Production and Heat Stability of Staphylococcal Nuclease

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No correlation existed between numbers of organisms and nuclease activity in laboratory-grown cultures of Staphylococcus aureus. Nuclease production was inhibited by anaerobic incubation and stimulated by aeration. Strains of S. aureus varied in the production of nuclease. The optimum pH for enzyme production was 8.3 and employment of a tris(hydroxymethyl)aminomethane buffer system resulted in increased production of the enzyme as compared with a phosphate buffer. The nuclease was extremely heat-stable and had a D value of 16.6 min at 130 C.

Although the production of a heat-stable nuclease by Staphylococcus aureus has been proposed as a characteristic for the identification of this species (6), the effect of various experimental conditions on the production of the enzyme has not been assessed. By using a rapid and sensitive assay procedure (3), the effect of pH, glucose, buffer system, and aeration on nuclease production was examined. Variation in enzyme production among different strains was also investigated.

Many previous investigators have observed a remarkable resistance of the enzyme to heat (1, 2, 7). It is generally agreed that the enzyme can withstand boiling in water for 30 min without appreciable loss of activity. In this study, we attempted to gain more precision in estimating the heat resistance of the enzyme by using the D-value (time at a given temperature to effect a 1-log decrease in enzyme activity) concept.

MATERIALS AND METHODS

Most of the materials and procedures employed in this study were described previously (3). The turbidimetric procedure for the assay of nuclease activity was used (3).

Thermal-destruction-rate curves for the S. aureus nuclease were estimated by using the supernatant fluid of a 24-h, brain heart infusion (BHI)-grown culture (250 ml in a 1-liter Erlenmeyer flask) of the desired strain as the enzyme preparation. Incubation was at 37 C on a gyratory shaker (New Brunswick Corp.). The cells were removed by centrifugation at 10,000 x g for 15 min in a RC2-B refrigerated centrifuge (Ivan Sorvall, Inc.). Because the strains produced large amounts of the enzyme under the above growth conditions, no concentration was necessary.

RESULTS

Production of nuclease by strains of S. aureus. Thirteen strains of Staphylococcus, including representative enterotoxin A-, B-, C1-, C2, D-, and E-producing isolates, were selected and screened for quantitative nuclease production. All strains were compared for their ability to produce nuclease within 24 h at 37 C under static-growth conditions (10 ml of BHI broth in 16-mm, screw-capped test tubes) and under aerated conditions (10 ml of BHI broth in a 50-ml shaker flask with incubation on a gyratory shaker).

After incubation, all cultures were centrifuged, and the supernatant fluids were collected and used as the enzyme preparation. Previous experiments demonstrated that the majority of the nuclease activity was in the supernatant fluid. Sonic disruption of cellular suspensions of nuclease-producing staphylococci failed to liberate demonstrable nuclease activity. As is shown in Table 1, all strains (except N9A, a coagulase-negative, non-enterotoxin-producing Staphylococcus sp.) produced detectable nuclease activity. Staphylococcus hyicus, as well as all of the enterotoxin-producing strains, showed some activity under both growth conditions tested. In all cases, nuclease production was stimulated by aeration. Increases in production ranged from 10- to 105-fold for these strains. No correlation between nuclease activity and enterotoxin type was evident.

Because optical density of the culture was not considered in the preceding experiment,
growth and nuclease production curves were estimated for *S. aureus* strains 100, 196E, S-6 and 334. All strains were grown in 250 ml of BHI broth in 1-liter, side-armed flasks and incubated on a gyrate shaker at 37°C. At various time intervals, the optical densities of the cultures were estimated, and samples of the growth medium were withdrawn aseptically. The cells were removed by centrifugation, and the supernatant fluids were assayed immediately for nuclease activity. Growth curves for the four strains coincided, whereas a marked difference in nuclease production was noticed (Fig. 1). In all cases, enzyme production paralleled logarithmic growth and continued at a high rate into the stationary-growth phase. Nuclease activity is plotted logarithmically in Fig. 1. If the values were plotted arithmetically, the differences in nuclease production among the strains would be even more evident, but difficult to plot. From these data it is evident that a quantitative estimation of staphylococcal nuclease is not a useful criterion for the quantitative estimation of *S. aureus* (1). Not only do individual strains vary in the relative amounts of nuclease produced under identical growth conditions, but environmental conditions have a marked effect on enzyme production.

