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Effect of Lysozyme on the Recovery of Heated *Clostridium botulinum* Spores

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Lysozyme in the recovery medium increased the recovery of heated spores, thereby raising the measured heat resistance of type E *Clostridium botulinum* spores about 1,800-fold and type A spores up to 3-fold.

In 1969 Cassier and Sebald (5) showed that plating medium containing lysozyme initiated germination of presumed nonviable heat-damaged *Clostridium perfringens* type A spores. Recently, Duncan et al. (6) showed that heat-injured *C. perfringens* type A spores were incapable of germination and outgrowth in complex media without addition of either lysozyme or a germination-inducing protein isolated from *C. perfringens*. Their findings indicated that alteration of the spores by heat or alkali inactivated the normal lytic system responsible for cortical degradation during germination. Since this work was completed, Sebald and Ionesco (7) have demonstrated outgrowth of *C. botulinum* type E spores heat shocked at 80 C for 10 min by having lysozyme in the recovery medium, whereas no survival occurred without lysozyme. However, they indicated that lysozyme did not have any effect on spore recovery of other *C. botulinum* types. In this investigation it was found that lysozyme in the recovery medium did increase type A spore recovery, although the effect was not as dramatic as it was with type E spores.

The object of this investigation was to determine quantitatively the effect of lysozyme on the recovery of heated calcium-form *C. botulinum* 62A and untreated *C. botulinum* 1304E spores. Like other spores (1-4), the thermal resistance of mature botulinum spores can be chemically manipulated between the heat-sensitive and the heat-resistant forms. The heat-resistant form is the calcium form, and the heat-sensitive form is the hydrogen form. Untreated 1304E spores are type E spores harvested directly from growth medium without chemical manipulation. The calcium-form type E spores were not used, since it had not yet been discovered how to load the spores with calcium without germinating them.

The heat resistance of washed, lyophilized spores was determined by using distilled water, phosphate buffer (pH 7.1), and strained asparagus food puree as heating media. Heat resistance was measured by the count reduction method using T-Best agar as the recovery medium (8). Heated spores were subcultured in this medium in parallel trials with and without added lysozyme. In preliminary experiments, the amount of lysozyme added to the medium was tested at 1, 5, and 10 μg per ml of recovery medium. All three levels of lysozyme gave essentially the same spore recoveries. A final concentration of 5 μg of medium per ml was adopted for all subsequent experiments.

For calcium-treated 62A spores, the effect of lysozyme in the recovery medium increased spore recovery as indicated by higher D values shown in Fig. 1a.

The log survivor versus time plot of the spores heated in M/15 phosphate buffer shows the D_{235} value of 1.9 without lysozyme and D_{235} value of 3.4 with lysozyme.

Similar survivor curves were obtained when the *C. botulinum* 62A spores were heated in strained asparagus puree. Here the D_{235} values were 3.0 and 1.10, with and without lysozyme, respectively (Fig. 1b). When the heating temperature was raised to 245 F (ca. 118 C), the difference in D values with and without lysozyme seemed to increase. Here the D_{245} was 0.2 without lysozyme and 0.8 with lysozyme (Fig. 1c).

However, the magnitude of D values, without lysozyme, depended on individual batches of commercial tryptone and beef extract in the T-Best recovery medium. Recovery on some batches of commercial media ingredients was improved only about 20 to 30%, by lysozyme, as measured by D value for calcium type A spores, whereas others were improved by about threefold, but all reached about the same final level when lysozyme was present. With the addition

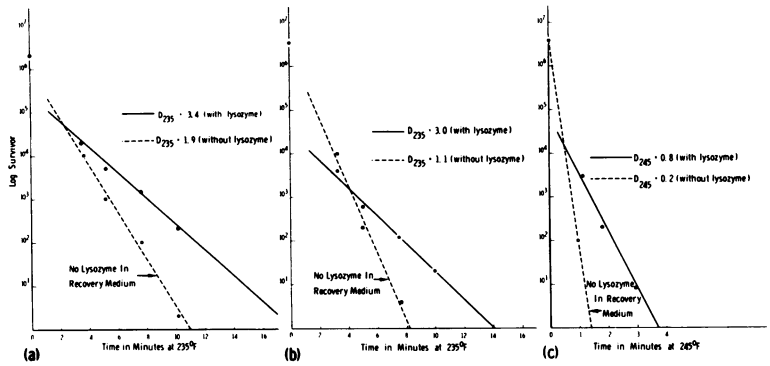

FIG. 1. *Effect of lysozyme on the recovery of heated C. botulinum 62A spores. Calcium C. botulinum 62A spores heated in (a) M/15 PO_4, pH 7.1, and (b, c) strained asparagus puree.*

of lysozyme to the recovery medium, colony outgrowth time was shortened by approximately half. In the case of recovery medium without lysozyme, frequently no visible colonies appeared until after 7 days of incubation at 30 C, and additional colonies may appear after 3 weeks of incubation. Colony counting may not be concluded for a month or more. In the case of recovery medium with lysozyme, visible colonies appeared in the tubes 48 h after subculturing. The maximum number of colonies usually appeared in 10 to 14 days.

