Influence of pH on the Heat Inactivation of Staphylococcal Enterotoxin A as Determined by Monkey Feeding and Serological Assay

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The effect of pH on the thermal inactivation of staphylococcal enterotoxin A was investigated. Analysis of heated toxin by immunodiffusion in gel indicated that enterotoxin A in beef bouillon was inactivated faster at pH 5.3 than at pH 6.2. The $z$ values (slopes) for the heat inactivation curves at pH 6.2 and 5.3 were 49.5 and 55 F (about 27 and 30 C), respectively. Enterotoxin produced and heated in dialyzed Casamino Acids medium and assayed by monkey feeding was more easily inactivated by heat at pH 5.3 than at pH 7.8. Thermal inactivation curves for enterotoxin A in beef bouillon (5 $\mu$g/ml, pH 5.3) were determined by two methods, monkey feeding and serological assay. The $z$ values for the curves obtained by these two methods were both 55 F, although loss of biological or toxic activity of the enterotoxin occurred before loss of serological activity.

In 1931 Jordan et al. (10) found, by feeding human volunteers, that sterile filtrates of an enterotoxigenic strain of Staphylococcus aureus could be boiled for 30 min without complete loss of toxic activity. Although staphylococcal enterotoxins are generally considered to be heat stable, it is evident that several factors may affect the heat resistance of these proteins.

Read and Bradshaw (13) reported that thermal inactivation of 30 $\mu$g of purified (99+%*) enterotoxin B per ml in veronal buffer as determined by serological assay was accomplished by heating for 16.4 min at 121 C as compared with 19 min required at this temperature for inactivation of an identical concentration of a crude enterotoxin preparation. Purified enterotoxin B in raw milk (30 $\mu$g/ml) was inactivated after being heated for 18.4 min at 121 C (14). Satterlee and Kraft (15) determined by serological assay that enterotoxin B was more easily inactivated in a beef slurry, pH 6.6, than in 0.013 M phosphate buffer, pH 7.4, at 100 C. In another investigation that used phosphate buffer as the heating menstrum, it was found that the heat inactivation time of enterotoxin B was shorter at pH 4.5 than at pH 6.4 when heated at 100 C (8). Denny et al. (5) noted from serological results that the heat resistance of enterotoxin A was dependent on both the concentration of the toxin at the time of heating and the menstrum in which the toxin was heated.

The purpose of this investigation was to determine by two different assay methods, serological gel diffusion and monkey feeding, the effect of pH on the heat inactivation of staphylococcal enterotoxin A (SEA) in two different heating substrates. SEA was chosen as the toxin to be studied because it has been reported to be the enterotoxin type most commonly associated with staphylococcal food poisoning (16). It was also of interest to compare the results of two assay methods (serological versus biological) used to measure the residual activity of heated enterotoxin.

MATERIALS AND METHODS

SEA production. S. aureus strain 100, provided by Merlin S. Bergdoll, Food Research Institute, University of Wisconsin, Madison, was used for SEA production. The organism was preserved on porcelain beads by the method of Hunt et al. (9) and stored at 4 C until time of use.

Activation of the culture was achieved by placing two of the coated beads in a screw-cap tube containing 6 ml of brain heart infusion broth (BBL). After 18 h of incubation at 35 C, 0.5 ml of this culture was added to a 2-liter flask containing 500 ml of brain heart infusion broth. The flask was placed on a mechanical shaker for aeration and incubated for 18 h at 37 C (8).

Cells were removed by centrifugation at 3,290 x $g$ for 30 min (4 C) and filtration (Millipore, 0.45-$\mu$m pore size). The cell-free material was concentrated by dialysis against polyethylene glycol as described by Hilker et al. (8) except that after concentration the interior of each sac was rinsed with 5 to 10 ml of 0.15 M phosphate buffer, pH 7.4. This crude SEA concentrate was stored in 10-ml portions at $-4$ C. SEA was also prepared as just described, but with...
the following exceptions: Casamino Acids medium (CAM) (1), a completely dialyzable medium, was used in place of brain heart infusion; strain 195-E (provided by M. S. Bergdoll) was substituted for strain 100; and dialysis sacs were rinsed with distilled water instead of phosphate buffer after dialysis against polyethylene glycol (6). The toxin concentrations of both preparations were determined by serological assay.

