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Rapid Screening Assay for Phospholipase C Activity in Mycoplasmas

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A screening assay for phospholipase C using a chromogenic substrate incorporated into agar medium is described. The assay directly visualizes phospholipase C activity of mycoplasma lysates and membranes on agar plates, or the activity may be measured by spectrophotometry. The results from the assay confirm the presence in *Ureaplasma urealyticum* of phospholipase C, which is predominantly localized in the membrane fraction. The procedure has the potential to screen phospholipase C activity in other mycoplasmas and microorganisms in general.

The pathophysiological mechanism of bacterial pneumonia may be related to the release of extracellular cytotoxins, such as phospholipases (3). The importance of bacterial phospholipases in infectious processes associated with prematurity has also been suggested (2). This has been attributed to the effect of phospholipase degradation of membrane phospholipids of the placental tissues or to the hydrolytic effect of lysophospholipids released by enzymes. One of the potent enzymes of phospholipid degradation is phospholipase C. We have reported that genital mycoplasmas, such as *Ureaplasma urealyticum*, show differences in phospholipase A activity when assayed by a radioisotopic method (4). In previous reports, detection of bacterial phospholipase activity was dependent on the appearance of an opacity in egg yolk media inoculated with the test organism (8, 9). However, the opacity reaction is not per se evidence of phospholipase activity; thus, such assay methods are nonspecific. More recently, thin-layer chromatographic techniques have been used for the rapid detection of bacterial phospholipases (6); again, these assays do not provide evidence as to the specific nature of the lecithinase activity involved in the hydrolytic reaction.

In this report, a simplified screening assay for phospholipase C using a chromogenic substrate is described for microorganisms such as mycoplasmas. Because the chromogenic substrate can be incorporated into agar medium, this assay can be performed on plates for direct visualization of phospholipase C activity and for measurement by spectrophotometry. A similar procedure has been adopted for exotoxin assays of *Legionella* bacteria (1). Our method is specific for phospholipase C and does not require a radioactive substrate. Large numbers of samples could therefore be screened in a relatively short time.

To assay for phospholipase C, the chromogenic compound *p*-nitrophenylphosphorylcholine, a water-soluble analog of phosphatidylcholine, was used as a substrate. Hydrolysis of this compound by the enzyme yields phosphorylcholine and yellow *p*-nitrophenol (5). The specificity of the site of action of phospholipase C on *p*-nitrophenylphosphorylcholine is the same as that of the normal substrate, phosphatidylcholine (Fig. 1). Detection of the reaction product, phosphorylcholine, was done by paper chromatography in the solvent system butanol/acetic acid/water (25/15/5, vol/vol/vol). The product was further identified by the Haines-Ischerwood spray on the chromatogram (blue spot on a white background) and gave an R_f value of 0.42 in the above-described solvent system. Detection of *p*-nitrophenol was done by spectrophotometry with an optimum A_{410}.

Three *U. urealyticum* serotypes were cultured separately in 500 ml of Trypticase soy broth base (BBL Microbiology Systems, Cockeysville, Md.) at pH 6.0 supplemented with 5% fetal bovine serum (Bocknek Organic Matters, Rexdale, Ontario, Canada), 5% fresh yeast extract (25%, wt/vol), 0.1% urea, 0.002% bromothymol blue, and 1,000 IU of penicillin per ml (11) as previously described (4). This medium is referred to as lean TS broth because of the reduced serum supplement. The organisms at a concentration of 10^7 to 10^8 color-changing units per ml were harvested by centrifugation at 35,000 × *g* for 45 min in a model RC-2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) and washed once in 30 ml of 0.25 M NaCl. The cell pellet was suspended in 2 ml of 0.25 M NaCl and used immediately for the preparation of whole-cell lysate, plasma membrane, and cell cytosol fractions by the standard method of osmotic lysis (10) with the following modifications. To lyse the washed cells, the cell suspension (in 0.25 M NaCl) was rapidly transferred into 20 to 25 ml of deionized water, preheated to 37°C, and then incubated for 30 min at 37°C. The membranes were collected by centrifugation at 35,000 × *g* for 30 min, and the supernatant was used to prepare the cytosol fraction by centrifugation at 100,000 × *g* for 1 h in a Beckman LS-65 Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The membrane fraction was washed twice consecutively in deionized water, suspended in 1 ml of β-buffer (10), and stored in plastic vials at −70°C or used immediately for the enzyme assays. The medium control was prepared by incubating uninoculated lean TS medium for 24 h and harvesting by centrifugation as described above. Protein was determined by the method of Lowry et al. (7).

