Tolerance of Staphylococcal Thermonuclease to Stress

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Remarkable tolerance to prolonged heating, prolonged storage, and bacterial proliferation was exhibited by staphylococcal thermonuclease in foods and broth. A purified enzyme preparation added to Brain Heart Infusion broth was unaffected by the growth of five bacterial species. Minimal inactivation was effected by Bacillus subtilis. Optimal growth of Streptococcus faecalis var. liquefaciens caused extensive inactivation of thermonuclease. However, storage at room temperature or the addition of 5% NaCl caused only minimal inactivation.

In the absence of a practical method for detecting staphylococcal enterotoxins in foods (1), the presence of a large number of Staphylococcus aureus is considered to be strong evidence of enterotoxin contamination (12). However, none of the available media for the quantitative determination of S. aureus in foods is completely satisfactory (2).

Recently, an enzymatic approach has been suggested. An extremely rapid, though complex, procedure for determining staphylococcal nuclease activity in foods was reported by Chesbro and Auburn (3). Subsequently, a convenient and inexpensive, yet rapid, method was developed in our laboratory (unpublished data). The specificity of the enzymatic procedure is based on the observations that the production of heat-resistant nuclease (thermonuclease) is a stable property of S. aureus (8, 10), and other microorganisms are not known to exhibit this property (3, 6, 7). Furthermore, the excretion of the enzyme from proliferating cells of S. aureus strains 234 and 5–6 in meats has been shown to vary in synchrony with elaboration of enterotoxins A and B (3, 9).

The present study was designed to investigate environmental conditions that might limit the value of detecting staphylococcal thermonuclease activity as an indication of staphylococcal proliferation in foods.

MATERIALS AND METHODS

Bacteria. Cultures of Lactobacillus casei, Streptococcus dicaryalactis, Micrococcus lactic, Sarcina lutea, Pseudomonas fragi, Bacillus subtilis, and Streptococcus faecalis var. liquefaciens were generously provided by E. B. Collins of the Department of Food Science, University of California, Davis. None of the cultures exhibited deoxyribonuclease activity. A coagulase-negative but enterotoxin-positive staphylococcus, S. aureus 698, was kindly provided by M. S. Bergdoll of the Ford Research Institute, University of Wisconsin, Madison.

Source of nuclease. Purified preparations of staphylococcal nuclease were purchased from Worthington (12,434 units/mg). Crude nuclease was obtained from an overnight culture of S. aureus 698.

Determination of nuclease activity. Staphylococcal thermonuclease was added to Brain Heart Infusion (BHI) broth, reconstituted nonfat dry milk, and saline. Enzyme activity was determined quantitatively by the methods of Lachica, Hoeprich, and Franti (unpublished data) in the nonfat dry milk samples; 0.5-g portions were suspended in 4.5 ml of water before assay of thermonuclease activity. With the other food products, activity was determined qualitatively (7).

Determination of growth. The optical density of cultures was determined by using a Bausch & Lomb Spectronic 20 colorimeter.

RESULTS

Prolonged heat treatment. The resistance of staphylococcal nuclease to heating (to 60 C and 97 C for up to 60 min) was evaluated by using milk, BHI broth, and saline containing 2.0 µg of a purified preparation of the enzyme per ml. In BHI broth and milk, nuclease was more resistant to thermal inactivation than in saline (Fig. 1). A similar observation was reported by Sulkowski and Laskowski (11), who found that the purified nuclease was resistant
to thermal inactivation in the presence of Ca$^{2+}$, nucleotides, and protein.

In contrast to the observation by Chesbro and Auburn (3) with S. aureus 234, we noticed no thermal activation of the nuclease from S. aureus 698. Although the nuclease is highly resistant, as is shown in Fig. 2, it is not completely refractory to denaturation by heat.

**Prolonged storage.** The resistance of thernonuclease in various foods to prolonged storage was determined. Measured quantities of nuclease (0.5 to 0.005 μg/g) were added to: butter, banana cream pie filling, and sterile reconstituted dry milk (stored at 5°C); colby cheese and meat spreads of beef, chicken, and turkey (stored at room temperature). Three different portions of nonfat dry milk with added thernonuclease (5 μg/g) were stored at 37, 25, and 5°C, respectively. All food samples were stored in air-tight containers and were examined at monthly intervals. After 10 months, all of the food samples exhibited nuclease activity before and after being steamed for 15 min. Of the milk samples, only the dried milk stored at 37°C exhibited a slight decrease in activity.

Another illustration of tolerance to storage is shown in Fig. 3. No decrease of nuclease activity was observed when a filtrate of a culture of S. aureus 698 was stored in screw-capped test tubes at 5°C for 4 months. A slight decrease was observed on storage at 25°C.

