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ABSTRACT

THOMPSON, T. L. (University of Nebraska, Lincoln), AND J. M. SHIVELY. Lethal action of ribonuclease for thermophilic bacilli. J. Bacteriol 91:673-676. 1966.—Exposure of a thermophilic bacillus, NU 47, to 0.2 μg/ml of pancreatic ribonuclease resulted in a drastic reduction of viable cells. The observed lethality is thought to be associated specifically with the ribonuclease, since heat treatment did not diminish activity and the addition of ribonucleic acid to the cells completely eliminated the action of the enzyme. Cells from 2-hr cultures, buffered between *p*H 6.0 and 7.0, were most sensitive. The lethal action was most pronounced when cells were exposed to the enzyme at temperatures between 25 and 45 C. A greater proportion of cells survived at higher temperatures. The release of ultraviolet-absorbing materials into the medium after exposure to enzyme could not be detected.

While testing the susceptibility of bacteriocins produced by thermophilic bacteria to various enzymes, it was noted that ribonuclease drastically reduced the numbers of survivors of the indicator strain. Further study revealed that one thermophilic strain, NU 47, rapidly succumbed to minute levels of ribonuclease. Many investigators (1, 2, 4) have reported that ribonuclease affects bacterial protoplasts. The action appears to be associated with lysis of the protoplasts.

Schlenk and Dainko (7) recently reported that exposure of yeast cells to purified ribonuclease resulted in a marked decrease in the numbers of viable cells and in the release of more than one-half of the ultraviolet-absorbing cellular material. Lethality of ribonuclease for viable bacterial cells has not previously been demonstrated.

This report describes conditions for the action of ribonuclease on whole cells of a thermophilic bacillus.

MATERIALS AND METHODS

Organisms. The organism used in this study was an obligate thermophilic bacillus, NU 47 (6), tentatively classified as *Bacillus stearothermophilus*.

Media. The liquid medium contained 2% Trypticase plus 0.2% glycerol. Glycerol was added after sterilization of the Trypticase, and 2% agar was added for the plating medium.

Preparation of cultures. Cells were grown in 50 ml of broth contained in 250-ml DeLong culture flasks in a New Brunswick shaker-incubator at 63 C. After an optical density of 0.2 had been reached (approximately 10^7 cells per milliliter), 0.5 ml of this suspension was employed as inoculum for a second temperature-equilibrated flask. Cells were again allowed to reach an optical density of 0.2 (approximately 2 hr). A portion of this culture was set aside in a refrigerator to serve as initial inoculum (0.5 ml) for the next day. The remainder was sedimented by centrifugation and was resuspended in 0.2% sterile Trypticase to one-half the original volume. An equal volume of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (*p*H 6.2) was added. This suspension was employed for treatment with ribonuclease.

Enzyme treatment. A 10-ml amount of the cell suspension was transferred to a 250-ml flask and allowed to equilibrate at the desired temperature (30 C). Stock ribonuclease (pancreatic, grade A, Calbiochem) was made up in distilled water to contain 1.0 mg/ml. The stock solution was diluted and added dropwise to yield a final concentration of 0.5 μg/ml of cell suspension. After 5 min of exposure, serial dilutions were made in 0.1% Trypticase, and survivors were determined by plate count.

Viable-cell count. Viable counts were made by the agar overlay technique. Plates were prepared which contained 35 to 40 ml of Trypticase-glycerol-agar. Prior to use, excess moisture was allowed to evaporate at room temperature by partially removing the covers for 1 hr. A 1.0-ml portion of cell dilution was transferred to a tube (13 by 100 mm) containing 1 ml of melted agar at 60 C. The entire content was spread over the agar surface of each plate. After the seed layer had solidified, the plates were incubated at 60 C with lids partially removed for 7 min. Counts were made after 15 to 18 hr of incubation at 60 C.

Required changes in the above procedures for the

study of certain parameters will be noted under Results.

RESULTS

Enzyme purity. Since the possibility exists that ribonuclease might contain contaminating proteolytic enzymes, the enzyme was heated for 10 min at 90 C (5). Heat treatment did not decrease activity. In fact, a slight stimulation of activity was noted. No attempts were made to purify the enzyme further.