Serological assay. Rabbit antiserum specific for SEA was prepared by the method described earlier (8). The single gel diffusion technique of Oudin (11) was used for serological analysis of SEA, gel diffusion tubes were incubated for 7 days at 34°C, and the length of the precipitin band was measured every 24 h. Absence of a visible band after 7 days was considered a negative reaction. For quantitation of results, a standard curve was prepared by using known concentrations of purified SEA (provided by E. J. Schantz, Ft. Detrick, Md.) in beef bouillon. Band length was plotted against toxin concentration on semilogarithmic graph paper, and all standard curve assays were made in duplicate.

Preparation of beef bouillon. Beef bouillon was prepared as described by Denny et al. (5). Final pH of the material was 6.2.

Heat treatment of SEA. The frozen crude SEA phosphatase buffer concentrate was thawed and diluted in beef bouillon to a concentration of 5 µg/ml. The pH of a portion of this material (SEA in beef bouillon, pH 6.2) and of SEA prepared in CAM (pH 7.8) was adjusted to 5.3 with 1 N HCl. The final concentration of SEA in CAM was 7 µg/ml. Aliquots of 2.25 ml of SEA in beef bouillon (pH 6.2 or 5.3) and in CAM (pH 7.8 or 5.3) were dispensed into individual Pyrex thermal-death-time tubes and heated in a thermostatically controlled oil bath as described earlier (6). Thermal inactivation curves were constructed according to the method of Denny et al. (6).

Monkey feeding assay. Frozen samples of heated SEA were sent to the Food Research Institute, University of Wisconsin, Madison, for determination of toxicity in monkeys. Samples were thawed and either 10 ml (SEA in beef bouillon) or 6.75 ml (SEA in CAM) of heated material was brought to a volume of 50 ml with distilled water and fed orally by stomach tube to young rhesus monkeys (Macaca mulatta). Each sample (50 µg of heated toxin) was fed in triplicate, and if at least one monkey gave an emetic response within 5 h after feeding, the assay was considered positive. Nonheated controls of all toxin preparations were fed in triplicate.

RESULTS AND DISCUSSION

Heat inactivation at pH 5.3 and 7.8. The heating times required to inactivate SEA in dialyzed CAM at pH 5.3 were all much less than those heating times required when the initial pH was 7.8, as determined by the emetic response in monkeys (Table 1). The z value (slope) of the heat inactivation curve when plotted for SEA in dialyzed CAM at pH 7.8 was 46°F (25°C). The shortest heating time given the toxin preparation at pH 5.3 was 1 min at each of the lowest four temperatures tested, and all emetic responses by monkeys were negative. The nonheated control samples at pH 5.3 and 7.8 gave positive emetic reactions.

Monkey feeding versus serological assay. Two methods were used to determine the heat inactivation end points of SEA (5 µg/ml) in beef bouillon at pH 5.3 (Fig. 1). End points resulting from emetic responses in monkeys were all

<table>
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<tr>
<th>Temp (°F)</th>
<th>pH 5.3</th>
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<td>250</td>
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* Enterotoxin A concentration in CAM at the time of heating was 7 µg/ml (2.25 ml/thermal-death-time tube).

b Positive in at least one of three monkeys each fed 6.75 ml of heated toxin (47.25 µg) made to 50-ml volume.

![FIG. 1. Heat inactivation curves of enterotoxin A in beef bouillon (pH 5.3) as determined by monkey feeding (△) and serological (●) assays. Enterotoxin concentration heated was 5 µg/ml. Symbols represent the longest heating period at each temperature giving a positive reaction. Three negative intervals (nine monkey feedings) were determined immediately above the positive points.](http://aem.asm.org)
lower than the serological end points. The \( z \) values for the heat inactivation curves as determined by monkey feeding and by serological assay were both 55 F (30 C), and at least three negative points were obtained above each end point presented. The thermal inactivation time curve obtained by feeding theseus monkeys represents SEA in beef bouillon heated at two temperatures, 212 F (100 C) and 250 F (121 C). Serological end points (last positive reaction) at 212, 220, 230, 240, and 250 F were 95, 84, 51, 36, and 24 min, respectively. The positive emetic end points in monkeys at 212 and 250 F were 75 and 15 min, respectively.

