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Specific Plate Assay for Bacterial Heparinase

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A procedure was developed for detecting heparinase activity on heparin agar plates. The method is based on the differential precipitation of heparin and heparinase-generated heparin fragments by protamine sulfate. Heparinase activity is detected by the presence of clear zones against a white background. This method can be used to screen for the expression of recombinant heparinase and to identify *Flavobacterium heparinum* mutants expressing heparinase constitutively.

Flavobacterium heparinum (3) and *Bacteroides* spp. (7, 13–15) produce enzymes capable of degrading the sulfated polysaccharide heparin. Of these, the *F. heparinum* enzymes have been studied most thoroughly. Heparinase (EC 4.2.2.7) is an eliminase (11) which catalyzes the first of several steps in the heparin catabolic pathway of *F. heparinum*, enabling the organism to utilize heparin as the sole source of carbon, nitrogen, and sulfur (3). Four of the enzymes in this pathway, heparinase and three sulfatases, have been purified and characterized (1, 2, 12, 16). The biosynthesis of these four enzymes is regulated by sulfate repression which is overridden by heparin induction (6). Heparinase itself has been used in several applications, including the preparation of low-molecular-weight anticoagulants (10) and antitumor agents (4). The recent development of an immobilized enzyme filter for blood deheparinization (9) has created a demand for larger quantities of heparinase than can currently be produced economically by *F. heparinum*. Therefore, the isolation of a more efficient producer, either by molecular cloning or by identification of a constitutive mutant, is of great importance.

An assay for heparinase activity based on the metachromatic shift of azure A from blue to red in the presence of heparin has been developed (5). Although this assay is useful in measuring activity in crude cellular extracts, it is not adaptable to screening large numbers of candidates, either in the form of an agar plate assay or in microtiter plates. A plate assay for heparin degradation based on the precipitation of free sulfate groups by barium has been reported (8). However, it appears that this assay requires the completion of several steps in the heparin catabolic pathway, particularly the sulfatase reactions, for detection.

Protamine sulfate is able to precipitate heparin from human blood by electrostatic association. This property was exploited in the development of a plate assay for heparinase activity. Heparinase assay plates contained 0.25 M sodium acetate, 0.0025 M calcium acetate, 1.0 g of porcine intestinal mucosal heparin per liter (Hepar Industries, Franklin, Ohio), and 1.5% agarose (Bethesda Research Laboratories, Inc.). The medium was adjusted to pH 7.0 and autoclaved before the plates were poured. Heparinase was isolated from *F. heparinum* by a variation of the method of Yang et al. (16) and was free of sulfatase activity. Various amounts of this enzyme (0.0, 0.1, 0.5, and 5 U, where 1 U is the amount of enzyme required to degrade 1 mg of heparin per h) were applied to a heparinase assay plate in 10 μl of 10 mM sodium phosphate–150 mM sodium chloride (pH 7.0) and incubated at 37°C for 1 h. Subsequently, a 2% protamine sulfate (salmon; Sigma) solution was poured over the surface of the plate. During a 1- to 2-h incubation period at room temperature, a white precipitate formed in the plates, with clear zones of increasing intensity at the areas where increasing amounts of heparinase were added (Fig. 1). A clear zone was similarly formed around *F. heparinum* grown on an LB agar plate containing 1.0 g of heparin per liter (Fig. 2). Furthermore, *Escherichia coli* JM83 grown on the same plate yielded no clear zone.

Detection of an *F. heparinum* constitutive heparinase producer requires growth of the organism on medium without heparin, which would override sulfate repression. *F. heparinum* was grown in 50 ml of minimal medium containing the following: K_2HPO_4, 3 g/liter; $KH_2PO_4 \cdot H_2O$, 1.5 g/liter; NaCl, 0.5 g/liter; NH_4Cl, 1.0 g/liter; $MgSO_4 \cdot 7H_2O$, 10 mM; L-histidine, 0.2 g/liter; L-methionine, 0.2 g/liter; glucose, 8.0 g/liter; and 10^{-4} M each $NaMoO_4 \cdot 2H_2O$,

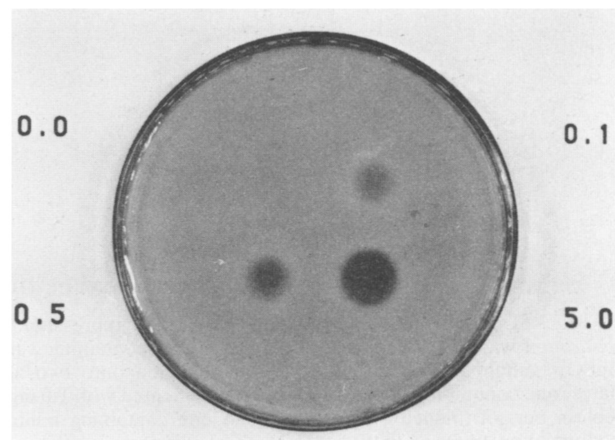

FIG. 1. Visualization of heparinase activity on heparin plates. Various amounts of purified heparinase from *F. heparinum* (0.0, 0.1, 0.5, and 5.0 U) were applied in 10-μl volumes onto 1.5% agarose plates containing 0.25 M sodium acetate–0.0025 M calcium acetate–1.0 g of porcine intestinal mucosal heparin per liter (Hepar Industries), pH 7.0. The plates were incubated for 1 h at 37°C. A 2% protamine sulfate solution (5 ml) was poured onto the plates and allowed to react with heparin for 1 h at room temperature. Clear zones appeared where heparin was degraded by the action of heparinase.

