Specific and Sensitive Plate Assay for Bacterial Lipases

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A plate assay to detect bacterial lipase (EC 3.1.1.3) in a medium containing trioleylglycerol and the fluorescent dye rhodamine B is presented. Substrate hydrolysis causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation. The logarithm of lipase activity from cell-free culture supernatants is linearly correlated with the diameter of halos, thereby allowing quantitation of lipase activities ranging from 1 to 30 nkat.

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are produced by various microorganisms either alone or together with esterases (carboxylic-ester hydrolases; EC 3.1.1.1) (2). Screening of lipase producers on agar plates is frequently done by using tributyrin (9) or Tween 80 (12) as a substrate. Modifications of these assays use Tween 80 in combination with Nile-blue (7) or neat’s-foot oil with Cu²⁺ salts (13). These substrates are not suitable to detect true lipases because they are hydrolyzed by esterases, too. The existence of lipases has to be verified by applying time-consuming methods, e.g., a titrimetric test (11) with trioleylglycerol, which is an ideal lipase substrate (6). Fungal lipase can be visualized after ultrathin-layer isoelectric focusing when the focusing gel is overlaid by an agar gel containing trioleylglycerol and the fluorescent dye rhodamine B (5). We adapted this technique to develop a specific and sensitive plate assay suitable either to identify lipase-producing bacteria or to quantify lipase activity in culture supernatants.

Olive oil as an inexpensive substitute for trioleylglycerol (6) was purified by passage through a column of neutral alumina in a solvent mixture of ether and petroleum ether (1:10 [vol/vol]) (2). Rhodamine B (1 mg ml⁻¹; Sigma Chemical Co., Munich, Federal Republic of Germany) was dissolved in distilled water and sterilized by filtration. Growth medium contained (per liter): nutrient broth, 8 g; sodium chloride, 4 g; and agar, 10 g. The medium was adjusted to pH 7.0, autoclaved, and cooled to about 60°C. Then 31.25 ml of olive oil (2.5% [wt/vol])-10 ml of rhodamine B solution (0.001% [wt/vol]) was added with vigorous stirring and emulsified by mixing for 1 min with an Ultra-Turrax homogenizer (Janke & Kunkel KG, Staufen, Federal Republic of Germany). After the medium was allowed to stand for 10 min at 60°C to reduce foaming, 20 ml of medium was poured into each plastic petri dish. Pseudomonas aeruginosa PAO was from B. W. Holloway, Clayton, Australia, P. aeruginosa PAC 1R was from P. Meadow, London, England, and mucoid P. aeruginosa strains were from J. Wingender, Bochum, Federal Republic of Germany. All other bacterial strains were from our own culture collection. Lipase-producing strains were identified on spread plates after incubation for 48 h at 37°C. To quantify lipase activity, 3-mm-diameter holes were punched into the agar and filled with 10 µl of cell-free culture supernatant. The plates were incubated for 16 h at 37°C. Lipase activity was assayed titrimetrically with purified olive oil as substrate (4), with 1 nkat representing 60 nmol of oleic acid released per min.

Agar plates containing trioleylglycerol and rhodamine B appear opaque and are pink colored. Lipase production is monitored by irradiating plates with UV light at 350 nm. After 16 h of incubation bacterial colonies began to show an orange fluorescence; with continuing incubation time orange fluorescent halos were formed around the colonies of lipase-producing strains, e.g., P. aeruginosa PAC 1R and Staphylococcus aureus (Fig. 1A, sectors 1 and 2). Strains not producing lipase, such as Escherichia coli, accumulated rhodamine B, i.e., formed pink colored colonies, but did not show orange fluorescence upon UV irradiation (Fig. 1A, sector 3). Examination of P. aeruginosa ATCC 9027, PAO, and PAC 1R, Serratia marcescens, S. aureus, and Bacillus subtilis on rhodamine B plates identified these strains as lipase producers, in accordance with the results of a titrimetric assay of culture supernatants. Production of exopolysaccharide did not interfere with a positive reaction on rhodamine B plates, as was shown when mucoid, i.e., alginase-producing, P. aeruginosa strains were screened. Plate methods to identify lipase-producing microorganisms with trioleylglycerol as substrate used pH indicators such as Nile-blue sulfate (3) or Victoria blue B (1) which respond to any changes in pH and inhibit growth of certain bacterial species; another method requires an additional developing step with OsO₄ (8). The rhodamine B plate method is insensitive to pH changes and allows reisolation of organisms which show no inhibition of growth or change of physiological properties.

A direct quantitation of lipase activity is difficult, presumably because of the low amounts of lipase molecules released by a single colony. Quantification can be done, however, by filling culture supernatants into holes punched into the agar, as was shown by testing a culture supernatant from P. aeruginosa PAC 1R (Fig. 1B). The logarithm of lipase activity is linearly related to the zone diameter (Fig. 2), thereby fulfilling the requirement of a valid agar diffusion assay (9). A linear regression analysis revealed a correlation coefficient of 0.991. The specificity of the assay was shown when esterase (carboxylic-ester hydrolase) was tested and no fluorescent halos were formed (Fig. 1B). The sensitivity of the assay is demonstrated by the fact that the lowest detection limit was about 1 nkat of lipase activity, whereas about 20 nkat was needed for the titrimetric assay.

The molecular mechanism underlying the formation of fluorescent products generated from trioleylglycerol hydrolysis by lipase is unknown. A method for determination of free fatty acids in solution has been described in which

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rhodamine B is used in the presence of uranyl ions, yielding orange fluorescent complexes with an excitation wavelength of 350 nm (10). This corresponds to our observations with immobilized rhodamine B, as does the detectable concentration range, which is given as 50 to 400 nmol of fatty acid. The mechanism suggested by the authors is a complex formation between cationic rhodamine B and the uranyl-fatty acid ion (10). We tested possible reaction products of trioleoylglycerol hydrolysis by lipase, i.e., mono- and dioleoylglycerol, oleic acid, and sodium oleate (data not shown). All products when solubilized in petroleum ether and filled into holes in rhodamine B plates gave a positive reaction, yielding orange fluorescent complexes. A conceivable mechanism may be the generation of excited dimers of rhodamine B which fluoresce at longer wavelengths than the excited monomer (excimer fluorescence).

FIG. 2. Relation between the logarithm of lipase activity and the diameter of halos formed on rhodamine B agar plates. Culture supernatant of P. aeruginosa PAC 1R (10 l) representing lipase activities in the range of 100 to 1,000 nmol of oleate min\(^{-1}\) (1.7 to 16.7 nkat) was pipetted into holes, and the plates were incubated for 16 h at 37°C. AX represents the diameter of the orange fluorescent halo minus the diameter of the hole. All values are mean values from triplicate determinations.

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LITERATURE CITED