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Purification and Characterization of an Extracellular 29-Kilodalton Phospholipase C from *Listeria monocytogenes*

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We purified and characterized an extracellular phospholipase produced by *Listeria monocytogenes*. This enzyme was separated as a homogeneous protein of 29 kDa by chromatography on DEAE-52 cellulose and Bio-Gel P100 columns. It is a zinc-dependent phospholipase C (PLC) that is mainly active at pH 6 to 7 and expresses lecinthinase activity and a weaker sphingomyelinase activity. The exoenzyme also hydrolyzed phosphatidylcholine, phosphatidylethanolamine, phosphatidylerine, and sphingomyelin but not phosphatidyl-inositol. It was distinct from the 36-kDa phosphatidylinositol PLC produced by *L. monocytogenes* and from the *L. ivanovii* sphingomyelinase. The pure protein expressed a weak, calcium-independent hemolytic activity and was not toxic in mice. Western immunoblot analysis using a rabbit immune serum raised against the enzyme showed that all virulent strains of *L. monocytogenes* tested produced in the culture supernatant a 29-kDa PLC. In contrast, no proteins antigenically related to the 29-kDa PLC were detected in supernatants of *L. ivanovii, L. seeligeri, L. innocua*, or *L. welshimeri*. The role in virulence of the 29-kDa PLC specifically produced by *L. monocytogenes* remains to be established.

To our knowledge, the first major contribution of the virulence of *Listeria monocytogenes* derives from the pioneering work by G. B. Mackaness, demonstrating that virulent bacteria survive and even multiply within macrophages during the infectious process (24). Since then, *L. monocytogenes* has been extensively used by immunologists as a model to study the induction of T-cell-mediated immunity against intracellular parasites (3, 20, 35). Recent reports have established that a cytolitic toxin, listerioliysin O, is a major virulence factor promoting intracellular multiplication of *L. monocytogenes* (5, 8, 16, 28, 36) and even of the nonpathogenic species *Bacillus subtilis* (2). Although intracellular growth appears as a crucial event in the course of infection, expression of virulence involves several other critical steps, including penetration and invasion of intestinal mucosa, dissemination of bacteria in host tissues by cell-to-cell spreading (13, 32, 40) to create abscesses, and passage across the blood-brain barrier to cause meningoencephalitis. Most likely, these steps require several unknown virulence factors which are expressed in vivo during infection.

Among the putative virulence factors, the lipase activity found in culture supernatants of all *L. monocytogenes* strains must be considered. As first observed long ago (7, 38), strains of *L. monocytogenes* produce an extracellular lipolytic activity that induces a zone of opalescence around the colonies on egg yolk or lecithovitellin agar (11, 14, 15, 16, 35, 39, 43). This lipase activity is distinct from listerioliysin O, having been recently identified as a 56-kDa thiol-activated cytolitic toxin devoid of any detectable enzymatic activity (9). It has been suggested that the lecinthinase released in the culture supernatant of *L. monocytogenes* might be due to a phospholipase C (PLC) (21), but to our knowledge, this enzyme has not been identified or purified. In this work, we purified and characterized the extracellular factor responsible for lecinthinase activity. It is a zinc-dependent 29-kDa PLC that hydrolyzes lecinthin and sphingomyelin but not phosphatidyl-inositol. This enzyme is weakly hemolytic and not toxic in mice. It is produced by all virulent strains of *L. monocytogenes* but has not been detected in related *Listeria* species.