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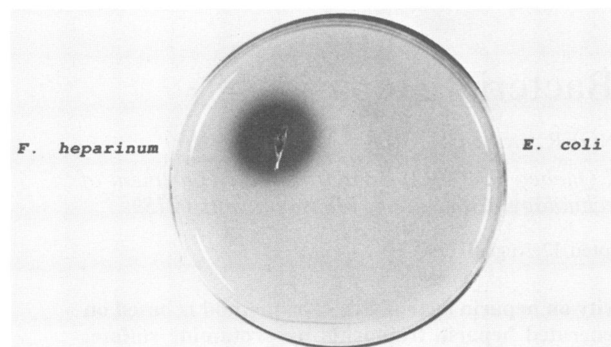

FIG. 2. Detection of heparinase in growing cells. *F. heparinum* and *E. coli* JM83 were grown on LB agar plates containing 1.0 mg of heparin per ml for 48 h at 30°C. The cells were washed from the plates with distilled water, and a 2% protamine sulfate solution (5 ml) was poured onto the plates and allowed to react with heparin for 1 h at room temperature. A clear zone was observed surrounding the area where *F. heparinum* had grown.

$CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $CaCl_2$. When the A_{600} reached 1.0, the culture was diluted and plated out onto two minimal medium agar plates (1.5%), one of which was supplemented with heparin (1.0 g/liter). The plates were incubated at 30°C for 36 h. The cells from each plate were transferred to nitrocellulose filter papers. *F. heparinum* colonies that had adhered to the filter papers were lysed by being exposed to chloroform vapors for 20 min. After cell lysis, the filter papers were placed on heparinase assay plates and incubated at 37°C for 1 h. The filter papers were discarded, and the plates were developed with 2% protamine sulfate. Clear zones appeared on the assay plate that had been incubated with lysed cells grown under heparinase-inducing conditions (heparin-supplemented plate), while no clear zones could be detected on the plate that had been incubated with lysed cells grown under sulfate repressing conditions (Fig. 3).

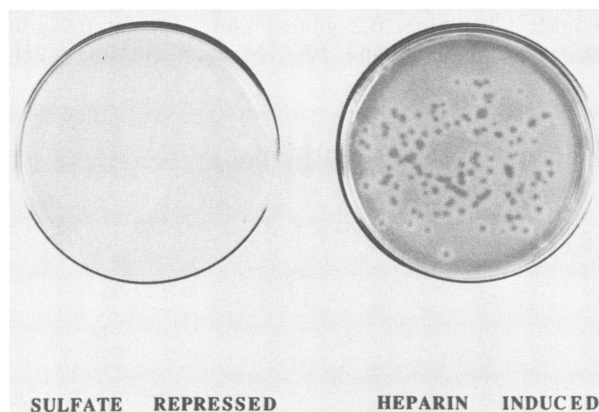

FIG. 3. Detection of heparinase induction and repression. *F. heparinum* was grown in liquid minimal medium containing 2 mM $MgSO_4$ (sulfate repressing conditions) and plated onto two agar plates, one containing minimal medium supplemented with 1.0 mg of heparin per ml (inducing conditions) and one containing minimal medium supplemented with 2 mM $MgSO_4$ (sulfate repressing conditions). The cells from each plate were transferred to nitrocellulose paper, exposed to chloroform vapors for 20 min, and overlaid onto assay plates containing 1.0 mg of heparin per ml, 0.25 M sodium acetate, 0.0025 M calcium acetate, and 1.5% agarose. The plates were incubated for 1 h at 37°C. A 2% protamine sulfate solution (5 ml) was poured onto the plates and allowed to react with heparin for 1 h at room temperature. Clear zones appeared only on the plate that had been incubated with lysed cells grown under heparinase-inducing conditions.

The plate assay described here appears sufficient for detecting heparinase activity and does not require the presence of other heparin-catabolic enzymes. This feature represents an improvement over previously reported methods and may prove useful in screening *E. coli* gene banks for the cloned heparinase gene. Additionally, the ability to differentiate *F. heparinum* grown under heparinase-repressing and -inducing conditions, by using nitrocellulose filters, indicates the usefulness of this technique in identifying constitutive mutants.

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