Whole-cell lysate, plasma membrane, and cytosol fractions of serotypes 3, 4, and 8 were incubated for 1 h in a shaker bath at 37°C with 80 mM *p*-nitrophenylphosphorylcholine (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Tris buffer (pH 7.4) containing 10 mM $CaCl_2$. The cells were then removed by centrifugation in a microfuge (Brinkman Instruments, Toronto, Ontario, Canada) at 12,000 × *g* for 5 min. The *p*-nitrophenol released into the supernatant was measured at 410 nm in a Beckman spectrophotometer

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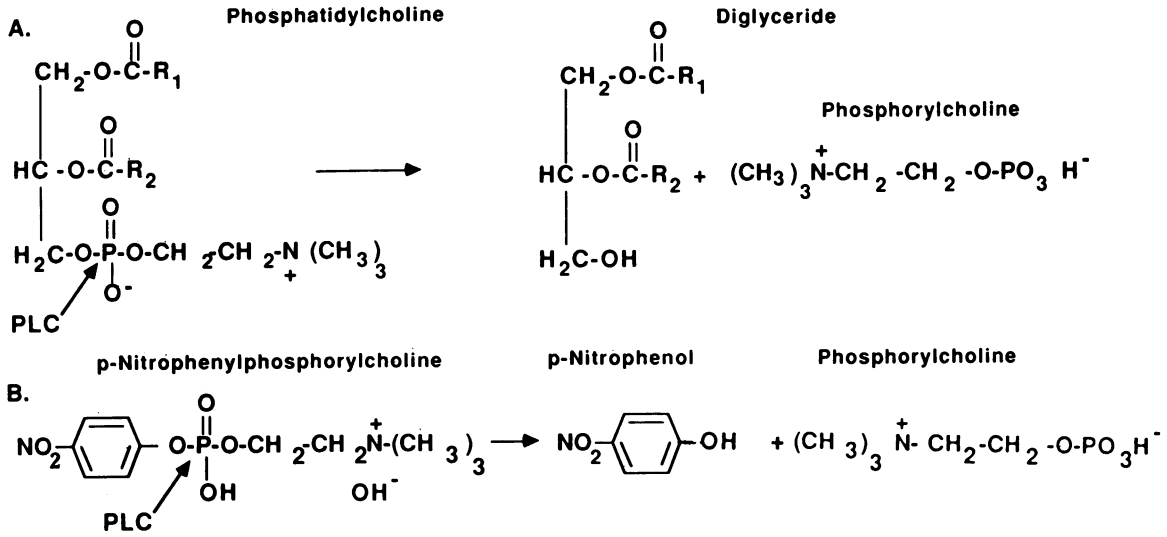

FIG. 1. Comparison of specificity of the site of action of phospholipase C (PLC) on substrates phosphatidylcholine (A) and *p*-nitrophenylphosphorylcholine (B). R_1 and R_2 refer to the fatty acids on the *sn*-1 and *sn*-2 carbons of the glycerol backbone of the phosphatidylcholine molecule.

(model 24D; Beckman Instruments). A standard curve was prepared with serial dilutions of *p*-nitrophenol (Fig. 2). Controls included buffer alone, mycoplasma cell lysate alone, and the substrate alone. To determine the specific activity, optical density values for buffer alone plus substrate were subtracted from the experimental values. The specific activity was then calculated based on units of protein.

The results confirmed the presence of phospholipase C activity in *U. urealyticum* (Table 1). Specific differences in enzyme activity in whole-cell lysates among the three serotypes of *U. urealyticum* are probably not significant. This result is in agreement with our previous data on the estimation of phospholipase C by the radioisotopic method (4). It is of interest that nearly the entire phospholipase C activity was confined to the membrane fraction in all three serotypes.

