APPLIED MICROBIOLOGY, Dec. 1973, p. 938-942

Vol. 26, No. 6
Printed in U.S.A.

Thermal Inactivation of Staphylococcal Enterotoxins B and C[1]

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Received for publication 23 August 1973

Thermal inactivation profiles of staphylococcal enterotoxins B (SEB) and C (SEC) at 80, 100, and 121 C showed that SEC is more resistant than SEB to heat. After 24 h of incubation at 25 C, some reactivation (recovery of serological reactivity) occurred in toxins that had been inactivated by heat. If the toxin was stirred during heating, reactivation did not occur. An examination of the reactivation kinetics of heat-treated SEC showed that reactivation was temperature dependent. At 25 C, the incubation temperature of heat-treated crude SEC (80 C for 10 min), 100% reactivation occurred after 24 h, whereas at 4 C only slight reactivation was observed. We and others observed that heat-treated toxins initially lost more serological activity when heated at a low temperature (80 C) than at a higher temperature (100 C); in the present study we demonstrate that this is a reversible phenomenon.

Since the initial observation by Dack et al. in 1930 (3) that staphylococcal enterotoxins were relatively stable to heat, many workers have studied a variety of parameters in relation to the heat stability of staphylococcal enterotoxins (1, 2, 5, 7). It is generally agreed that the heat stability of an enterotoxin varies with the type of toxin and the chemical and physical nature of the suspending medium. Recently, Satterlee and Kraft (8) and Jamlang et al. (6) reported that enterotoxin B was inactivated faster at 80 C than at 100 C after short-time initial inactivation (about 30 min). They attributed this phenomenon to low-temperature aggregation of protein molecules. In a preliminary study of inactivation of crude staphylococcal enterotoxins B (SEB) and C (SEC) at 80 and 100 C, we observed the same phenomenon. When heat-treated enterotoxins were left at 25 C for 24 h, however, they regained some activity (were reactivated). The purpose of this report is to present evidence of this phenomenon in SEB and SEC.

MATERIALS AND METHODS

Organisms and toxins. *Staphylococcus aureus* S-6 and 137, purified SEB and SEC, and their specific antiserum were obtained from M. S. Bergdoll (Food Research Institute, University of Wisconsin). SEB and antiserum B were also obtained from Makor Chemicals Ltd., Jerusalem, Israel. Crude SEB and SEC were obtained by growing *S. aureus* S-6 and 137 in PHP-NZ-Amine medium, as previously described (9).

Twenty-four h culture supernatants of *S. aureus* S-6 (SEB) and 137 (SEC) were collected and toxin levels in the supernatants were measured by the method of Fung and Wagner (4). The supernatants were then diluted with sterile, fresh culture medium to adjust the toxin concentration to 100 μg/ml (crude SEB and crude SEC). The pH values of the SEB and SEC preparations were 7.6 and 7.4, respectively. Purified SEC was suspended in sterile, fresh culture medium as well as phosphate-buffered saline (10) at a concentration of 100 μg/ml.

Heat inactivation and reactivation procedures. To study heat inactivation of the enterotoxins, 1.2 ml of toxin (crude or purified) was added to each of a number of Pyrex ampoules (7 cm by 10 mm); the ampoules were heat-sealed. Sets of sealed ampoules were then placed into an oil bath maintained at 80, 100, or 121 C. Heat-up times were estimated by using specific temperature-sensitive paper strips (Paper Thermometer Co., Natick, Mass.) sealed in vials containing 1.2 ml of sterile culture medium. The time required for the paper strip to change color (white to black) after immersion in the oil bath was estimated as the heat-up time for that particular test temperature. It was previously determined that toxin activity was unchanged before and after the heat-up time. Heat-up times were estimated to be 70, 40, and 40 s, respectively, for the 80, 100, and 121 C oil baths.

After predetermined time intervals of from 10 min to 5 h, ampoules were removed from the oil bath and opened, and a 1-ml sample was withdrawn. A 0.1-ml amount of heated sample was charged into each of two

[1] Journal series paper 4506 of the Pennsylvania Agricultural Experiment Station, July 27, 1973.

Pasteur pipettes containing monovalent antiserum B or C agar as described by Fung and Wagner (4). After 24 h of incubation at 37 C in a moist chamber, the lengths of the precipitin bands in the agar were measured (4). The quantities of toxin in the samples were then estimated by matching precipitation band lengths with those plotted on a standard curve. An average of the two determinations was used as the estimate of toxin activity after heat inactivation.