**MATERIALS AND METHODS**

*Bacterial strains and culture media.* For the purification of PLC, we used strain EGD (serovar 1/2a), originated from the Trudeau Institute (1982, Saranac Lake, N.Y.). The other bacterial strains used were *L. monocytogenes* N62262 (serovar 1/2a), N8018 (serovar 4b), N74217 (serovar 1/2b), N2661 (serovar 4b) (all wild strains from patients, from the Necker Hospital Collection, Paris, France), SLCC5136 (serovar 1/2a), SLCC5351 (serovar 4b), SLCC5132 (serovar 1/2a), and CIP82110T (serovar 1/2a); *L. ivanovii* CIP4141T (serovar 5); *L. seeligeri* CIP100100T (serovar 1/2); *L. innocua* CIP8011T (serovar 6); and *L. welshimeri* SLCC5334T (serovar 6) (CIP, Collection de l’Institut Pasteur; SLCC, Special *Listeria* Culture Collection, Würzburg, Germany).

For subcultures, brain heart infusion (BHI) broth or agar (bioMérieux, Marcy l’Etoile, France), trypticase soy agar (Diagnostics Pasteur, Marnes la Coquette, France), and 5% horse blood BHI agar were used. For production of the extracellular lecinthinase, bacteria were grown on trypticase-glucose-yeast (TGY) broth prepared as follows: Biotrypticase (bioMérieux), 30 g; yeast extract (Difco Laboratories, Detroit, Mich.), 20 g; NaHPO₄, 12H₂O, 8.3 g; KH₂PO₄, 0.7 g; and quartz-distilled water to 1,000 ml. This broth was supplemented with 0.1 mM ZnSO₄ and vegetable-activated charcoal (Prolabo, Paris, France) at 0.2% final concentration. In some experiments, ZnSO₄ was replaced by CuSO₄, NiSO₄, Co(NO₃)₂, or FeCl₃ (0.1 mM final concentration).

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After the pH was adjusted to 7.5, the culture broth was autoclaved at 115°C for 20 min. Sterile glucose was then added to a final concentration of 1% before inoculation. Lipolytic activity was detected on charcoal-treated TGY broth supplemented with 0.1 mM ZnSO4 to BHI agar supplemented with 2.5% fresh egg yolk previously diluted in 0.15 M NaCl or in phosphate-buffered saline (PBS) (1:2, vol/vol). Colonies producing lipolytic activity were surrounded with a large opalescent zone revealing egg yolk degradation.

**Purification of PLC.** Bacteria were grown under shaking conditions for 9 h at 37°C in 2 liters of charcoal-treated TGY broth supplemented with 0.1 mM ZnSO4 prepared as described above. After centrifugation at 10,000 × g for 15 min at 4°C, the supernatant fluid was 10-fold concentrated by rapid ultrafiltration at 4°C in an Amicon DC2 (hollow fiber H1 × 10:10-kDa cutoff). To purify the enzyme, we used a DEAE-52 (Whatman Biosystems, Maidstone, United Kingdom) column (40 by 2.6 cm) and Bio-Gel P100 (Bio-Rad Laboratories, Richmond, Calif.) columns (100 by 2.6 cm).

**Titration and characterization of PLC.** Phospholipase activity was titrated as described by Möllby et al. (30). Briefly, 100 μl of supernatant was incubated for 1 and 4 h at 37°C with 200 μl of lecithin (lecithin, 3.6 g [Merck Laboratories, Darmstadt, Germany]; sodium cholate, 2.4 g; ZnSO4, 1 mM final concentration; distilled water, 100 ml) and 700 μl of 0.15 M NaCl. Enzymatic activity was estimated turbidimetrically at 510 nm with an M25 spectrophotometer (Beckman, Brea, Calif.). One turbidimetric unit was arbitrarily defined as the amount of enzyme that causes an increase of absorbance of 0.100 within 1 h or, for very weak activity, 4 h. Protein concentration was determined by the method of Bradford (4) with Bio-Rad reagents. The isoelectric point of purified PLC was assessed in a pH gradient by isoelectric focusing (Ampholine column type 8100; LKB Instruments Inc., Gaithersburg, Md.) according to the method of Hagnerlund (12).

