Turbidimetric Assay of Staphylococcal Nuclease

ALAN ERICKSON AND R. H. DEIBEL

*Department of Bacteriology and Food Research Institute, College of Agricultural and Life Sciences,
University of Wisconsin, Madison, Wisconsin 53706*

A simplified turbidimetric procedure was developed to assay staphylococcal nuclease activity. The ease of performance and sensitivity to nanogram quantities enhance the utilization of the method for the quantitative or qualitative estimation of the enzyme. Unlike plating methods, the turbidimetric procedure affords the differentiation between heat-stable and heat-labile nuclease activity.

The exocellular nuclease produced by strains of *Staphylococcus aureus* has been studied extensively since its discovery by Cunningham et al. (2). Its remarkable thermostability, dependence on calcium ions for optimal activity, and hydrolysis of nucleic acid chains yielding fragments terminated by 3'-phosphate groups has made the enzyme a subject of interest to enzymologists as well as to bacteriologists (1, 2, 10).

Weckman and Catlin (12) observed high deoxyribonuclease activity in clinical isolates of *S. aureus* and also demonstrated a close correlation between nuclease and coagulase production. The use of the heat-stable staphylococcal nuclease as an indicator of potentially pathogenic staphylococci (7) prompted an investigation into the various DNase assays available to determine which was most suitable for this particular application.

Jeffries et al. (5) incorporated deoxyribonucleic acid (DNA) in an agar medium to study DNase production by fungi and bacteria. After first streaking with a test organism and then incubating, unhydrolyzed DNA was precipitated by flooding the plates with 1 N HCl. A clear zone around a colony indicated the presence of DNase. A recent modification of this method by Smith et al. (11) involved the addition of 0.005% methyl green to the medium. Since methyl green combines only with highly polymerized DNA, nuclease activity is detected readily by a clearing of the dye around a DNase-producing colony. This method is used widely for the detection of DNase production in semi-solid growth media.

The quantitative procedures to estimate DNase activity are laborious and involved, and in some cases they are affected by the purity of the enzyme and substrate preparations employed. The two methods most often used are the viscometric method described by Laskowski and Seidel (8), and the spectrophotometric method of Kunitz (6).

The method presented in this paper is a modification of Houck's procedures for the turbidimetric estimation of ribonuclease (3) and DNase (4). The method is sensitive and rapid, and it requires no special apparatus or technical skill. It is relatively unaffected by the purity of the enzyme, and, unlike the agar-plate methods, it lends itself to the routine determination of the heat stability of a nuclease preparation.

MATERIALS AND METHODS

Cultures. All enterotoxin-producing strains of *Staphylococcus aureus* were obtained from the Food-Research-Institute Collection, Madison, Wisc. *Staphylococcus* strain N9A, a coagulase-negative, non-enterotoxin-producing isolate (from an outbreak of anaerobic nitrite burn in fermented sausage), and *Staphylococcus hyicus*, a coagulase-negative strain implicated in certain skin infections (enterotoxin has not been tested), were obtained from the collection of the Department of Bacteriology, University of Wisconsin.

Propagation and storage of cultures. All cultures were maintained by daily transfer in Brain Heart Infusion broth (Difco). Stock cultures were made by freezing a drop of a 24-h culture in Brain Heart Infusion broth. All cultures were grown at 37 C.

Deoxyribonucleic acids and ribonucleic acids. The DNA used as the substrate in the nuclease assay was DNA from Difco. Other nucleic acid preparations used were DNA highly polymerized, DNA not highly polymerized, ribonucleic acid (RNA), and soluble RNA (all from Nutritional Biochemicals Corp., Cleveland, Ohio).

Crystalline micrococcal nuclease. Micrococcal nuclease (source was *S. aureus*) was obtained from the Worthington Biochemical Corp., Freehold, N.J.

The enzyme was dissolved in boric acid-borax buffer, pH 8.6, for use in the experiments.

Growth and turbidity estimations. All growth and turbidity estimations were made with a Spectronic-20 spectrophotometer (Bausch & Lomb, Inc.) at a wavelength of 600 nm.

Preparation of reagents and buffers to be used in the nuclease assay. Salmon sperm DNA (Difco) was dissolved in 100 ml of distilled-deionized water to give a final concentration of 1,000 μg/ml. This nucleic acid solution was placed in a boiling water bath for 30 min to effect denaturation. The solution was cooled immediately in an ice bath and stored at 4 C. As will be discussed subsequently, staphylococcal nuclease exhibits greater activity with denatured substrate. The final DNA solution was viscous and had a pH of about 6.9.