For the reactivation studies, 0.4-ml samples of heat-treated toxin were quickly placed into duplicate petri dishes (10 cm diameter by 1.5 cm height). The petri dishes were then sealed by tape. It was previously determined that evaporation of liquid in the petri dishes was negligible and that no viable organisms were found in the heat-treated liquids. One petri dish was incubated at 25 C and the other one at 4 C; after 24 h of incubation, toxin activities were measured as described in the previous paragraph. Data were plotted as percent activity of SEB and SEC versus heating time and percent activity after a 24-h reactivation time. All experiments were repeated two to four times.

Reactivation kinetics. A set of ampoules containing crude SEC (100 μg/ml) were heat treated for 10 min at 80 or 100 C. Toxin activities were measured, and then equal numbers of the ampoules were stored at 25 and 4 C. After reactivation time intervals ranging from 15 min to 24 h, toxin activities were measured. The data were plotted as percent toxin activity versus reactivation time.

Effect of stirring. Since heat inactivation and subsequent reactivation of SEB and SEC seemed to relate to unfolding and refolding of toxin molecules, mechanical agitation of toxin solutions during heating should effect the stability of the toxins. Portions (25 ml) of crude SEC solutions were placed in 100-ml beakers containing magnetic stirring bars. Each beaker was sealed with a rubber stopper equipped with a thermistor (Mettler TM 15, Arthur H. Thomas Co., Philadelphia, Pa.) for temperature measurement and a syringe for removal of samples. The SEC solutions were heated at 80 or 95 C with or without stirring. After 10 and 60 min of heating, 1-ml samples were withdrawn; toxin activities were determined as described in the previous section. As a control, SEC solutions were stirred for up to 5 h at 25 C; samples were withdrawn hourly to determine the effect of stirring alone on toxin activity. Experiments were conducted using both "slow" and "rapid" stirring. No quantitative rates of stirring (i.e., revolutions per second) were available for the instrument, except that slow and rapid stirring created small and large vortexes, respectively. The speed of the slow stirring was approximately half that of the rapid stirring.

RESULTS AND DISCUSSION

It should be emphasized that activities of heat-treated toxins and reactivated toxins were measured only by a single gel-diffusion technique. Although the serological activities of toxins cannot be used to directly indicate their biological activities, good correlations of these two parameters were reported by Read and Bradshaw (7) and Jamlang et al. (6).

The thermal inactivation and reactivation curves of crude SEB and SEC are shown in Fig. 1 and 2. After 5 h of heating at 80 C, both SEB and SEC retained some (5 to 10%) residual serological activity. Heating SEB and SEC at 100 and 121 C totally inactivated the toxins in 3 h and 30 min, respectively. In agreement with the observations of other workers (6, 8), both SEB and SEC were inactivated slightly more rapidly at 80 C than at 100 C during the initial heating period (about 10 to 30 min). Thereafter, heating at 100 C resulted in more rapid inactivation than heating at 80 C. Considering the similarities of pH, toxin concentration, and suspending medium composition, SEC was more heat resistant than SEB (compare Fig. 1 and 2).

After 24 h of reactivation at 25 C, both SEB and SEC treated at all three temperatures regained a certain amount of activity, except that the toxin preparations that were heated until no serological activity remained did not regain any activity. Figures 1 and 2 also show that the degree of reactivation decreased as toxins achieved greater degrees of inactivation. Although 80 C provided more rapid inactivation of toxins than 100 C after 10 min of treatment, the reactivated toxin activity of 80 C-treated

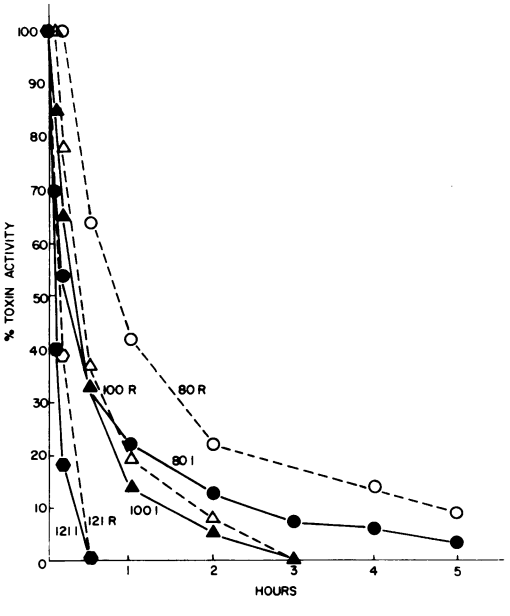

FIG. 1. *Heat inactivation and subsequent reactivation of crude SEB. Solid and open hexagons, triangles, and circles represent heat inactivation and subsequent 24-h reactivation curves at 121, 100, and 80 C, respectively. Abbreviations: I, Inactivation curve; R, 24-h reactivation curve.*