**Hemolytic assays.** The hemolytic activity of the purified PLC was estimated by incubating, for 18 h at 37°C in microtiter plates, 2 μl of serial twofold dilution of the enzyme in 0.15 M NaCl (eventually supplemented with 1 mM CaCl2) with 50 μl of 2% erythrocyte suspensions (3 × 108 to 6 × 107 cells per ml) from various animal species (human, sheep, horse, guinea, pig, and rabbit). Hemolytic activity was expressed as the amount of pure PLC required to totally lyse 50 μl of 2% erythrocyte suspension after 18 h of incubation at 37°C.

**TLC.** The enzymatic specificity of the pure PLC preparation was analyzed by thin-layer chromatography (TLC) on Silica Gel 60 plates (Merck), using as substrates egg yolk lecithin, synthetic lecithin (L-α-phosphatidylcholine-dimyristoyl), sphingomyelin, L-α-phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylserine (Sigma Chemical Co., St Louis, Mo.). We also used L-α-lysophosphatidylcholine (Sigma), obtained by the action of phospholipase A2 on egg yolk, phosphorylcholine chloride, 1-monopalmitoylglycerol, and 1,2-dipalmitoylglycerol (Sigma) as controls. Briefly, 200 μl of substrate (36 mg/ml) dispersed in sodium cholate (24 mg/ml) diluted in 700 μl of 0.15 M NaCl supplemented with 100 μl of 10 mM ZnSO4 was incubated for 1 and 4 h at 37°C with 5 μl of pure enzyme (75 μg/ml ~ 2,000 U/ml). In some experiments, EDTA (1 to 2 mM final concentration) was added to the reaction mixture. Then the phospholipids were extracted with 1 ml of chloroform-methanol-water (65:25:4, vol/vol/vol) or in hexane-diethylether-acetic acid (70:30:1, vol/vol/vol) to detect the release of diacylglycerol. Plates were then stained with iodine vapors or with ammonium molybdate to visualize the PO4 components (29). Pure phospholipase C (alpha-toxin) from Clostridium perfringens, kindly provided by Collette Jolivet-Reynaud (Institut Pasteur, Paris), was used as a control.

The optimum pH of enzymatic activity was determined as described above by incubating for 1 h at 37°C. 5 μl of purified PLC (2,000 U/ml) were dispersed in 100 μl of 0.15 M NaCl containing 1 mM ZnSO4 adjusted at various pHs ranging from 5.0 to 8.5. Related phospholipids were then analyzed by separating chromatography in chloroform-methanol-water and iodine staining as described above.

**Liposomes.** Liposomes were prepared by sequentially mixing 154 mg of phosphatidylcholine (Merck), 68 mg of cholesterol (Sigma), 25 ml of CHCl3, and 5 ml of methanol. After evaporation of the organic solvents at 31°C, lipids were dissolved in 3 ml of diethyl ether (Prolabo) and then added with 15 ml of distilled water. The mixture was then ultrasonicated for 4 min at 4°C and emulsified at 3,000 rpm/min with an Ultra-Turrax T25 (BioBlock Scientific, Illkirch, France). The molar ratio (phosphatidylcholine/cholesterol) was 7:4. Fifty microliters of liposomes was injected in 940 μl of 0.15 M NaCl at various pHs (5 to 8.5) and incubated with 10 μl of purified enzyme (2,000 U/ml) for 4 h at 37°C. The A530 was then measured with a Beckman M25 spectrophotometer.

**Amino acid composition and sequencing.** Sequence analysis was carried out with a gas-phase sequencer (model 470A; Applied Biosystems, Foster City, Calif.) equipped with an on-line phenylthiodyantoin amino acid derivative analyzer (model 120A) and run with the sequencing program recommended by the manufacturer as described previously (1). The pure 29-kDa PLC was electrotransferred on Immobilon (Millipore, Bedford, Mass.) and hydrolyzed by trypsin (Sigma). The peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC) (Phenomenex) and 0.1% acetic acid-acetonitrile gradient and analyzed by a diode array detector (model 100S; Applied Biosystems). One apparently homogeneous peptide was further sequenced (1).