The addition of 100 μg of ribonucleic acid (RNA) per ml (Torula, grade B, Calbiochem) to the cells prior to the addition of 0.5 μg of ribonuclease per ml completely reversed lethal action. However, RNA added 2 min after the enzyme reduced lethality only slightly.

Culture age. Cells from cultures of different ages (0 to 6 hr) were harvested by centrifugation and suspended in buffered Trypticase to yield a final concentration of 10^7 cells per milliliter. Cells from 2-hr-old cultures were most susceptible to the action of ribonuclease (Fig. 1). Younger cultures demonstrated marked resistance. This observation was unexpected, since ribonuclease-sensitive cells (0.5 ml of a 2-hr culture) were employed as an inoculum for the preheated medium. Repeated washings did not alter this resistance. Increased ribonuclease concentrations, however, resulted in a decrease in the number of survivors.

pH. Several buffers were employed to determine the effect of *p*H on ribonuclease action. The lethal action of ribonuclease on cells suspended in distilled water or in 0.05 M phosphate or Tris buffers at *p*H 7.0 was greatly enhanced over that obtained when cells were exposed in 0.1% Trypticase. However, when treatment was carried out at 60 C, a loss of from 10 to 40% of the viable cells in the control flasks occurred. The viability of control cells could be maintained for long periods at 60 C when they were suspended in 0.1% Trypticase plus 0.025 M Tris (*p*H 8.0 to 9.4) or Tris-maleate (*p*H 5.8 to 8.0) buffers. For this reason, Trypticase buffered with Tris or Tris-maleate was employed as suspending fluid for *p*H studies (Fig. 2). Cells treated at *p*H values between 6.0 and 7.0 were most susceptible to enzyme action. Between *p*H 7.6 and 8.2, cells were markedly resistant. In fact, almost 65% of the cells survived exposure at *p*H 8.0 compared with less than 1% at *p*H 6.4. A rapid decline in survivors occurred between *p*H 8.2 and 8.4.

Enzyme concentration. As shown in Fig. 3, 0.05 μg/ml of ribonuclease reduced the number of survivors by over 50%. Concentrations of enzyme greater than 0.2 μg/ml decreased survivors to 1% or less. During these studies, it was noted that increasing the concentration of Trypticase above 0.1% while maintaining the *p*H at 6.2 led to a suppression of ribonuclease activity, as evidenced by the number of survivors (Fig. 4). This protective effect of Trypticase could be overcome by doubling the enzyme concentration with each twofold increase in Trypticase concentration.

Exposure time. Over 80% of the cells were rendered nonviable by 30-sec exposure to the enzyme (Fig. 5). Exposure of cells beyond 2 min resulted in only a slight decrease in the number of survivors. Several seconds necessarily lapsed be-

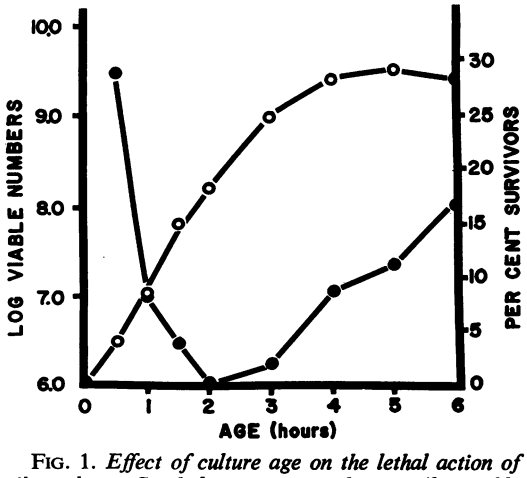

FIG. 1. *Effect of culture age on the lethal action of ribonuclease. Symbols:* ○ = *growth curve (log viable numbers);* ● = *per cent survivors after ribonuclease treatment.*