**Effect of oxygen tension on nuclease production.** Vigorous aeration was shown to have a marked stimulatory effect on nuclease production in all strains used in this investigation. To determine if small changes in oxygen tension would influence nuclease production, atmospheres of 0, 5, 10, 15, and 20% oxygen were examined for their effect by using *S. aureus* strains 100 and S-6. Erlenmeyer flasks (50 ml) containing 10 ml of BHI broth were inoculated with 0.05 ml of a 10⁻¹² dilution of washed cells obtained from a 24-h BHI culture (standard inoculum). The cultures were placed in desiccators which were flushed (three times) and filled with the appropriate gaseous mixture. Commercially prepared oxygen-nitrogen mixtures (Matheson Gas Products) were used for the 5, 10, and 15% oxygen atmospheres, whereas helium (trace of CO₂) was employed for anaerobic conditions. Atmospheric conditions were used for the “20%” oxygen tension. All cultures were incubated for 48 h at 37°C. Immediately upon removal from the desiccators, the optical density of all samples was estimated, followed immediately by boiling for 10 min to prevent further nuclease production. As shall be discussed, boiling does not interfere with nuclease activity. All cultures were centrifuged, and the supernatant fluids were collected and assayed for nuclease activity. The results indicated a definitive oxygen requirement for nuclease production (Fig. 2). A marked increase at 5% oxygen tension, followed by modest but detectable increases at the successively higher oxygen tensions, characterized the results. These data indicate that

**Table 1. Nuclease production by various Staphylococcus strains**

| Strain | Enterotoxin (serological type) | Coagulase production | Nuclease (units/ml)* |
|--------|-------------------------------|-----------------------|----------------------|
|        |                               |                       | Static               |
|        |                               |                       | Aerated              |
| 100    | A                             | +                      | 104                   |
| 95     | A                             | +                      | 72                   |
| 196E   | A                             | +                      | 29                   |
| S-6    | B (some A)                    | +                      | 177                   |
| 272    | B                             | +                      | 21                   |
| 334    | B                             | +                      | 4                    |
| 379    | B                             | +                      | 36                   |
| 137    | C₁                            | +                      | 16                   |
| 361    | C₂                            | +                      | 193                  |
| 472    | D                             | +                      | 13                   |
| 326    | E                             | +                      | 18                   |
| Hycus  | O*                            | –                      | 14                   |
| N9A    | None                          | –                      | 0                    |

*Activities expressed are an average of three to five trials.

*No enterotoxin has been identified.

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**Fig. 1.** Growth response (optical density, solid line) and nuclease production (dashed lines) for *S. aureus* strain 334 (O), 100 (●), 196 E (△), and S-6 (▲).
anaerobic incubation radically diminishes the production of staphylococcal nuclease.

**Effect of glucose and buffering capacity on nuclease production.** Privat de Garilhe (7) reported that glucose inhibited production of staphylococcal nuclease. Because this phenomenon would be significant when relating the presence of heat-stable nuclease with the degree of *S. aureus* contamination in a food product, it was investigated further. BHI broth was prepared with final glucose concentrations of 0.2 to 0.8%. One set was prepared with a succinate-phosphate buffer system (0.75% KH$_2$PO$_4$ plus 0.50% sodium succinate, pH 7.2). Another series with identical glucose concentrations but with no added buffering capacity was also prepared. The glucose was autoclaved separately and added aseptically to the basal medium to prevent the destruction of the glucose at higher pH values if autoclaved in the medium. Ten milliliters of each medium in a 50-ml shaker flask was inoculated with *S. aureus* strain S-6 (standard inoculum). After incubation for 24 h at 37°C on a gyratory shaker, final pH values and nuclease activities of the cultures were estimated. No attempt was made to adjust the pH value of the culture supernatant fluid prior to assay for nuclease activity, because all samples were diluted in the borate buffer. Moreover, separate experiments indicated sufficient buffer capacity to override nominal pH variation in the sample. The results indicated that the glucose concentration not only influenced the final pH value of the culture, but that nuclease production was dependent on pH (Fig. 3). The growth response in the cultures approached maximal values with an optical density range from 1.8 to 2.0. With the succinate-phosphate-buffered media, maximal nuclease activity was observed at a 0.6% glucose concentration and at a corresponding final pH of approximately 8.3. In the unbuffered flasks, final pH values of the media decreased with increased glucose concentration, and concomitant decreases in nuclease production were also observed. These results indicate that glucose favors increased production (most probably associated with increased growth), and an inhibitory effect can be associated with a decrease in pH value because of fermentation of the sugar.

**Effect of initial pH of the medium on nuclease production.** A series of 0.4 M KH$_2$PO$_4$-NaOH buffer solutions, ranging from pH 6.0 to 8.0, and 0.4 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer solutions, ranging from pH 8.0 to 9.0, were prepared and autoclaved. A concentrated basal medium was prepared (37 g of BHI broth in 750 ml of water) and 12 ml was dispensed into 50-ml shaker flasks. After autoclaving, 4 ml of the desired buffer was added aseptically to the flasks to give a final buffer concentration of 0.1 M. A 1-ml sample was withdrawn aseptically to determine the initial pH value of each medium. The flasks were inoculated with *S. aureus* strain S-6 and incubated with shaking at 37°C. After 24 h of incubation, final pH values and nuclease activities of the various cultures were estimated (Table 2). Again, the optimal pH for nuclease production was shown to be approximately 8.3, as seen in the Tris-hydrochloride buffer system. With the KH$_2$PO$_4$-NaOH buffer system (initial pH range 6.4 to 7.7), an increase in pH was accompanied by a decrease in nuclease production throughout the entire range of the buffer. Perhaps phosphate had an inhibitory effect on enzyme production, possibly due to chelation of calcium, but this possibility was not investigated further. A series of 0.2 and 0.4 M Na$_2$CO$_3$-NaHCO$_3$ buffers ranging from pH 8.7 to 10.0 was prepared and similarly examined.

