Convenient Assay for Staphylococcal Nuclease by the Metachromatic Well-Agar-Diffusion Technique

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The metachromatic agar-diffusion (MAD) microslide technique was adapted for quantitative assay for staphylococcal thermonuclease in heterogeneous systems, such as milk and broth. When an enzyme-containing solution was placed in a well cut in the agar, a bright pink halo was obtained. The diameter of the pink zone of hydrolysis was related to time and temperature of incubation and to nuclease concentration. Concentrations of nuclease as low as 0.005 µg/ml and as high as 2.0 µg/ml were conveniently determined after 3 hr at 37 C.

Of methods available for assay of deoxyribonuclease activity (3–6, 8, 9), only the procedures using methyl green (5, 6, 8) are directly applicable with impure systems. The methyl green methods require a highly polymerized deoxyribonucleic acid (DNA) substrate. For opaque systems, only the disc-agar-diffusion methyl green procedure (8) and the HCl-flood- ing technique (4), as modified by Jarvis and Lawrence (3), are available. The latter method is limited by interference from proteins.

The well-agar-diffusion method described in this paper is convenient, simple, inexpensive, and rapid. Low-cost, standard equipment is used; a highly polymerized DNA is not required; and quantitatively reproducible results are obtainable with impure, opaque systems that contain high concentrations of protein. The method is based on the metachromatic effect of agar and DNA in Toluidine Blue (7). Hence, the method is designated as the metachromatic agar-diffusion (MAD) microslide technique. Measurements of the diameters of zones of DNA hydrolysis were related to the enzyme activity of a known staphylococcal nuclease standard by using brain heart infusion (BHI) broth and homogenized whole milk as diluents. Several parameters which may influence the nuclease reaction were investigated.

MATERIALS AND METHODS

Determination of nuclease activity. The MAD reagent, consisting of Toluidine Blue (Matheson, Coleman and Bell), DNA (Difco), and Ionagar no. 2 (Colab Laboratories), was prepared as described by Lachica et al. (7). Small volumes (3.0 ml) of the molten reagent were pipetted onto plastic immunoplates (2.5 by 7.5 cm, Hyland) on a leveling table. The depth of the MAD reagent was 1.6 mm. After gelling, as many as 10 equidistant wells (2 mm in diameter) were cut; fewer wells may be cut for samples with high nuclease activity. Initially, the wells were filled with 3-µliter samples dispensed with a 10-µliter syringe (Hamilton Corp.). Subsequently, a simple procedure was adopted in which each well was filled with three loopfuls (platinum, 2 mm in diameter) of enzyme solution. The cover for each slide was replaced to minimize desiccation. After incubation, bright pink zones of nuclease activity developed. The diameters of these zones of DNA hydrolysis were measured with an enlarger-viewer (Universal projector, National Instrument Laboratories, Rockville, Md.).

Experimental design. Three factors were studied for the MAD assay of staphylococcal nuclease in BHI broth: enzyme concentration (nine levels: 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 µg/ml); duration of incubation (four levels: 1, 2, 3, 4 hr); and incubation temperature (two levels, 25 and 37 C). For each time and temperature of incubation, MAD slides were set up containing each of the nine concentrations of enzyme in duplicate. To accommodate larger zones of reaction, exception was necessary for incubation at 37 C for 4 hr—the nine levels of enzyme were distributed in two MAD slides. The slides were numbered in the order in which they were filled and were evaluated in the same order after incubation.

To determine the effect of salt and pH levels, triplicates of BHI broths containing various levels of enzyme (0.005 to 2.0 µg/ml) were adjusted to three salt concentrations (0.5, 5.0, 10.0% NaCl) and three
pH values (pH 4.5, 7.5, 9.5) by using NaOH and HCl solutions. The wells were filled as described above and incubated for 3 hr at 37 C.

For the assay of staphylococcal nuclease in milk, seven levels of enzyme concentration (0.1 to 1.0 \( \mu g/ml \)) were added to homogenized whole milk and subsequently placed in the wells of the MAD slide in triplicate and incubated for 3 hr at 37 C. This experiment was repeated 1 week later.

The data obtained from these factorial experiments were analyzed by the methods of Snedecor and Cochran (10).

RESULTS

The statistical analysis (Table 1) validates the feasibility of assay of staphylococcal nuclease by using the MAD microslide technique. The precision of the assay was good, as evidenced by the small value of the residual error mean square (MS = 0.08) relating to well-to-well variation within an experiment.

Day-to-day variation was small, as indicated by the nonsignificant value of the replications mean square (MS = 0.03). This small day-to-day effect probably reflects the fact that the experiments were run twice—three wells per experiment giving a total of six wells per point. Although, the day-to-day variation was small, the careful investigator may wish to calculate a standard curve for each day’s work. Because the reliability of the standard becomes greater with a greater number of points, the actual number of points necessary for such a curve will vary with the purpose of the assay.