The buffer used in the staphylococcal nuclease assay was a boric acid-borax buffer (0.2 M in terms of borate) adjusted to pH 8.6. This was composed of 10.49 g of $Na_2B_4O_7 \cdot 10 H_2O$ and 5.57 g of H_3BO_3/L. If additional pH adjustment was necessary, 0.2 M solutions of borax and boric acid were added. The hydrolysis of DNA by staphylococcal nuclease is activated by the presence of calcium ions (2), and $CaCl_2$ was added to a final concentration of 10^{-3} M to satisfy this cofactor requirement.

RESULTS

Preparation of the standard curve, procedure for enzyme assay, and calculation of units. To prepare the standard curve, 0.25 to 1.50 ml (250 to 1,500 μg) of the DNA stock solution was dispensed to 18-mm matched test tubes. The volume was adjusted to 9.0 ml in each tube by the addition of borate buffer, and the temperature was equilibrated to 37 C in a water bath. To the nucleic acid buffer solution, 1.0 ml of 4 N HCl was added to precipitate the DNA. The turbidity was allowed to develop for 10 min at 37 C and it was estimated at 600 nm.

To determine the activity of a sample of nuclease, 1.0 ml of DNA stock and 7.0 ml of borate buffer were mixed and allowed to equilibrate at 37 C. One milliliter of the desired dilution of the enzyme was added and incubated with the substrate. A 15-min incubation period gave satisfactory results, but other time periods may be used before the acid is added to stop enzyme activity and develop the turbidity.

The amount of substrate depolymerized per milliliter was estimated using the standard curve. The units of nuclease present in the enzyme sample were calculated using the following equation: nuclease units per milliliter = (substrate depolymerized per milliliter) × dilution factor/time of incubation (minutes).

Conditions for turbidity development. This assay was based on the relationship between turbidity and the amount of precipitable nucleic acid present. Therefore, various conditions were examined to determine their effect on turbidity development.

The effect of incubation time at 37 C on the turbidity produced following the addition of acid is illustrated in Fig. 1. Maximum optical density was observed after approximately 8 min and the suspension was stable for another 10 min. Decreasing the temperature from 37 to 25 C caused a slight decrease in the rate of turbidity development, but the maximum optical density remained unaffected. Ten minutes of development time at 37 C was chosen for standard assay conditions.

The turbidity produced by precipitating varying concentrations of DNA is shown in Fig. 2. The standard curve is essentially linear in the range of 20 to 100 μg/ml, where 100 μg/ml represents 1 ml of DNA stock diluted to 10 ml by assay buffer (8 ml) and 4 N HCl (1 ml). Denatured DNA (30 min at 100 C), native DNA (unboiled stock solution), and sonically treated DNA (denatured DNA sonically treated for 4 min at a setting of 75 on a Biosonic Oscillator [Bronwill Scientific]) all gave identical standard curves.

Four other commercial nucleic acid preparations were examined for acid precipitability. All differed in slope but displayed linearity similar to that observed with DNA (Difco). Since 100 μg of DNA/ml (and lower concentrations) was in the linear range of the curve, this was chosen as the initial concentration in the enzyme assay.

The molarity of the HCl used to develop the turbidity was insignificant as long as it could override the buffering capacity of the particular system used in the assay.

Conditions for enzyme assay using crystalized staphylococcal nuclease. Several factors were examined to determine their effect on the enzyme assay using crystalline micrococcal nuclease. The nuclease was dissolved in borate buffer at pH 8.6, and appropriate dilutions in this buffer were made for use as in the previously described assay.