**SDS-PAGE and Western immunoblot analysis.** Proteins from the fractions obtained at various steps of the purification procedure were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Samples of 50 μl (5 to 10 μg of protein) were boiled for 2 min in 2% (wt/vol) SDS–5% (vol/vol) 2-mercaptoethanol–10% (vol/vol) sucrose–0.002% (wt/vol) bromophenol blue in 0.1 M Tris hydrochloride buffer (pH 6.8). Electrophoresis was performed in 7.5% to 20% (wt/vol) gradient polyacrylamide-0.1% SDS gels at 0.07 M Tris buffer (pH 8.8) for 18 h at 6 mA. The proteins were stained by Coomassie blue or silver nitrate.

For Western blot analysis, supernatants from bacterial strains grown for 9 h at 37°C in charcoal-treated TGY broth were passed through a 0.22-μm-pore-size filter and precipitated with 10% trichloroacetic acid. The precipitate was centrifuged at 10,000 × g at 4°C for 30 min and dissolved in 0.12 M Tris buffer (pH 6.8). Samples were boiled for 2 min as described above. Electrophoresis was performed in 13% (wt/vol) polyacrylamide–0.1% SDS in 0.37 M Tris buffer (pH 8.8) for 2 h at 150 mA. Proteins were electrophoretically transferred (1 h, 50 mA) onto nitrocellulose sheets (PH 79; Schleicher & Schuell, Dassel, Germany), in 0.2 M glycine-20% (vol/vol) isopropanol 0.025 M Tris buffer (pH 8.3). The sheets were incubated for 1 h at room temperature with shaking in chloroform-methanol-water (65:25:4, vol/vol/vol) or in hexane-diethylether-acetic acid (70:30:1, vol/vol/vol) to detect the release of diacylglycerol. Plates were then stained with iodine vapors or with ammonium molybdate to visualize the PO4 components (29). Pure phospholipase C (alpha-toxin) from Clostridium perfringens, kindly provided by Collette Jolivet-Reynaud (Institut Pasteur, Paris), was used as a control.
PBS-10% Tween supplemented with 5% (wt/vol) skimmed milk (Regalait; France-Lait, Saint Marin-Belle Roche, France) prior to 1 h of incubation with rabbit anti-PLC diluted 1:2,000 in the buffer described above and then washed eight times in buffer before addition of 20 ml of milk buffer containing a peroxidase-labeled goat anti-rabbit immunoglobulin (Organon-Teknika-Cappel, Malvern, Pa.) diluted 3:1,000. Shaking was continued for 1 h at room temperature, and then the filters were washed six times in buffer supplemented with 0.1% Triton X-100. Enzymatic activity was revealed by addition of diaminobenzidine tetrahydrochloride (Sigma) supplemented with 0.02% hydrogen peroxide.

Rabbit anti-29-kDa PLC serum and immunoadsorbed antibodies. Female albino rabbits (3 kg) supplied by IFFA-Credo (Saint German sur l'Arbresle, France) were immunized by injecting subcutaneously 25 μg of purified PLC from L. monocytogenes emulsified in complete Freud's adjuvant on days 0, 7, and 14 and in incomplete Freund's adjuvant on day 90. Blood was collected before immunization and 2 weeks after the last injection. This antisera was immunoadsorbed on purified 29-kDa PLC as follows. The pure 29-kDa PLC (75 μg/ml) was incubated on nitrocellulose sheets (BA85; Schleicher & Schuell) for 30 min at 22°C. After adsorption, the sheets were saturated with 5% skimmed milk as described above and then rinsed in PBS (pH 7.2). Rabbit anti-29-kDa PLC serum diluted 1:10 (PBS, pH 7.2) was incubated for 30 min at 22°C on nitrocellulose sheets in a volume of 5 ml. After extensive washings in PBS (pH 7.2), immunoadsorbed antibodies were eluted three times with 5 ml of glycine-HCl (pH 2.2) for 15 min at 22°C. Eluates were rapidly neutralized by addition of 1 M PO₄H₂O₄ and concentrated on a Centricon PM30 filter (Amicon) to a volume of 4 ml, corresponding to an optical density at 280 nm of 0.40. This immunoadsorbed anti-29-kDa PLC was then used for Western blot analysis.