As shown in Fig. 2a, for heated *C. botulinum* type E spores, the effect of lysozyme in the recovery medium was more dramatic. When the spores were heated in M/15PO_4 buffer using recovery medium without lysozyme, D_{175} was 1.3.

With lysozyme in the recovery medium, the heating temperature had to be raised to obtain

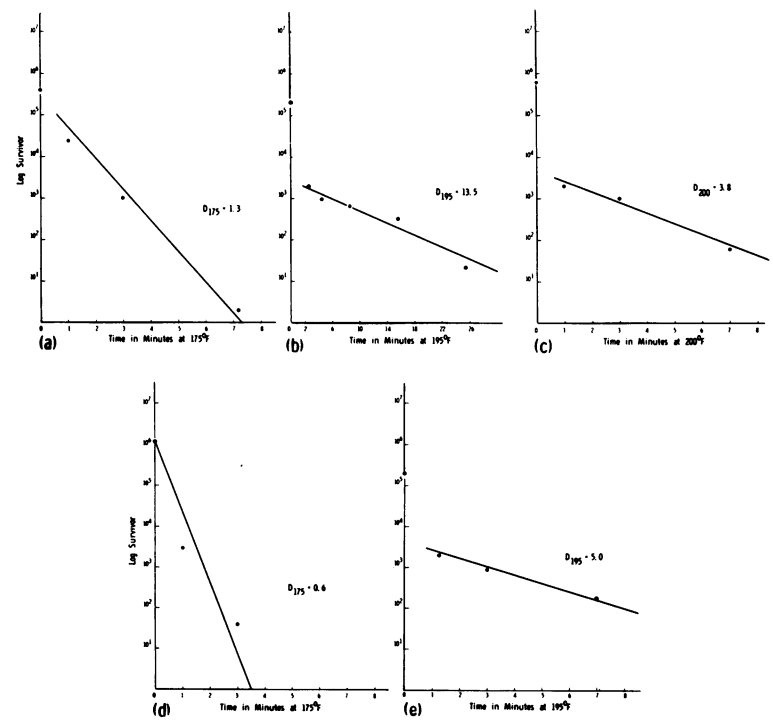

FIG. 2. *Effect of lysozyme on the recovery of heated untreated C. botulinum 1304E spores with (a, d) no lysozyme in the recovery medium and (b, c, e) 5 μg of lysozyme per ml of recovery medium. Heating menstruum: (a, b, c) M/15 PO_4, pH 7.1; (d, e) distilled water.*

measurable destruction. At 195 F (ca. 91 C), D_{195} was 13.5 min (Fig. 2b). At 200 F (ca. 93 C), a D_{200} value of 3.8 was obtained (Fig. 2c). This gave about an 1,800-fold increase in apparent heat resistance due to the addition of lysozyme in the recovery medium. When distilled water was used as a heating menstruum, (Fig. 2d), D_{175} without lysozyme was 0.6. With lysozyme in the recovery medium, the heating temperature needed to be raised to 195 F where a D_{195} value of 5.0 was obtained (Fig. 2e). Assuming a z value of 10 as obtained in phosphate buffer, this would calculate to approximately a 900-fold increase in heat resistance.

However, for type E spores, lysozyme in the recovery medium did not seem to have any effect on the colony outgrowth time. Colony outgrowth was very rapid as compared with type A spores. At 30 C, colonies appeared 24 to 48 h after subculturing. Colony counting was normally concluded within 7 days with or without added lysozyme.

In summary, lysozyme in the recovery medium enhanced and speeded outgrowth of heated *C. botulinum* spores. With added lysozyme in the recovery medium, the spore recovery effect, as measured by colony formation, was more dramatic for type E spores than for type A spores. In both cases, higher D values were obtained due to increased spore recovery. Although type E spores with lysozyme in the recovery medium are much less resistant than type A, they are still significantly more resistant than had been generally considered.

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