To develop a method for screening or visualization of phospholipase C activity on agar plates, lean TS agar medium was supplemented with 80 mM *p*-nitrophenylphosphorylcholine as substrate. Whole-cell lysate, plasma membrane, and cytosol fractions (50 to 100 μg of protein) were placed on the surface of the agar plate, evenly spread, and incubated aerobically at 37°C for 24 h. Controls included TS agar plate with no substrate but with lysate, TS agar plate with substrate only, and TS agar plate with no substrate. A definite bright yellow developed after 24 h of incubation in the agar medium with whole-cell lysates or membrane fractions of *U. urealyticum* serotypes. Controls developed a very slight yellow as the result of a change in the agar medium during incubation (Table 2).

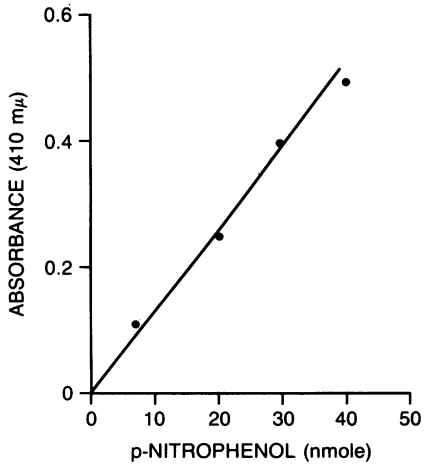

FIG. 2. Relationship between A_{410} and *p*-nitrophenol in a standard assay. The assay was linear up to 60 nmol of *p*-nitrophenol. Values are means of two separate assays.

Agar plate assays were also done with (i) TS agar medium and substrate (80 mM) without 5% serum and 5% yeast extract, and (ii) 1% saline agar alone with substrate (80 mM). Both these assays were negative. Thus, the complete medium is essential for enzyme activity and color development on the agar plates, indicating an absolute requirement for certain factors in the serum and yeast supplements.

The results obtained by this assay are in agreement with those of our earlier studies of phospholipase C activity in *U. urealyticum* serotypes (4). In that study, in which radiolabeled substrates were used, the cell lysates of all three serotypes indicated the presence of phospholipase C activity during exponential- and stationary-phase growth. Thus, both assay systems confirm the presence of phospholipase C activity in *U. urealyticum*. However, the results from the two assays are not directly related, due to the different molecular structure of the substrates used (Fig. 1).

The procedure, therefore, has the potential to rapidly

TABLE 1. Hydrolysis of *p*-nitrophenylphosphorylcholine by cellular fractions of *U. urealyticum* serotypes

Cellular fraction	*p*-Nitrophenol liberated[a] by serotype:		
	3	4	8
Lysate	0.61 ± 0.10	1.10 ± 0.3	3.20 ± 0.5
Plasma membrane	180.1 ± 11.0	223.2 ± 21.0	249.0 ± 18.0
Cytosol	<0.01 ± 0.05	<0.01 ± 0.05	<0.01 ± 0.05

[a] Expressed as nanomoles per milligram of protein per hour. The values are means ± standard deviation of three separate experiments.

TABLE 2. Screening assay for phospholipase C activity of *U. urealyticum* on agar plates containing *p*-nitrophenylphosphorylcholine as substrate

Sample	Yellow intensity[a] (liberated *p*-nitrophenol)
Whole-cell lysate	++++
Plasma membane	++++
Cytosol fraction	++
Controls. Agar medium with:	
No substrate plus lysate	(+)
Substrate only	(+)
No substrate	(+)

[a] Color intensity is indicated in arbitrary units as follows: ++++, very intense yellow; ++, medium yellow; (+), pale yellow.

screen for phospholipase C activity. It can be used for studies on enzymological characteristics of the metal ion and detergent requirements of this enzyme. The major advantage of this system is that it does not require expensive radioactive substrates for the assay of phospholipase C. With the spectrophotometric method, quantitative measurement of the enzyme activity can also be obtained. The agar plate procedure provides a simple screening method for phospholipase C activity of ureaplasmas and other species of mycoplasmas or microorganisms.

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