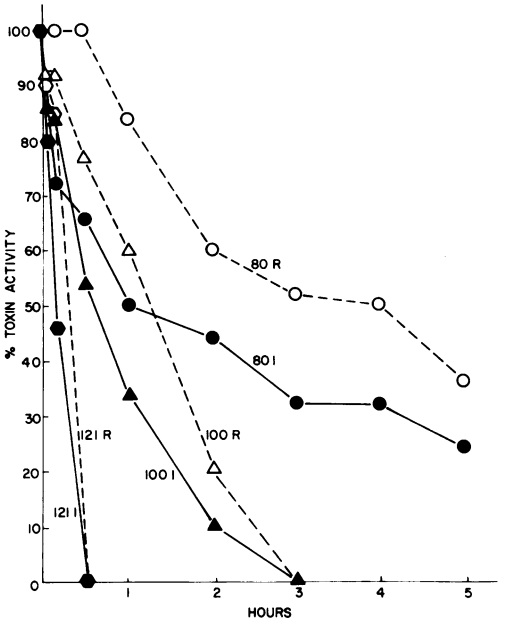

FIG. 2. *Heat inactivation and subsequent reactivation of crude SEC. Legend identical to Fig. 1.*

toxin was higher than that exhibited by 100 C-treated toxins. No reactivation, or negligible reactivation, was observed in heat-treated toxins stored at 4 C (data not shown).

The 80 C heat-inactivation and reactivation profiles of purified SEC diluted in culture medium and phosphate-buffered saline are presented in Fig. 3. It is evident that the medium (a casein hydrolysate solution) increased the heat stability of SEC. Reactivation occurred in purified, heat-treated SEC similar to the crude SEC (compare Fig. 2 and 3). Purified SEC in buffer was totally inactivated after 5 h of heating at 80 C, but a substantial amount of activity remained when SEC was protected by the culture medium (Fig. 3). When similar experiments were performed at 100 C (data not shown), total inactivation was achieved after 1 h of heating when SEC was suspended in buffer; however, when purified SEC was suspended in culture medium, 3 h was needed to inactivate the toxins. Reactivation of heat-treated SEC also occurred after 100 C treatment.

Figure 4 shows that 80 C-treated crude SEC (10 min) regained about 10% of its initial activity when incubated for 2 h at either 25 or 4 C. No further reactivation was observed in heat-treated SEC stored at 4 C; however, reactivation continued at 25 C until the heat-treated SEC had regained 100% of its initial activity. Similar reactivation profiles of 100 C-treated SEC (10 min) were obtained (data not shown).

The effect on crude SEC of stirring during heating is presented in Table 1. Heating and stirring of SEC at 95 C for 10 and 60 min resulted in more inactivation of the toxin compared to the unstirred SEC. Moreover, no reactivation of SEC was observed for the heat-stirred SEC, indicating that permanent damage to the toxin molecules occurred in this combined process. The unstirred toxins exhibited reactivation after heat treatment similar to those toxins reported in other sections of this report.

Contrary to the 95 C heat treatment data, after 10 min of heating at 80 C the unstirred SEC lost more activity than the stirred SEC (Table 1). Stirring of toxin apparently prevented the formation of low temperature-induced protein aggregates, as was shown in Fig. 1 and 2 and reported by others (6, 8). The 80 C heat treatment data also show that heat-stirred SEC after 10 and 60 min did not reactivate after 24 h of incubation time, whereas unstirred SEC had the ability to reactivate. Stirring of toxin at room temperature for 5 h did not result in loss of toxin activity. The data

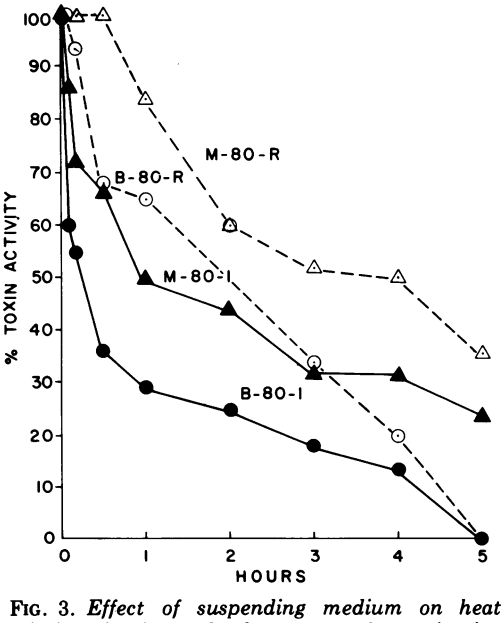

FIG. 3. *Effect of suspending medium on heat (80 C) inactivation and subsequent 24-h reactivation of SEC. Solid and open circles and triangles represent inactivation and subsequent 24-h reactivation of SEC suspended in buffer solution and sterile medium, respectively. Abbreviations: M, sterile medium; B, phosphate saline buffer; I, inactivation; R, reactivation.*