The effect of time on substrate depolymerization is shown in Fig. 3. Two different enzyme concentrations, 5.8 and 29.0 ng (total enzyme/10 ml of reaction volume), were compared. A distinct linear region was observed for both concentrations, and a comparison of the slopes showed that 29.0 ng of enzyme degraded the substrate exactly five times faster than did the 5.8 ng level. The turbidity of the enzymatically treated solutions was translated into micrograms per milliliter of DNA using the stan-
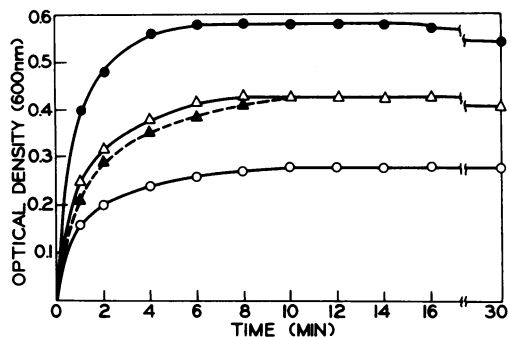
FIG. 1. *Effect of time after the addition of acid on the turbidity of 50* (O), *75* (Δ, ▲), *and 100* (●) *µg of DNA/ml. All estimations were made at 37 C except* (▲), *which was incubated at 25 C.*
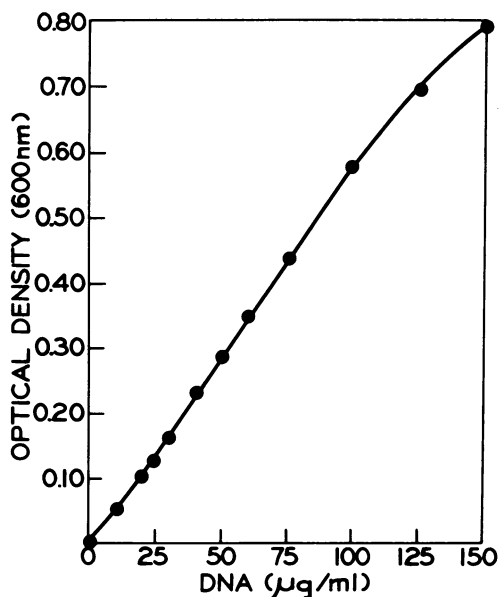

FIG. 2. *Standard curve for nuclease assay (10-min developing time at 37 C).*

dard curve, and the substrate depolymerized was determined by difference.

Using standard assay conditions (borate buffer at pH 8.6; 15-min incubation time at 37 C; 10-min developing time with 1 ml of 4 N HCl at 37 C), the extent of depolymerization using 5 to 30 ng (total enzyme/10 ml of reaction volume) of the purified nuclease was examined (Fig. 4). A linear correlation between nuclease concentration and substrate depolymerization was observed. In the 15-min incubation period, 2.8 µg/ml of the substrate was depolymerized per nanogram of enzyme.

Although native and denatured DNA were found to give identical standard curves in

regard to turbidity development upon acidification, a comparison of the activity of the purified nuclease on these two substrates showed that they differed significantly (Fig. 5). A comparison of the slopes in the linear regions showed that the enzyme displays five times greater activity on the boiled (denatured) substrate. Twenty-nine nanograms of enzyme/10 ml of reaction volume hydrolyzed the denatured substrate at a rate of 6.0 µg per ml per min, whereas the native substrate was hydrolyzed at a rate of only 1.2 µg per ml per min. These data suggested that the enzyme has a definitive affinity for the single-stranded substrate, and the enzyme may preferentially attack RNA and single-stranded DNA. A previous investigation has demonstrated that the enzyme displays both exo- and endonucleolytic activity with the native substrate (9).

A series of borate buffers was prepared and used to determine the effect of pH on the activity of the crystallized micrococcal nu-
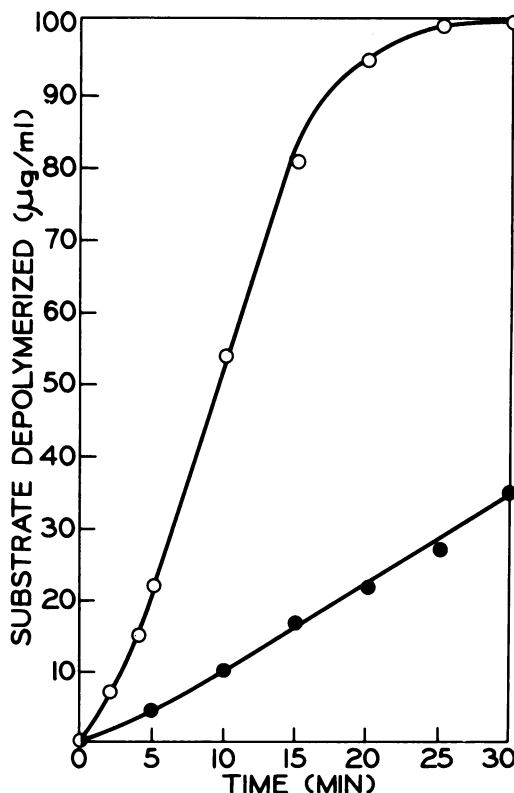