Toxicity in mice. Specific-pathogen-free female ICR Swiss mice, 6 to 8 weeks old (Charles River, Saint Aubin les Elbeuf, France), were used. Groups of five mice were injected intravenously (i.v.) with increasing dose of purified PLC (1 to 25 μg per mouse), and mortality was observed over a period of 1 week.

RESULTS

Production of extracellular lecithinase in the culture supernatant of L. monocytogenes. We first defined the optimal conditions to produce lecithinase activity in the culture supernatant from L. monocytogenes. Bacteria were grown at 37°C under shaking conditions, using TGY broth or charcoal-treated TGY broth supplemented or not with 0.1 mM ZnSO₄. Enzymatic activity was then titrated at successive times in the culture supernatant by a turbidimetric method, using lecithin as the substrate (30). Bacterial growth was identical in the four different culture conditions (Fig. 1). Enzymatic activity was undetectable when TGY broth was used and remained very weak when ZnSO₄ was added to this broth. In contrast, enzymatic activity was significantly enhanced in charcoal-treated TGY broth, with a peak at 6 h (15 U/ml) and then a rapid decrease. When both charcoal and ZnSO₄ were added to TGY broth, enzymatic activity reached 70 U/ml by 9 h, at a time when the stationary phase of bacterial multiplication was reached (Fig. 1). The lecithinase activity was not stimulated when Zn²⁺ was replaced by Fe³⁺, Cu²⁺, Co²⁺, or Ni²⁺ (data not shown).

Purification of the 29-kDa PLC. The purification procedure was used on 9-h cultures made on charcoal-treated TGY broth supplemented with ZnSO₄. The enzyme was purified from the crude extracellular concentrated supernatant by a three-step procedure. Fractions obtained were checked for phospholipase activity (turbidimetric method) and by SDS-PAGE (Fig. 2). The concentrated supernatants were dialyzed against 0.05 M Tris hydrochloride buffer (pH 7.45) (Fig. 2, lane 1) and applied onto a DEAE-52 cellulose column equilibrated with this buffer at a flow rate of 0.7 ml/min. The unretracted material containing about 90% of the initial phospholipase activity was concentrated by ultrafiltration in a stirred cell equipped with a PM10 membrane (Fig. 2, lane 2), and applied onto a Bio-Gel P100 column and eluted with PBS (pH 6.8). The fractions containing enzymatic activity (Fig. 2, lane 3) were pooled, concentrated, and applied onto

FIG. 2. SDS-PAGE (Coomassie blue staining) of proteins associated with lecithinase activity during the three-step purification procedure of phospholipase of L. monocytogenes (see Materials and Methods). Lanes: 1, concentrated supernatant; 2, after DEAE-52 column (step 1); 3, after Bio-Gel P100 column (step 2); 4, after Bio-Gel P100 column (step 3); 5, markers (5 to 10 μg of protein per well). The purified exoenzyme is a 29-kDa protein.
TABLE 1. Purification of an extracellular PLC from *L. monocytogenes*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Protein (µg/ml)</th>
<th>Activity (U/ml)</th>
<th>Sp act (U/mg of protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture supernatant</td>
<td>2,000</td>
<td>75</td>
<td>50</td>
<td>666</td>
<td>88</td>
</tr>
<tr>
<td>2. Concentrated supernatant</td>
<td>250</td>
<td>175</td>
<td>355</td>
<td>2,028</td>
<td>14</td>
</tr>
<tr>
<td>3. DEAE-52</td>
<td>10</td>
<td>650</td>
<td>6,500</td>
<td>10,000</td>
<td>65</td>
</tr>
<tr>
<td>4. Bio-Gel P100</td>
<td>4</td>
<td>180</td>
<td>3,500</td>
<td>19,400</td>
<td>14</td>
</tr>
<tr>
<td>5. Bio-Gel P100</td>
<td>6</td>
<td>75</td>
<td>2,000</td>
<td>26,000</td>
<td>12</td>
</tr>
</tbody>
</table>

