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Lecithin Agar for Detection of Microbial Phospholipases[1]

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Lecithin agar was developed on which phospholipase C produced turbid zones and phospholipase A produced clear zones. Reactions on lecithin agar agreed 74% of the time with reactions in egg yolk broth. On lecithin agar, interpretation was easier, phospholipase A was detectable, and opaque zones were visible 1 or 2 days earlier than on egg yolk agar. All constituents of the medium can be autoclaved.

Egg yolk broth and egg yolk agar are the media most commonly used to screen bacteria for production of phospholipase C. However, maintenance of sterility is difficult because of contaminants from eggs, and interfering reactions may make interpretation difficult. Therefore, we undertook to develop agar containing lecithin for detection of phospholipase activity.

The medium was prepared in three fractions as follows. Fraction A contained 3.0 g of crude soy lecithin (Eastman Kodak Co.) and 45 ml of distilled water. Fraction B contained 1.0 g of tryptone, 0.5 g of yeast extract, 0.5 g of glucose, 1.8 g of agar, and 50 ml of distilled water. Fraction C contained 5 ml of 0.1 M $CaCl_2$. Fraction A was brought to boiling and swirled to form a viscous sol, which was sonicated until homogeneous. Blending of unheated fraction A in a Waring blender for 2 min at high speed was also satisfactory. The fractions were autoclaved separately for 15 min at 121 C. After equilibration of temperature to 42 to 44 C, the fractions were combined with gentle swirling, A + (B + C). To prevent separation, plates were poured immediately with 12 to 15 ml of the mixture.

Pseudomonads were streaked onto surfaces of lecithin agar plates. After incubation for up to 9 days at 21 C, plates were observed for changes in appearance of the medium.

A culture of *Pseudomonas fluorescens* 178 produced an opaque zone, suggesting phospholipase C activity (formation of water-insoluble diglyceride). Strain 157 of an unknown species of *Pseudomonas* produced a clear zone, which was attributed to activity of phospholipase A_1 or A_2 (formation of water-soluble lysolecithin; see McMurray and Magee [3]).

Products of lecithin degradation were determined by thin-layer chromatography using prepared plates (5 by 20 cm; Analtech, Inc.). Neutral lipids were determined on Silica Gel G plates using hexane-ethanol-acetic acid (80:20:1) as solvent, and phospholipids were separated on Silica Gel HR using chloroform-methanol-acetic acid-0.9% NaCl (50:25:8:4). Silica gel plates were activated at 110 C for 1 h. Equal quantities of medium were excised from modified and unmodified zones of plates with care to exclude bacteria. Samples were extracted (1) in 8 ml of 1:1 chloroform-methanol and 1 ml of distilled water. After centrifugation, the bottom layer, containing lipids, was removed by Pasteur pipette, evaporated to dryness, and dissolved in 2 ml of chloroform.

Plates were spotted (8 μl) with extract from the zone of hydrolysis, extract from unmodified (control) media, and lipid standards. Spots were allowed to dry before plates were placed in the solvent. Spots were visualized by spraying with 10% H_2SO_4 and charring. Figure 1 is a chromatogram of material from the opaque zone produced by *P. fluorescens* 178. Lecithin was degraded as evidenced by disappearance of its spot on the phospholipid chromatogram. Also, the quantity of 1,2-diglyceride on the neutral lipid plate was greater than that in the unmodified agar. Therefore, it was concluded that the lecithin was hydrolyzed by phospholipase C.

The chromatograms (Fig. 2) of the clear zone produced by *Pseudomonas* sp. 157 indicated activity of phospholipase A_1 or A_2. Both lysolecithin (phospholipid plate) and free fatty acids (neutral lipid plate) were observed in extracts from the modified medium but not in extracts of the unmodified medium. The fact that 0.01 M sodium lauryl sulfate failed to inhibit hydrolysis is further evidence that the enzyme was phospholipase A (2).

Tests were made of the effects of varying

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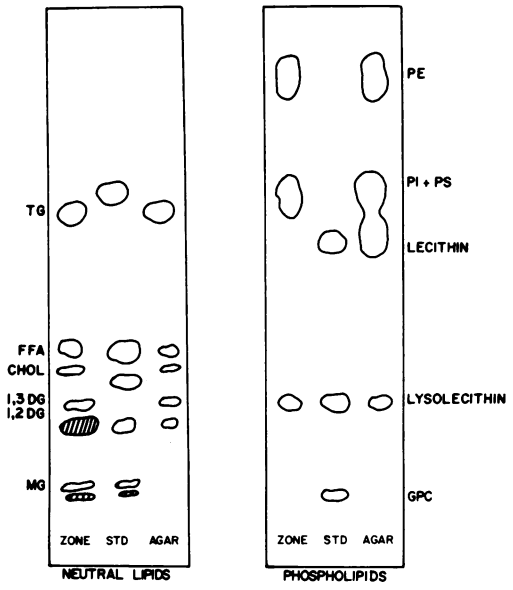

FIG. 1. *Thin-layer chromatograms (Silica Gel G for neutral lipids and Silica Gel HR for phospholipids) of extracts of lecithin agar and of the opaque zone surrounding colonies of microorganism 178 (P. fluorescens) grown on lecithin agar. TG, Triglyceride; FFA, free fatty acids; CHOL, cholesterol; DG, diglyceride; MG, monoglyceride; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; GPC, α-glycerolphosphorocholine.*

concentrations of calcium on reactions produced by phospholipase-producing bacteria on lecithin agar. The medium was formulated as previously described except that quantities of 0.01 M $CaCl_2$ were varied as follows: 0, 0.1, 0.5, 1.0, 2.5, and 5.0 ml per 100 ml of agar. Production of phospholipase C by *P. fluorescens* 178 and *Bacillus cereus* 146 was detected only at the highest concentration of calcium. However, activity of phospholipase A by *Pseudomonas* sp. 157 was most apparent in media with 2.5 ml of added $CaCl_2$ solution. Zones were considerably smaller when 5.0 ml of $CaCl_2$ solution was added. Also, a cloudy zone, which developed within and subsequent to formation of the clear zone at lower concentrations of Ca^{2+}, failed to appear within the medium containing the highest concentration. This turbidity was apparently produced by activity of lysophospholipase which hydrolyzed lysolecithin. The fatty acids thus released would have precipitated as calcium salts, forming the turbid zones.