FIG. 3. *Effect of incubation time at 37 C on the substrate depolymerized by 5.8* (●) *and 29* (O) *ng of crystalline S. aureus nuclease (per 10 ml of reaction volume) acting on 100 µg of DNA/ml at pH 8.6.*
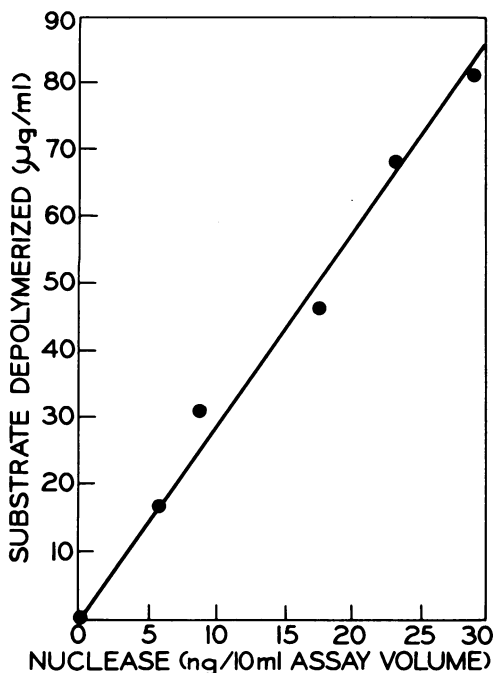
FIG. 4. *Effect of 5 to 30 ng of crystalline S. aureus nuclease acting on 100 µg of DNA/ml for 15 min at 37 C and pH 8.6.*
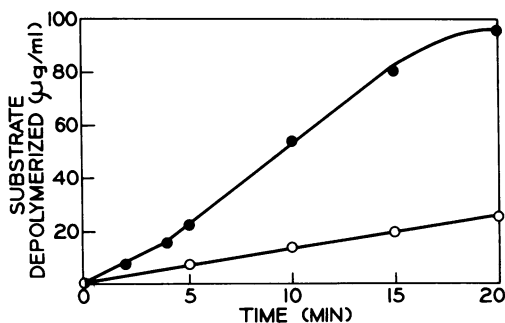

FIG. 5. *A comparison of native (O) and denatured (●) DNA as substrate for 29 ng (per 10 ml of reaction volume) of staphylococcal nuclease.*

of DNA) and 7.0 ml of borate buffer were mixed in matched 18-mm test tubes and equilibrated at 37 C. The spent growth medium of the isolates (the cells were removed by centrifugation or filtration) was heated in a boiling water bath for 15 min and cooled in ice. One milliliter was added to the substrate-buffer mixture, and, following a 30-min incubation period, 1.0 ml of acid was added. Optical densities were determined following the 10-min development time, but in nearly all cases this was unnecessary since the undiluted enzyme preparation and extended incubation time were sufficient to hydrolyze all 100 µg of substrate/ml, provided the isolate was a nuclease producer. Strains of various staphylococcal species were examined in this manner, and the results were compared with those obtained when each was tested on DNase test agar (Difco). As seen in Table 1, an absolute correlation between the two procedures was obtained. The tube turbidity test also affords the determination of the heat stability of the nuclease in contrast to the agar-plate procedure. Thus, we can see the difference between *S. aureus* cultures that characteristically produce a heat-stable nuclease (7) and other bacteria which possess nuclease activity in which the nuclease is heat-labile.
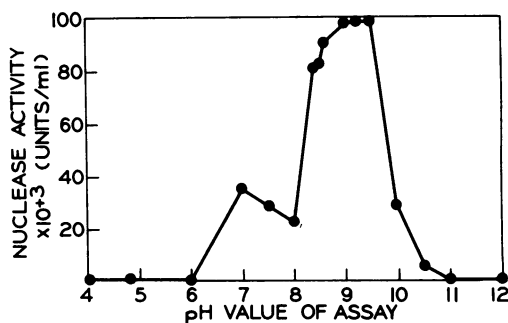

FIG. 6. *Effect of pH on the activity of 29 ng (per 10 ml of reaction volume) of S. aureus nuclease acting for 15 min on 100 µg of DNA/ml at 37 C.*

clease under assay conditions. As is shown in Fig. 6, two optima were observed: a minor peak at pH 7, and a relatively broad major peak centering at about pH 9.2. A pH of 8.6 has been reported as optimal (2), but apparently for this particular system a slightly higher pH is favored. In all experiments dealing with enzyme production and characterization, however, borate buffer at pH 8.6 was used.