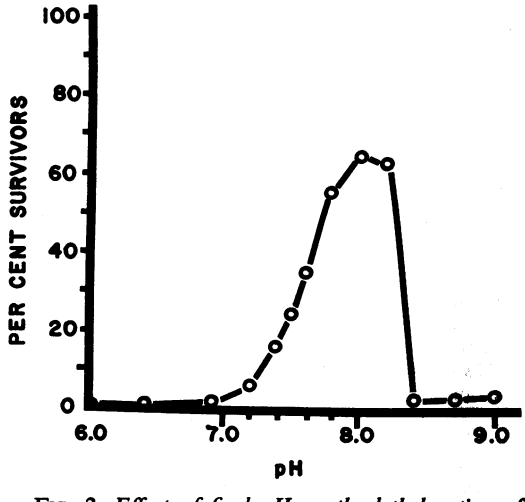

FIG. 2. *Effect of final pH on the lethal action of ribonuclease.*

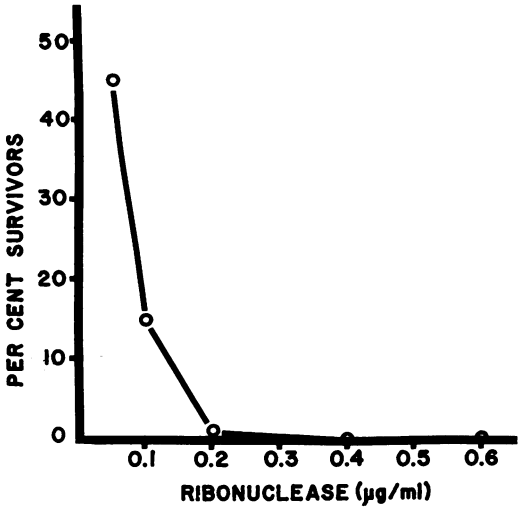

FIG. 3. *Effect of enzyme concentration on the lethal action of ribonuclease.*

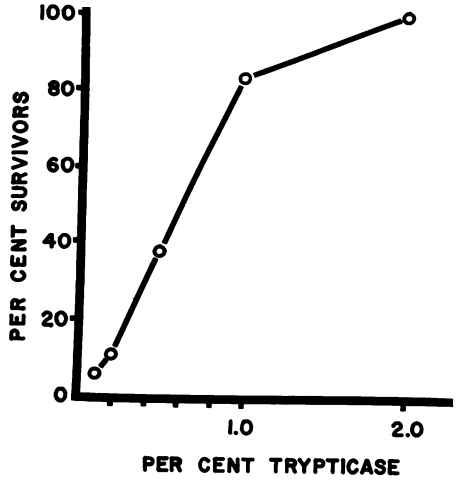

FIG. 4. *Effect of Trypticase concentration on the lethal action of ribonuclease.*

tween termination of exposure and final dilution for plating. To reduce this additional exposure somewhat, cells and enzyme were reacted in a Millipore filter containing a 0.45-μ filter pad. At intervals, the suspending fluid was pulled through the filter and the trapped cells were washed several times with 0.1% Trypticase. Cells were then eluted from the pad, and viable counts were made. The results were similar to those in Fig. 5.

Temperature. The organism employed in this study is incapable of growth at temperatures below 42 C. As the incubation approached the optimal growth temperature (60 C), sensitivity to ribonuclease (0.2 μg/ml) decreased (Table 1).

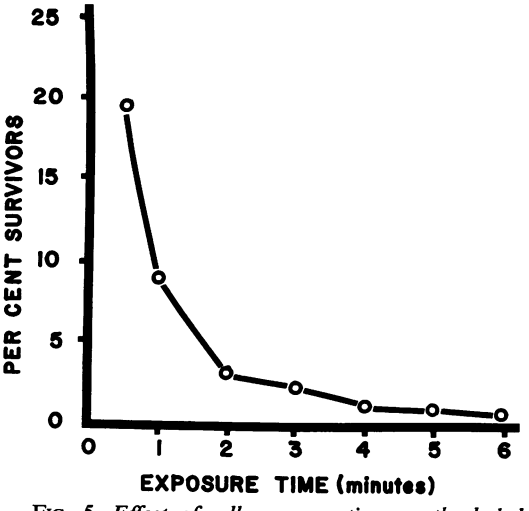

FIG. 5. *Effect of cell exposure time on the lethal action of ribonuclease.*

TABLE 1. *Effect of temperature on the lethal action of ribonuclease*

Exposure temp	Survivors
C	%
25	4.2
35	3.4
45	11.5
55	36.8
65	33.0

TABLE 2. *Effect of $MgSO_4$ and KH_2PO_4 on the lethal action of ribonuclease*

Concn	Per cent survivors*	
	$MgSO_4$	KH_2PO_4
M		
0.1	55.0	38.6
0.01	24.5	0.1
0.001	0.1	0.2

* A control without $MgSO_4$ or KH_2PO_4 showed 0.4% survivors.