a second Bio-Gel P100 column, which allowed to recover the purified enzyme (26,000 U/mg of protein), as evidenced by the single band observed by SDS-PAGE (Fig. 2, lane 4). A summary of the purification steps, all carried out at 4°C, is presented in Table 1. The pure enzyme appears as a single polypeptide chain of *M.* 29,000, visualized as one sharp band on SDS-PAGE after staining with Coomassie blue. Increasing the load of pure toxin up to 5 µg of protein did not reveal any additional band after silver staining (not shown). The isoelectric point of the pure enzyme was determined to be 8.4.

The 29-kDa enzyme expresses a zinc-dependent lecithinase and sphingomyelinase activities. The enzymatic activity of the 29-kDa protein was studied by TLC (Fig. 3). After incubation of lecithin or sphingomyelin for 1 and 4 h at 37°C with 10 U of pure enzyme, the migration patterns were analyzed after iodine staining. Lecithin and sphingomyelin were hydrolyzed after 1 h of incubation by the enzyme in the presence of 1 mM ZnSO₄ (Fig. 3, lanes 2 and 6), whereas sphingomyelin required 4 h for complete hydrolysis. The enzymatic activity was abrogated by addition of 1 to 2 mM EDTA (Fig. 3, lanes 3, 4, and 7), indicating that the lipolytic activity is zinc dependent. The pH range of lecithinase activity was evaluated by incubating for 1 h at 37°C the purified enzyme (10 U) with lecithin at various pHs between 5.0 and 8.5. The released phospholipids were then analyzed by TLC (Fig. 4A). The optimum pH of phospholipase activity was between pH 6 and 7. Similar results were obtained on phosphatidylcholine-cholesterol liposomes treated with the enzyme (Fig. 4B).

**Characterization of the enzymatic activity.** The enzymatic activity of the 29-kDa protein was further characterized by TLC. It is known that lecithin is hydrolyzed by PLC into diacylglycerol and phosphocholine and that sphingomyelin is hydrolyzed by sphingomyelinase into ceramide and phosphocholine. We first demonstrate that the migration hydrolysis profile of egg yolk lecithin obtained with the 29-kDa protein of *L. monocytogenes* was similar to that observed with pure phospholipase C from *C. perfringens*, giving rise to diacylglycerol (Fig. 5A, lanes 2 and 3), as shown by iodine staining. In contrast, the lecithin hydroly-
obtained by digestion of egg yolk lecithin with phospholipase A₆ migration in chloroform-methanol-water; iodine staining. The same migration patterns were observed when lecithin was hydrolyzed by the 29-kDa protein and PLC from C. perfringens. (B) TLC of synthetic lecithin (l-α-lysophosphatidylcholine-dimyristoyl) revealing the release of dimyristoylglycerol (incubation for 4 h at 37°C). Lanes: 1, 1-monopalmitoylglycerol; 2, 1,2-dipalmitoylglycerol; 3, lecithin; 4, lecithin plus 29-kDa enzyme; 5, phosphorylcholine (TLC was directly loaded after enzymatic reaction without extraction; migration in hexane-diethyl ether-acetic acid, using synthetic lecithin; phosphorylcholine; lecithin plus 29-kDa PLC, lecithin; LPC, l-α-lysophosphatidylcholine; DPG, dipalmitoylglycerol).

Amino acid composition and partial peptide sequence of the enzyme. We repeatedly failed to determine the NH₂-terminal sequence of the protein, which was probably blocked. The 29-kDa protein was then electrotransferred onto Immobilon

2% suspensions of these erythrocytes were 70, 280, and 200 ng, respectively. Finally, the toxicity tests were performed by injecting i.v. groups of five mice with increasing doses of pure enzyme. Mice survived when receiving up to 25 μg i.v.