The assay was modified and used as a qualitative test to screen staphylococcal isolates for the presence of heat-stable nuclease. One milliliter of DNA stock solution (1,000 µg

TABLE 1. *Comparison of agar-plate and tube-turbidity methods for the detection of DNase activity in cultures of S. aureus*

Species of *Staphylococcus* tested	No. strains positive/no. strains examined	
	Plate DNase test	Tube-turbidity test
S. aureus	11/11	11/11
S. epidermidis	0/20	0/20
S. hyicus	1/1	1/1
Staphylococcus sp. N9A	0/1	0/1

DISCUSSION

The method described in this paper is based on the assumption that the turbidity formed upon acidification is related directly to the amount of unhydrolyzed nucleic acid present. The linear relationship between optical density and DNA concentration, and the proportional decreases in turbidity due to the activity of varying amounts of enzyme, permit a rapid and quantitative estimation of approximately 5 to 30 ng of crystalline staphylococcal nuclease in the assay system. The nuclease was found to hydrolyze denatured DNA at a much faster rate than native DNA. Although denatured DNA and an equivalent amount of native DNA have identical optical densities when precipitated with acid, the use of the denatured substrate substantially increased the activity of the enzyme.

It would appear that this assay should be a useful tool for the quantification of the nuclease produced by strains of *S. aureus*. The assay is easy to perform, it does not require expensive equipment or reagents, and it is sensitive to nanogram quantities of the enzyme. Furthermore, with minor modifications, the assay can be applied to the estimation of other DNase and RNase as well as for assaying the heat stability of the enzymes.

ACKNOWLEDGMENT

LITERATURE CITED

1. Cunningham, L. 1958. Natural 3'-deoxyribomononucleotides. J. Amer. Chem. Soc. **80**:2,546–2,549.
2. Cunningham, L., B. W. Catlin, and M. Privat de Garilhe. 1956. A deoxyribonuclease of *Micrococcus pyogenes*. J. Amer. Chem. Soc. **78**:4,642–4,645.
3. Houck, J. C. 1958. The microdetermination of ribonuclease. Arch. Biochem. Biophys. **73**:384–390.
4. Houck, J. C. 1959. The turbidimetric determination of deoxyribonuclease activity. Arch. Biochem. Biophys. **82**:135–144.
5. Jeffries, C. D., D. F. Holtman, and D. G. Guse. 1957. Rapid method for determining the activity of microorganisms on nucleic acids. J. Bacteriol. **73**:590–591.
6. Kunitz, M. 1950. Crystalline deoxyribonuclease. I. Isolation and general properties. J. Gen. Physiol. **33**:349–362.
7. Lachica, R. V. F., K. F. Weiss, and R. H. Deibel. 1969. Relationships among coagulase, enterotoxin, and heat-stable deoxyribonuclease production by *Staphylococcus aureus*. Appl. Microbiol. **18**:126–127.
8. Laskowski, M., and M. K. Seidel. 1945. Viscometric determination of thymonucleodepolymerase. Arch. Biochem. Biophys. **7**:465–473.
9. Prival de Garilhe, M. 1967. Microbial nucleases, p. 170–179. *In* M. Prival de Garilhe (ed.), Enzymes in nucleic acid research. Holden-Day, Inc., San Francisco.
10. Privat de Garilhe, M., L. Cunningham, U. Laurila, and M. Laskowski. 1957. Studies on isomeric dinucleotides derived from deoxyribonucleic acid. J. Biol. Chem. **224**:751–758.
11. Smith, P. B., G. A. Hancock, and D. L. Rhoden. 1969. Improved medium for detecting deoxyribonuclease-producing bacteria. Appl. Microbiol. **18**:991–993.
12. Weckman, B. G., and B. W. Catlin. 1957. Deoxyribonuclease activity of micrococci from clinical sources. J. Bacteriol. **73**:747–753.