**Effect of bacterial overgrowth.** Proteolytic enzymes elaborated by growing bacteria might degrade staphylococcal thernonuclease in foods. Accordingly, fresh cultures were inoculated into BHI broths (10$^6$ cells/ml) containing 2.0 μg of purified staphylococcal nuclease per ml. Except for P. fragi, which was incubated at 25°C, all cultures were incubated at 37°C for 24 hr, followed by incubation at 25°C for the next 3 days. Of the seven cultures, five had no effect on the nuclease (Fig. 4). The culture of B. subtilis effected minimal inactivation. However, activity decreased nearly 1,000-fold within 24 hr in the presence of a growing strain of S. faecalis var. liquefaciens. Enterotoxin B was unaffected by enterococcal growth (Genigeregis and Lachica, unpublished data).

Further studies revealed that a decrease of the incubation temperature to 25°C diminished considerably the inactivating potency of the growing enterococcal population (Fig. 5), despite the heavy growth attained in 48 hr. A similar effect was observed with the addition of 5% NaCl. Figure 6 shows that the degradation of the nuclease occurred during the late

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**Fig. 1.** Thermal inactivation curves for purified staphylococcal nuclease in milk (O), Brain Heart Infusion broth (●), and saline (Δ) heated at 60°C (broken line) and 97°C (solid line).

**Fig. 2.** Thermal inactivation curves for the nuclease in the filtrate of Staphylococcus aureus 698.

**Fig. 3.** Inactivation of the nuclease in the filtrate of Staphylococcus aureus 698 during storage for 4 months.
exponential and early stationary phase of enterococcal growth at 37 C.

DISCUSSION

The following conditions are recognized as limiting the value of the viable count technique in screening for enterotoxin-contaminated foods (12): (i) heating of processed foods destroys the staphylococci but the foods may still cause food poisoning because of the heat-resistance of staphylococcal enterotoxins; (ii) staphylococci may die off during storage while the enterotoxins persist; (iii) microbial growth may mask the presence of staphylococci which initially produced enterotoxin in the food.

In the present study, the tolerance of staphylococcal thermonuclease to stress permitted enzymatic detection of staphylococcal proliferation, i.e., the determination of potential enterotoxin contamination. Thus, testing for heat-stable nuclease may circumvent the limitations encountered with the use of viable count procedures.

The observed resistance of staphylococcal thermonuclease is consistent with the observations of Anfinsen and co-workers who have studied the physicochemical properties of the enzyme intensively (4). It is a globular protein consisting of a single polypeptide chain containing 149 amino acids; the molecular weight is 16,807. Rapid renaturation, even at high dilution after heat treatment, is facilitated by the small size and the absence of stabilization i through disulfide bonds. In addition, the presence of Ca$^{2+}$ and nucleotides, especially deoxythymidine 3',5'-diphosphate, protects the more susceptible bonds in the vicinity of the binding site from cleavage by proteolytic enzymes.

In our experiments, late exponential- and early stationary-phase growth of a strain of S.

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**Fig. 4.** Inactivation of staphylococcal nuclease in growing cultures of bacteria. Pseudomonas fragi was incubated at 25 C throughout the period of observation, whereas all of the other cultures were incubated at 37 C for the first 24 hr, followed by incubation at 25 C thereafter.

**Fig. 5.** Effects of incubation temperature and NaCl content of BHI broth on the growth of Streptococcus faecalis var. liquefaciens (solid line) and the inactivation of staphylococcal thermonuclease (broken line). Symbols: (O) 37 C, 0.5% NaCl; (□) 25 C, 0.5% NaCl; (△) 37 C, 5.0% NaCl; (●) 25 C, 5.0% NaCl.
faecalis var. liquefaciens at 37°C brought about significant inactivation of staphylococcal nuclease. However, comparably dense growth either at 25°C or in the presence of 5% NaCl did not result in comparable inactivation. Yet, it might be supposed that staphylococcal contamination of foods could be missed by nuclease testing if an enterococcal contaminant were also present and conditions favored its outgrowth. However, high densities of bacteria other than staphylococci generally render foods inedible by grossly altering the appearance and texture of foods while contributing offensive odors and taste, e.g., $10^6$ to $475 \times 10^4$ cells per ml of milk (5). Also, in the authors' experience with a limited number of samples of foods implicated in outbreaks of staphylococcal food poisoning, the presence of thermostable nuclease was readily demonstrable (9). Although enterococci in foods are testimony to contamination with enteric microflora, it is doubtful that concurrent staphylococcal contamination would be obfuscated on this basis.

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