Western blot analysis of culture supernatants of L. monocytogenes and related species. Trichloroacetic acid-precipitated proteins obtained from culture supernatants of charcoal-treated TGY supplemented with 0.1 mM ZnSO₄ were migrated by SDS-PAGE. Electrophoresed proteins were probed with a rabbit anti-29-kDa protein serum (dilution, 1:2,000) except in lane 10, for which an immunoadsorbed anti-29-kDa protein serum was used (dilution, 1/10). (A) L. monocytogenes. Lanes: 1, CIP82110T; 2, SLCC5132; 3, SLCC3551; 4, SLCC5156; 5, N2661; 6, N742177; 7, N8018; 8, N62662; 9, EGD; 10, EGD. (B) L. monocytogenes and related species. Lanes: 11, L. monocytogenes EGD; 12, L. innocua CIP80115T; 13, L. welshimeri SLCC53345; 14, L. seeligeri CIP100100; 15, L. ivanovii CIP 4141T. All strains of L. monocytogenes produced the 29-kDa enzyme as well as a 33-kDa antigenically related protein that was also recognized by the immunoadsorbed antiserum. Related Listeria species do not produce extracellular proteins antigenically related to the 29-kDa PLC from L. monocytogenes proteins.

FIG. 5. Characterization of enzymatic activity. (A) TLC of egg yolk lecithin as described for Fig. 3 (incubation for 4 h at 37°C). Lanes: 1, lecithin; 2, lecithin plus 29-kDa enzyme; 3, lecithin plus purified PLC from C. perfringens; 4, l-α-lysophosphatidylcholine obtained with digestion of egg yolk lecithin with phospholipase A₂. (Migration in chloroform-methanol-water; iodine staining). These results show that the 29-kDa protein and PLC from C. perfringens migrate in the same lane of the TLC plate. (B) TLC of synthetic lecithin (l-α-lysophosphatidylcholine-dimyristoyl) revealing the release of dimyristoylglycerol (incubation for 4 h at 37°C). Lanes: 1, 1-monopalmitoylglycerol; 2, 1,2-dipalmitoylglycerol; 3, lecithin; 4, lecithin plus 29-kDa enzyme; 5, phosphorylcholine (TLC was directly loaded after enzymatic reaction without extraction; migration in hexane-diethyl ether-acetic acid, using synthetic lecithin; phosphorylcholine, lecithin plus 29-kDa PLC, lecithin; LPC, l-α-lysophosphatidylcholine; DPG, dipalmitoylglycerol).
and hydrolyzed by trypsin. Peptides were further purified by HPLC. The sequence of one homogeneous peptide, corresponding to an internal part of the molecule, was determined to be Tyr-Phe-Asn-Gln-Ser-Val-Thr-Asp-Tyr.

DISCUSSION

In this work, a 29-kDa PLC was purified to homogeneity from the supernatant of L. monocytogenes EGD. This exoenzyme expresses lecithinase activity and weaker sphingomyelinase activity (Fig. 3), as does the PLC of C. perfringens (19, 37). The exoenzyme hydrolyzes phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine but not phosphatidylinositol, thus displaying a wide substrate specificity. These results clearly distinguish the 29-kDa PLC from the PI-PLC recently described in L. monocytogenes (22, 26). This PI-PLC is encoded by a gene located upstream from hlyA and is strongly homologous to the PI-PLC produced by Bacillus thuringiensis and Bacillus cereus (22, 26).

We found that the 29-kDa enzyme is inactive on phosphatidylinositol and differs in molecular weight from the 36-kDa PI-PLC produced by L. monocytogenes (22, 26).

