).

The highly purified enzyme showed no activity on phosphatidylethanolamine, phosphatidylcholine, phosphatidyl-

serine, PIP, or PIP_2. These results are consistent with those obtained for other PI-PLCs isolated from bacterial sources (15) and place this PI-PLC in the type II category defined by Low and Saltiel (30). The specific activity of the purified enzyme is also similar to that found for the enzymes from *B. cereus* and *B. thuringiensis* (15). In distinction from the type II enzyme from *B. cereus*, which has a pI of 5.4 (20), and that from *B. thuringiensis*, with a pI close to 5.1 (19), the pI of the *L. monocytogenes* enzyme is ~9.4. The products of hydrolysis of PI catalyzed by the crude enzyme from *L. monocytogenes*, diacylglycerol (4, 33) and inositol 1,2-cyclic phosphate (4), have been identified previously. As observed with crude supernatants of *L. monocytogenes* (33), the homogeneous PI-PLC is also capable of cleaving a PI-glycan protein, the mfVSG of *T. brucei*. It yields the same lipid product, diacylglycerol; however, its activity at pH 7.2 was only 8% of that of the enzyme from *B. thuringiensis* when comparable PI hydrolysis activity units (nanomoles of PI hydrolized per minute) were tested.

Initial studies on transposon insertion mutations in *plcA*, which caused greatly decreased virulence, were complicated by the polar effects of these mutations on the downstream gene *prfA*. Accordingly, an in-frame deletion mutation of *plcA* has been constructed, and this mutant was found to be much less affected in its virulence for mice than the transposon insertion mutants. Electron microscopic comparisons of the *plcA* deletion mutant with the wild-type strain have demonstrated delayed escape from the primary phagocytic vesicle in bone marrow-derived macrophages, suggesting that PI-PLC is needed for efficient lysis of host phagosome membranes (5).

The inability of the enzyme from *L. monocytogenes* to cleave PIP and PIP_2 decreases the number of substrates that could be cleaved during the passage of this organism through a mammalian host cell. Nevertheless, its ability to cleave the PI-glycan of PI-anchored proteins and PI affords several potential targets. PI-anchored proteins are usually found on the external surface of the cell (29, 30) and would therefore be present in the lumen of the endocytic vesicle, offering protracted opportunities for cleavage of the protein anchors. Although PI is generally believed to be present in the inner monolayer of the cell membrane, recent studies have provided evidence for the presence of approximately 20% of total PI in the outer monolayer of the human erythrocyte membrane (3, 12) and for as much as 50% in the outer monolayer of the rat hepatocyte plasma membrane (18). Hydrolysis of PI both in the lumen of the endocytic vesicle and on the cytosolic faces of cellular membranes (18) would produce diacylglycerol, an activator of protein kinase C, and inositol 1,2-cyclic phosphate, a potential intracellular growth signal (42), but there is at present no evidence for effects on host intracellular signalling. The activity of PI-PLC on PI-Triton X-100 micelles at pH 5.5 and 6.0 (Fig. 5B) suggests that the enzyme, if expressed, would be active in acidified phagocytic vesicles and in the cytosol. Listeriolysin O, in contrast to other related hemolysins, is optimally active at pH 5.5 and is inactive at pH 7.0, suggesting that an acidified phagosome is the site of its activity in the host (13). The definitive role of PI-PLC in pathogenesis and the intracellular substrates, whether bacterial or host, remains to be clarified. It is important to note, however, that only pathogenic species in the genus *Listeria* secrete PI-PLC (33, 38).

L. monocytogenes also secretes a broad-specificity PLC (1) of 29 kDa, conventionally designated PC-PLC, which has recently been purified (14). Strains with mutations in the gene for this enzyme, *plcB*, make small plaques on 3T3 fibroblast monolayers, and electron microscopic studies of these mutants suggest that the PC-PLC is involved in lysis of the double membrane vacuole formed after infection of a cell by engulfment of the bacterium within a pseudopodlike structure formed in infected cells during cell-to-cell spread (50). It is possible that the two phospholipases act sequentially or in concert both during escape from the initial single-membrane endocytic vesicle and during escape from the double membrane vesicle found after cell-to-cell spread.

In this study, purification and characterization of the PI-PLC has established its size, pI, requirements for activity, and substrate specificities. Additional studies on its ability to hydrolyze lipids in natural membranes under a variety of conditions are needed. Other studies on the expression of the enzyme in host tissues will be aided by the availability of specific antibodies. A combined biochemical, cell-biological, and genetic approach to the study of its pathogenesis should lead to a clearer understanding of the importance of PI-PLC during infection.

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