It seems evident from the data in Fig. 1 that the toxicity of SEA in monkeys is eliminated more readily by heat than is the serological activity. This difference in heat lability may be a result of the difference in the sensitivity of the two assay methods, the serological assay being able to detect smaller quantities of heated enterotoxin.

In the food industry, the most widely used methods for detection of staphylococcal enterotoxins are based on serological reactions (2, 3) which are, in fact, much more sensitive than the monkey feeding test. Although humans are probably more sensitive to enterotoxin than monkeys (12), it is possible that a positive serological test reaction for enterotoxin A in a heated food, for example, may not be a valid indication of the toxic level of enterotoxin present. A food sample that has been judged by serological tests to contain enterotoxin A might not produce symptoms of toxicity in humans.

**Heat inactivation at pH 6.2 and 5.3.** The influence of pH on the thermal inactivation of 5 \( \mu g \) of SEA per ml in beef bouillon, as determined serologically, is demonstrated by the results given in Table 2. The first negative serological reactions for heated toxin, pH 6.2, were recorded after heating for 160, 114, 65, 44, and 27 min at 212, 220, 230, 240, and 250 F, respectively. Corresponding data for enterotoxin heated at pH 5.3 are given in Table 2. When the serological end points were plotted on semilogarithmic graph paper as described earlier, the heat inactivation curve of SEA in beef bouillon at pH 6.2 had a \( z \) value of 49.5 F as compared with 55 F for the curve representing SEA heated at pH 5.3.

These results agree with the data presented in Table 1 in that a lowered pH in both cases caused a reduction in the heat resistance of SEA, although the influence of pH seemed to be much less pronounced in beef bouillon than in dialyzed CAM. In this respect, the effect of pH on the thermal inactivation of enterotoxin is probably greatest when the toxin preparation is relatively free of large-molecular-weight proteins or other materials that might bind or in some way protect the toxin molecule from heat inactivation.

The effect of pH on the heat inactivation of SEA in beef bouillon was greatest at the lower temperatures (Table 2). For example, there was 55 min of heating time between the end points of the two pH levels at 212 F compared with a difference of 2 min at 250 F.

The results from this investigation would indicate that, within the pH ranges and conditions tested, (i) the heat inactivation of SEA is pH dependent, (ii) the lower the pH, the less the amount of heat required to inactivate a given concentration of SEA, and (iii) the amount of heat required to reduce the toxic or biological activity of SEA to levels undetectable by monkey feeding is less than the amount needed to inactivate the serological activity of the toxin by gel diffusion analysis.

Conformational changes in the enterotoxin molecule may have also occurred as a result of the lower pH, making the toxin more susceptible to the effects of heat. Chu et al. (4) reported the isoelectric point of SEA to be 6.8, and the difference in charge created by the lowered pH may have been sufficient to increase the heat lability of the toxin. It should also be pointed out that the levels of SEA added in this investigation, both in beef bouillon and CAM, were greater than the concentrations of SEA that would be found in foods naturally contaminated by strains of *S. aureus* producing type A toxin (2). Because toxin concentration has been found to affect the heat inactivation of SEA (5), it is probable that the end points of toxin inactivation reported here are higher than the heating times that would be needed to inactivate the

**Table 2.** Heat inactivation of enterotoxin A (5 \( \mu g/ml \)) in beef bouillon at pH 5.3 and 6.2 as determined by serological gel diffusion

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<thead>
<tr>
<th>Temp (F)</th>
<th>pH 5.3</th>
<th>pH 6.2</th>
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<td>250</td>
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*Presence of a visible precipitation band after incubation of gel diffusion tubes for 7 days.*
lower concentrations of enterotoxin normally found in typical food poisoning outbreaks.

ACKNOWLEDGMENTS

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LITERATURE CITED