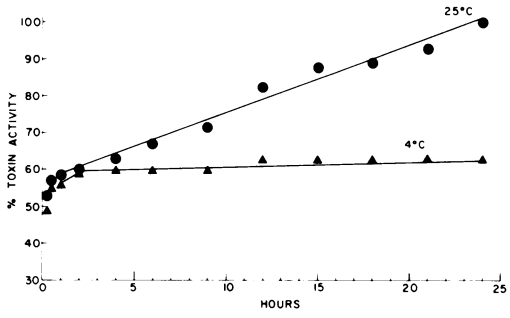

FIG. 4. *Reactivation kinetics of 10 min, 80 C-treated crude SEC. Circles and triangles represent reactivation curves at 25 and 4 C, respectively.*

obtained during slow and rapid stirring were almost identical, indicating that the stirring rate was not crucial; slow stirring was sufficient to augment heat effect during toxin inactivation. The mechanism of stirring effects are not clear, but stirring may affect oxidation of the denatured molecule. We did not examine this phenomenon further, but stirring in the presence of reducing agents might provide a clue as to the nature of this process.

The anomaly found by Jamlang et al. (6), Satterlee and Kraft (8), and Soo et al. (H. M. Soo, S. R. Tatani, and R. W. Bennett, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 1, 1973), that heat-treated toxins initially lost more activity at a low (80 C) than at a higher (100 C) temperature, was shown in the present study to be a reversible phenomenon. After 24 h of reactivation, 100 C-treated toxin lost more activity than 80 C-treated toxin, both at the initial (10 to 30 min) and prolonged heating times.

Several conclusions can be made. First, crude SEC was more heat stable than crude SEB under similar experimental conditions. Second, heat-treated toxins at "sublethal" time intervals have the ability to reactivate; the degree of reactivation is dependent on time, temperature, and the amount of inactivation incurred. Totally inactivated toxins did not have the ability to reactivate. Last, mechanical stress or oxidation (stirring) during heat treatment resulted in permanent damage to the toxin; reactivation of heat-stirred toxins did not occur.

Based on the data obtained, we attempted to develop a hypothesis to explain the mechanisms of heat inactivation of staphylococcal enterotoxins. When toxins are subjected to heat under conditions that lead to incomplete inactivation, some toxin molecules totally unfold, some partially unfold, and some molecules remain intact. Our serological assay detected only the intact molecules. Under suitable conditions (25 C for 24 h), the partially unfolded toxins reassociated and serological reactivity was regained. This is the phenomenon that we have termed "reactivation." Mechanical stress or oxidation can cause the partially unfolded toxin to become totally unfolded, or they can prevent renaturation; thus, reactivation cannot occur. During low-temperature heating (80 C), protein-protein aggregation may also occur, in addition to the three degrees of folding of toxins mentioned above. However, we have some reservations about aggregation as a mechanism of toxin inactivation. Serological assays detect only active toxins; since our assay method relies on diffusion, aggregates would not be detected to the extent that individual toxin molecules are detected. Therefore, it is possible that unstable aggregates dissociated during the reactivation process (6).

It should be noted that data collected in this study were obtained using high levels of toxins (100 μg/ml) as an experimental system. Further studies using small amounts of toxins (0.1–0.01 μg/ml) in various food systems are needed to obtain information on inactivation and reactivation of toxins more closely related to actual staphylococcal intoxication cases.

The heat stability of staphylococcal enterotoxins has obvious practical implications in food safety. It is important to prevent *S. aureus* from contaminating foods and subsequent proliferation and production of enterotoxins. Once the toxins have formed, it is impractical to try to eliminate them from food, even by prolonged heating. Also, Dangerfield (H. G. Dangerfield, Program Annu. Meet. Amer. Soc. Microbiol., Session 36—seminar, 1973) reported that heat may potentiate the toxicity of staphylococcal enterotoxin A in human volunteers.

TABLE 1. *Effect of stirring on heat inactivation and subsequent reactivation of crude SEC*

Heating temp (C)	Activation of SEC	Toxin activity[a] (%)			
		10-min heating		60-min heating	
		Stirred	Unstirred	Stirred	Unstirred
95	Inactivation	68	84	19	30
	24-h reactivation	66	95	21	39
80	Inactivation	93	75	53	55
	24-h reactivation	93	99	52	64

[a] Original toxin activity: 100 μg/ml.

ACKNOWLEDGMENTS

We thank M. S. Bergdoll of the Food Research Institute, University of Wisconsin, for enterotoxins and their specific antiserum. The discussion and suggestions of P. A. Hartman and H. E. Snyder are appreciated.

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