![Graph 1](image1)

**Fig. 2.** The effect of oxygen tension on nuclease production by *S. aureus* strains 100 (O—O) and S-6 (●—●). The optical densities of the cultures at 48 h. Strain 100 (O—O) and strain S-6 (●—●) are compared.

![Graph 2](image2)

**Fig. 3.** Final pH of the medium at 24 h (dashed lines) and nuclease activity (solid lines) for *S. aureus* strain S-6 grown in BHI with various initial glucose concentrations. Unbuffered media (▲) and succinate-phosphate buffered media (□) are compared.
TABLE 2. Effect of initial and final pH value of growth medium on nuclease production by Staphylococcus aureus S-6

| Buffer system     | pH value of medium | Nuclease activity (units/ml) |
|------------------|-------------------|-----------------------------|
|                  | Initial | Final   |                  |
| KH₂PO₄-NaOH      | 6.4     | 6.8     | 12,400           |
|                  | 6.7     | 7.1     | 8,800            |
|                  | 7.1     | 7.5     | 3,900            |
|                  | 7.5     | 7.7     | 3,200            |
|                  | 7.7     | 8.0     | 2,000            |
| Tris-hydrochloride| 7.9     | 8.0     | 7,860            |
|                  | 8.3     | 8.3     | 18,000           |
|                  | 8.6     | 8.3     | 14,000           |
| Na₂CO₃-NaHCO₃    | 8.8     | 8.8     | 280              |
|                  | 9.1     | 9.5     | 0e               |
|                  | 9.5     | 9.7     | 0e               |
|                  | 9.8     | 9.8     | 0e               |

* No growth was evident.

No appreciable nuclease was produced in any of these cultures, and the organism failed to initiate growth above an initial pH of 9.1.

Thermostability of staphylococcal nuclease. The nuclease of *S. aureus* is an extremely heat-stable protein (2). To obtain a quantitative estimation of this property, thermal-destruction-rate curves were prepared for the enzyme. *S. aureus* strain S-6 was grown in 250 ml of BHI in a 1-liter Erlenmeyer flask incubated on a gyratory shaker for 24 h at 37°C. The culture was centrifuged, and the supernatant fluid was collected and used as the enzyme preparation. The final culture pH was 8.2. The enzyme preparation (1.5 ml) was placed in sections of 9-mm Pyrex tubing, and the ends were sealed with an oxygen torch. A constant-temperature oil bath (E.H. Sargent and Co.) was equilibrated at 100°C, and the sealed Pyrex tubes were immersed in the oil and withdrawn at various time intervals. Immediately upon removal from the oil, the samples were cooled in an ice bath and stored in ice until assayed for nuclease activity. Similar determinations were made at temperatures of 120 and 130°C. The logarithm of the nuclease activity versus the time of heat treatment at the various temperatures is illustrated in Fig. 4. The D value at 100°C was 180 min, at 120°C was 34 min, and at 130°C was 16.6 min. Identical curves were obtained when enzyme preparations from two other *S. aureus* strains (100 and 334) were examined similarly under approximately the same conditions. These data reinforce previous observations regarding the remarkable heat stability of the staphylococcal nuclease as well as afford quantitation of the enzyme's thermostability.

A Z value (increase in degrees Fahrenheit required to cause a 1-log decrease in the D value) was calculated from the D values obtained in the preceding section. A Z value of 51°F (28.3°C) was obtained for the enzyme. This is indicative of unusually high heat stability, because many of the more heat-resistant bacterial spores have Z values of approximately 18°F (4).

DISCUSSION

The examination of several enterotoxin-producing strains of *S. aureus* revealed that no correlation existed between enterotoxin type and quantitative nuclease production. Although similar growth curves were obtained, there was great variation in the amount of nuclease produced by the various strains.

A definitive oxygen requirement was demonstrated for enzyme production, and aeration was shown to have a marked stimulatory effect on nuclease production. Glucose was found to inhibit the production of nuclease and was probably due to the production of acidic end products during growth. Because of the variation in nuclease production associated with strains of *S. aureus*, as well as the effect of oxygen tension and pH value of the medium, caution must be exercised when relating nuclease activity to numbers of organisms in a food product or the relative food-poisoning potential of a food sample. The qualitative presence of the enzyme in a food product could indicate a history of staphylococcal contamination. However, too many other factors negatively influence a quantitative relationship between the amount of enzyme present and the number of staphylococci in the sample.

The unusual thermostability of the enzyme (10% of the original activity remains after 34 min at 120°C) can be used to great advantage in the identification of *S. aureus* isolates. The rapid and relatively simple qualitative test for

![Fig. 4. Thermal-destruction-rate curves for S. aureus nuclease at 100°C (▲), 120°C (〇), and 130°C (●).](image-url)
the detection of this enzyme allows the testing of the heat-stable nature of the nuclease. Previous tests have failed in this respect (5, 8).

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