Also, lethal action could be partially reversed by incubating ribonuclease-treated cells at 60 C for a few minutes. The reversal of lethality will be discussed in a subsequent communication.

Ions. Schlenk and Dainko (7) reported that cations and phosphate inhibited the action of ribonuclease on yeast cells. Both magnesium and phosphate in relatively high concentrations reduced the lethal action of ribonuclease on the bacillus studied (Table 2).

Adsorption. The enzyme appears to be rapidly and firmly bound to the cells since, after a 1-min exposure, little or no lethal action could be detected in the filtrate. Heat-killed cells (10 min at

80 C) or ultraviolet-killed cells, in concentrations approximating the number of viable cells normally employed, effectively removed ribonuclease.

Heat-killed cells were somewhat less efficient in adsorbing ribonuclease than were ultraviolet-killed cells. Partially purified cell walls also reduced the lethal action of ribonuclease. The amount of contaminating membrane, if any, was not determined for the cell wall preparation.

Release of ultraviolet-absorbing materials. Various buffers, enzyme concentrations, cell numbers, and exposure times were employed to determine whether ribonuclease resulted in the release of ultraviolet-absorbing constituents from the cells. No significant amount of 260 mμ absorbing material was detected in the supernatant fluid after enzyme treatment.

DISCUSSION

The possibility that a contaminant, such as a proteolytic enzyme, in the ribonuclease preparation might be responsible for the observed lethality cannot be excluded. This appears unlikely, however, because of the low concentrations of the enzyme which effected cell inactivation and because of the enzyme's stability to high temperatures. Furthermore, the addition of RNA to the cell suspension prior to the enzyme eliminated lethal action.

The most probable site for binding of ribonuclease by bacterial cells would be the cell membrane, which has been shown to contain relatively high concentrations of RNA (8). The availability of membrane RNA to ribonuclease was demonstrated by Brenner (1), who observed that protoplasts of *B. megaterium* were lysed by ribonuclease. The deterrence to such an interpretation would be the presence of an intact cell wall. Gerhardt and Judge (3) have, however, determined the threshold limit for polymer penetration through *B. megaterium* cell walls to be of a molecular weight of 57,000. The largest openings were calculated to be approximately 107 A. If one assumes that the thermophilic bacillus possesses a wall structure similar to *B. megaterium*, then the wall would offer no insurmountable obstacle to a molecule of the dimensions of ribonuclease (molecular weight, 14,000).

The cause for resistance of newly transferred cells is difficult to assess. It is possible that the cells, when inoculated into fresh medium, accumulate a ribonuclease-inhibiting material(s) which is present in low concentrations. As division proceeds, the amount of accumulated material per cell decreases to a point where it is no longer inhibitory to the enzyme. Our results bear out the fact that Trypticase contains materials which inhibit ribonuclease.

The optimal *p*H range for the lethal action of ribonuclease is below the *p*H of 7.2 to 8.2 for optimal enzyme activity. In fact, the thermophilic cells are quite resistant to the enzyme between *p*H 7.4 and 8.4. It is probable that the optimal *p*H range observed represents the range most conducive to the passage of the enzyme through the cell wall or for binding of the enzyme to its active site.

The observed resistance of cells to ribonuclease at elevated temperatures may be due to the restricted penetration of the enzyme through the cell wall, the rapid repair of cellular damage, or the inhibition of enzyme action. It appears that the temperature effect occurred after enzyme penetration, since cells exposed at 30 C could be partially recovered on subsequent incubation at 60 C. Either rapid cellular repair or inhibition of enzyme action at the elevated temperatures would best explain the decreased sensitivity.

Whether susceptibility to ribonuclease is characteristic of a few thermophilic bacilli or whether it is widespread among bacteria was not ascertained. One other thermophilic strain, *B. stearothermophilus* NU 10, was found to be susceptible to the action of this enzyme. Several strains of mesophilic bacilli screened were found to be resistant. The conditions for screening mesophiles, however, were those giving optimal lethal action against the thermophilic strain. It is possible that conditions were not optimal for activity against mesophilic bacilli.

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