The biological properties of the 29-kDa PLC were further investigated. The exoenzyme was active at a wide pH range (6.0 to 7.0), as shown by TLC and liposome hydrolysis at various pHs (Fig. 4). It was weakly hemolytic for erythrocytes from various animal species, including guinea pig, horse, and human, but was not hemolytic for sheep erythrocytes. Interestingly, sheep erythrocytes are practically devoid of phosphatidylcholine, whereas guinea pig, horse, and human erythrocytes contain 29 to 42% of this compound (33). Moreover, the exoenzyme was not toxic in mice injected i.v. with 25 μg of pure protein, in contrast to, for example, C. perfringens PLC (25, 41).

The enzymatic activity was strongly enhanced in culture supernatants when bacteria were grown in charcoal-treated TGY broth supplemented with ZnSO₄ but was not stimulated in the presence of Fe²⁺, Cu²⁺, Co²⁺, or Ni²⁺. These data suggest that the 29-kDa PLC produced by L. monocytogenes is zinc dependent. Zinc may act directly on the 29-kDa exoenzyme, as shown on Fig. 3. Interestingly, zinc also plays a critical role in the enzymatic activities of PLCs from C. perfringens (19, 31) and B. cereus (23), which are metalloenzymes containing two zinc ions essential to their catalytic activity. The 29-kDa PLC from L. monocytogenes may also be a metalloenzyme, as is the zinc-dependent metalloprotease recently described in this pathogen (6, 27).

We found that the 29-kDa PLC was produced by all virulent strains of L. monocytogenes tested, as shown by Western blot analysis using a rabbit polyclonal immune serum raised against the pure 29-kDa protein. An antigenically related double band at ~33 kDa, as well as a minor 66-kDa band, was also detected in L. monocytogenes supernatant. We can eliminate the possibility that these additional bands are due to contamination of rabbit antiserum. However, the 33-kDa band is still recognized when antiserum is highly diluted (1:2,000 to 1:4,000), whereas the other bands disappear. The 29- to 33-kDa proteins are also detected by a 29-kDa PLC immunoadsorbed antiserum diluted 1:10, suggesting that the 33-kDa protein is antigenically closely related to PLC. That the 33-kDa protein is an inactive precursor of the 29-kDa PLC remains to be established.

The anti-29-kDa PLC serum did not detect any signal in L. ivanovii supernatant, which also produces an opalescent zone on egg yolk agar. It has been demonstrated that L. ivanovii produces a 24- to 27-kDa sphingomyelinase (18, 42) devoid of lecithinase activity (42). On the basis of the enzymatic activity, molecular weight, and absence of antigenic cross-reactivity, the 29-kDa PLC of L. monocytogenes appears to be different from this sphingomyelinase. With regard to the other species (L. innocua, L. seeligeri, and L. welshimeri) which do not produce the opalescent zone of egg yolk agar, the anti-29-kDa PLC also failed to detect any signal in culture supernatants of these bacteria. Moreover, no cross-reactivity has been detected by Western blot analysis between the 29-kDa PLC and PLCs from C. perfringens and B. cereus (10). The partial peptide sequence of an internal fragment of the 29-kDa PLC was obtained, revealing the nonapeptide Tyr-Phe-Asn-Gln-Ser-Val-Thr-Asp-Tyr.

This nonapeptide was found into the structural gene encoding the 29-kDa PLC which has been recently located and sequenced (41a).

In conclusion, L. monocytogenes appears to produce at least two extracellular PLCs, a 29-kDa PLC characterized in this work and a 36-kDa PI-PLC. It has recently been found that a 32-kDa protein expresses a phospholipase activity, as shown on SDS-PAGE overlaid with egg yolk agar (17). Whether this enzyme is different from the 29-kDa PLC described here remains unknown. Interestingly, it has been shown that there exists a common genetic control between listeriolysin O and other determinants, including lipolytic phenotype on egg yolk agar (17). These data suggest that the 29-kDa PLC may be coregulated with other virulence factors, but the role of this enzyme in virulence remains unknown.

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