Concentration, time, and temperature significantly affected the microslide agar-diffusion assay of staphylococcal nuclease (respective F values: 2,313, 4,132, and 4,898). It is therefore important that these factors be standardized for the assay.

The impact of change in concentration was essentially linear—note F = 6 for deviation from linearity, as compared with F = 18,000 for linear evaluation (Table 1). This is graphically illustrated in Fig. 1, a semilogarithmic plot of concentration of nuclease added to milk versus the diameters of zones of hydrolysis of DNA. The curve borders at both sides of the regression line represent the confidence limits of the zone diameters for each enzyme concentration (10).

Deviation from linearity was decidedly favored by increased periods of incubation. The effect was most pronounced beyond 3 hr (Fig. 2). In addition, the sharpness of the margins of zones of hydrolysis diminished with longer incubation periods. Thus, a 3-hr period of incubation is preferred.

When the assay was carried out at 25 C, the edges of the zones of hydrolysis were indistinct, and zone diameters could not be measured accurately. At 37 C precise measurement was possible.

The interactions of concentration with time and temperature (Ct \( \times \) Ti and Ct \( \times \) Tp), time with temperature (Ti \( \times \) Tp), and the three factors with each other (Ct \( \times \) Ti \( \times \) Tp) were significant; the F values were (Table 1): 24.00, 10.80, 82.80, and 3.84, respectively. However, such interactions appeared to be of less importance than the effects of each factor individually (F values: Ct = 2,313; Ti = 4,132; Tp = 4,898).

Neither salt content as high as 10% NaCl nor

| Source of variation                  | df | SS   | MS   | F    |
|-------------------------------------|----|------|------|------|
| Replication (days)                  | 1  | 0.03 | 0.03 | 0.34NS |
| Concentration (Ct)                  | 8  | 1498.45 | 187.31 | 2,313* |
| Linear                              | 1  | 1495.00 | 1495.00 | 18,000* |
| Dev.                                | 7  | 3.45 | 0.49 | 6* |
| Time (Ti)                           | 3  | 1003.87 | 334.62 | 4,132* |
| Linear                              | 1  | 963.73 | 963.73 | 12,000* |
| Quadratic                           | 1  | 40.11 | 40.11 | 446* |
| Cubic                               | 1  | 0.02 | 0.02 | <1NS |
| Temperature (Tp)                    | 1  | 396.67 | 396.67 | 4,898* |
| Ct \( \times \) Ti                  | 24 | 46.83 | 1.94 | 24.00* |
| Ct \( \times \) Tp                   | 8  | 7.01  | 0.88 | 10.80* |
| Ti \( \times \) Tp                   | 3  | 19.98 | 6.66 | 82.80* |
| Ct \( \times \) Ti \( \times \) Tp   | 24 | 7.46  | 0.31 | 3.84* |
| Error (residual)                    | 71 | 5.75  | 0.08 |      |

* df, Degrees of freedom; SS, sum of squares; MS, mean square; F, Snedecor “F” ratio; NS, not significant.
* Significant at 0.001 level.
* Dev, Deviation from linearity.

TABLE 1. Analysis of variance: three factors affecting the metachromatic agar-diffusion assay of staphylococcal nuclease
of nuclease action and the logarithm of enzyme concentration with the MAD microslide technique. The variability of the method was evaluated by using the commercially available enzyme from Worthington. Results of the linear regression analysis indicated that the standard curve does not need to be plotted separately for each slide when an unknown is tested.

The sensitivity of the MAD microslide method (0.005 μg/ml of sample) exceeds that of other methods applicable to impure, opaque samples. Thus, with the paper disc method of Le Talaer et al., the minimal detectable amount of nuclease is 0.125 μg/9-mm disc (8).

The noninterference of pH and salt levels in the BHI broth may be attributed to the small size of the samples (ca. 3 μliters) applied to the wells and the well-buffered, high pH (9.0) of the MAD reagent.

The assay of the nuclease in milk demonstrates that neither protein nor opacity of the experimental material interferes with the measurements. Our technique therefore eliminates the need for the additional manipulations of extraction and purification.

The method is suitable for the rapid, routine screening of large numbers of samples by a single laboratory worker. Moreover, the MAD reagent is so stable that slides may be dried and preserved for documentation, thereby eliminating the necessity for a photographic record. The method is also amenable to automation by using an automatic zone-size reader (1, 2).

It should also be noted that the technique is applicable to the assay of other deoxyribonucleases such as pancreatic deoxyribonuclease I and streptococcal deoxyribonucleases (Lachica and Hoeprich, unpublished data).

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