Colonies of *P. fluorescens* 178 swarmed over the agar surface of the control plates without added calcium. Their swarming, hence motility, decreased as calcium concentration increased. In contrast, the ability of this bacterium to produce green, water-soluble pigment increased with calcium concentration.

It took as long as 6 days for zones to appear on lecithin agar. This relatively long time was unexpected because most investigators, including ourselves, have demonstrated synthesis of phospholipase C in the early logarithmic growth phase.

We observed that 0.5% glucose was responsible for decreased enzyme production in a liquid medium. The lecithin agar also contained 0.5% glucose. Therefore, the delayed reaction on the solid medium might have been caused by the presence of the carbohydrate. To test this hypothesis, lecithin agars were formulated with and without 0.5% glucose and were streaked with 25 known phospholipase-producing bacteria. The plates were observed daily, and the time to produce a moderate zone of precipitation (at least 1 to 2 mm) was recorded.

Glucose accelerated the appearance of the precipitate with five of the isolates, delayed it with 12, and made no difference with the remainder. The average time to produce a reaction of moderate degree was 3.0 ± 1.6 days when glucose was present and 2.2 ± 1.2 days when it was absent.

Because egg yolk agar and egg yolk broth have been used for detection of production of phospholipase C, we compared results of reac-

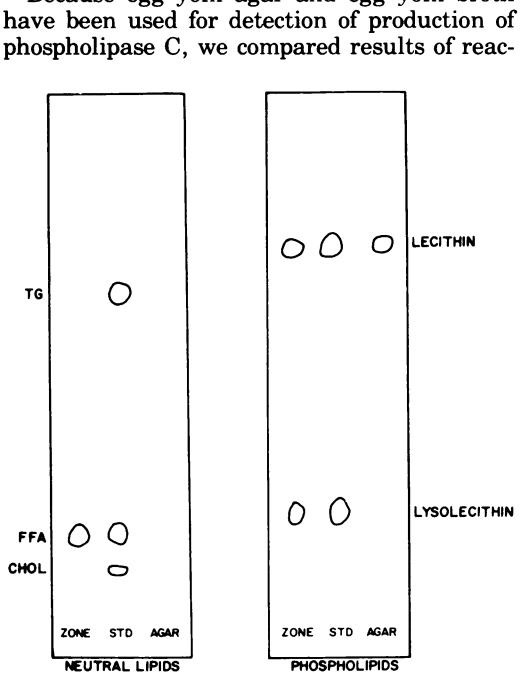

FIG. 2. *Thin-layer chromatograms (Silica Gel G for neutral lipids and Silica Gel HR for phospholipids) of extracts of lecithin agar and of the clear zone surrounding colonies of microorganism 157 (Pseudomonas sp.) grown on lecithin agar. TG, triglyceride; FFA, free fatty acids; CHOL, cholesterol.*

tions on them to those on lecithin agar. Nineteen gram-negative isolates from rinse tests of cleaned milk processing equipment in a commercial dairy plant were tested. The assumption was made that an egg yolk reaction (curd formation) was indicative of phospholipase C activity (4). An opaque zone on solid media and a positive egg yolk broth reaction occurred in most cases, whereas a negative egg yolk broth reaction was usually observed when the phospholipase A reaction (clear zones) occurred on solid media. Reactions confirmed each other in egg yolk agar and egg yolk broth (+ or −) 84% of the time, whereas reactions in lecithin agar and egg yolk broth were the same 74% of the time. However, interpretation was easier on lecithin plates because of fewer interfering reactions in the agar. Also, the opaque zone of phospholipase C usually occurred 1 or 2 days earlier on lecithin agar. Additionally, it was found that egg yolks must be fresh to yield opacity in the agar upon hydrolysis by phospholipase C. The 50% egg yolk emulsion of Difco is adequate when fresh, but upon storage yields an atypical clearing after phospholipase C activity. Sterility is certainly easier to maintain in preparations of lecithin agar since all components can be autoclaved.

Some cultures produced an opalescent zone on egg yolk agar and a corresponding clear zone on lecithin agar. Since these zones occurred within the opaque zone characteristic of phospholipase C, it is probable that a lipase was present that was able to hydrolyze the diglycerides produced by the former enzyme.

LITERATURE CITED

1. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.
2. **Haberman, E., and K. L. Hardt.** 1972. A sensitive and specific plate test for the quantitation of phospholipases. Anal. Biochem. 50:163-173.
3. **McMurray, W. C., and W. L. Magee.** 1972. Phospholipid metabolism. Annu. Rev. Biochem. 41:129.
4. **van Heyningen, W. E.** 1941. The biochemistry of the gas gangrene toxins. I. Estimation of the α-toxin of *Cl. welchii* Type A. Biochem. J. 35:1246-1256.