STUDIES ON THE SPORES OF AEROBIC BACTERIA

IV. A Heat Resistant Catalase from Spores of *Bacillus Terminalis*[1]

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In the course of investigating the enzyme content of spores of *Bacillus terminalis* (since identified as a strain of *Bacillus cereus*), the observation was made that well cleaned spores exhibited considerable catalase activity. Such activity in a spore preparation can be attributed in most cases to improper cleaning, as stressed by Hardwick and Foster (1953). In our case, however, it was found that the catalase activity remained unchanged after repeated washings, indicating that the enzyme was associated intimately with the spore. This was inferred also by the finding that the activity of the spores remained unaltered by heating to 80 C for 30 minutes or to 100 C for 5 minutes. The catalase activity of the vegetative cells is destroyed completely by such treatment. The high heat resistance of this enzyme associated with spores is analogous to the alanine racemase previously reported by Stewart and Halvorson (1953). This report is concerned with a study of the heat resistant characteristics of this enzyme.

MATERIALS AND METHODS

Preparation of spores. Spores of *B. terminalis* were obtained as described previously, washed six times, and dried under vacuum from the frozen state. Microscopic examination of stained preparations showed no vegetative cells or debris.

Determination of catalase activity. Catalase activity was determined in the conventional Warburg apparatus at 30 C. The test suspension was placed in the side arm and, after thermal equilibration, was mixed with 441 μM H_2O_2 buffered at pH 7.0. Reduction of the peroxide concentration to 44 μM per ml did not change the rate or extent of oxygen evolution.

The activity of the preparation was inhibited 50 to 60 per cent by 0.01 M KF, NaN_3, or NaCN; 0.02 M disodium versenate had no effect on the activity.

The maximum amount of gas was given off after 20 to 25 minutes on the Warburg apparatus, and as shown in figure 1 the total gas given off was proportional to the amount of cell preparation added. For this reason, catalase activity is reported as μL gas evolved per mg dry weight after 23 minutes reaction time on the Warburg apparatus.

Preparation of cell extracts. Dried spores were ground with an equal volume of 325 mesh alumina. Powdered dry ice was added to keep the mixture chilled and to assist in the grinding which was continued in a mechanical mortar and pestle for 6 to 8 hours. Plate counts indicated that 40 per cent of the spores were rendered inviable after one hour grinding, and 85 per cent in six hours. Then the mass was suspended in water and the alumina removed by low speed centrifugation. For cell-free preparations, the alumina-free suspension was spun at higher speed and the slightly viscous supernatant liquid frozen and stored.

EXPERIMENTAL RESULTS AND DISCUSSION

In order to show differences in heat stability of the catalase of spores and vegetative cells, suspensions of 5 mg intact spores and of 1 mg dried vegetative cells per ml of 0.033 molar phosphate buffer (pH 7) were heated in a water bath at 80 and 100 C. Aliquots were removed after various periods of time, chilled immediately in an ice bath, and tested for catalase activity. The results are shown in figure 2. The relatively low activity of the vegetative cell preparation is due to the fact that the cells were dried and had been stored for nearly a year. Young vegetative cells (3.5 hours old) exhibited considerably greater activity (1,360 μL per mg) which was destroyed by 3 minutes heating at 80 C.

It can be seen that 10 minutes heating at 80 C, which has no effect on the activity of the intact

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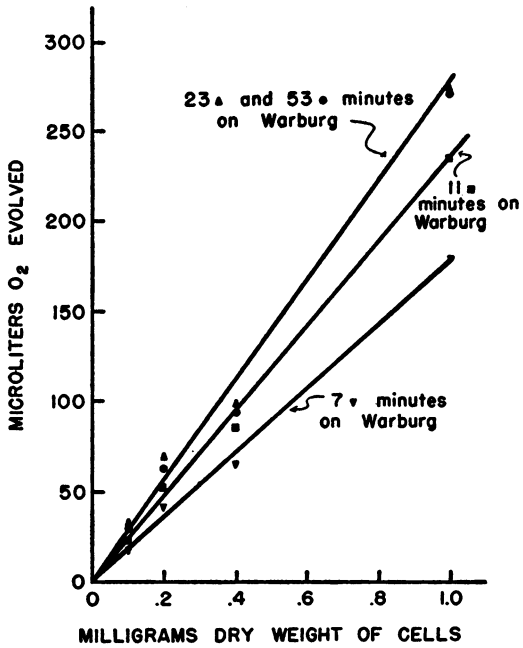

Figure 1. Oxygen evolution as a function of the weight of spore preparation.

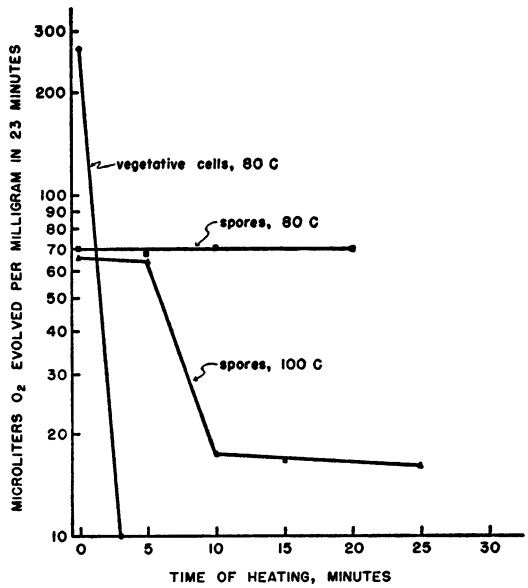

Figure 2. Thermal inactivation of catalase of intact spores and vegetative cells.

spores, completely destroys the activity of the vegetative cells.

The results are analogous to the findings reported by Stewart and Halvorson (1953) regarding alanine racemase. Consequently, cell-free preparations of spores were used in an attempt to separate the heat stable from the heat sensitive enzyme.

TABLE 1

Activity of cell-free suspension and of resuspended particles of disrupted spores, before and after heating to 80 C for 10 minutes

	μL GAS EVOLVED PER 0.5 ML OF SUSPENSION IN 23 MINUTES	
	Supernate	Particles
Unheated	530	340
Heated	90	200
Per cent activity remaining	17	59

After grinding the cells by the method described, the large particles and intact spores were removed by centrifuging at approximately 2,000 rpm for 20 minutes. The residue was resuspended in a volume of water equal to the volume of the supernate, and the supernate and the suspension of particles were tested for total activity and for activity resistant to 80 C for 10 minutes. The results are shown in table 1.

Since the suspension of particles contained an unknown number of intact spores which had withstood the grinding, as well as the larger particles resulting from the grinding, the values in table 1 merely show that both a heat resistant and a heat sensitive catalase is present in the cell-free supernate, and that the heat resistant catalase forms a larger fraction of the particles than of the supernate.

Attempts were made to fractionate the cell-free suspension by the use of ammonium sulfate precipitation. It was found that 90 per cent of the activity came down at 40 to 60 per cent saturation, and that both the precipitate and supernate contained the same ratio of heat sensitive to heat resistant enzyme.

Differential centrifugation of the cell-free material was attempted at various gravitational intensities (20,000 G for 25 minutes, 80,000 G for one hour, 140,000 G for 4 hours). The thermal inactivation curves at 80 C of the supernates and residues from each of these preparations are shown in figure 3.

It can be seen that the two enzyme fractions cannot be separated by the centrifugation procedure employed. This indicates that the resistance does not involve mere association of the

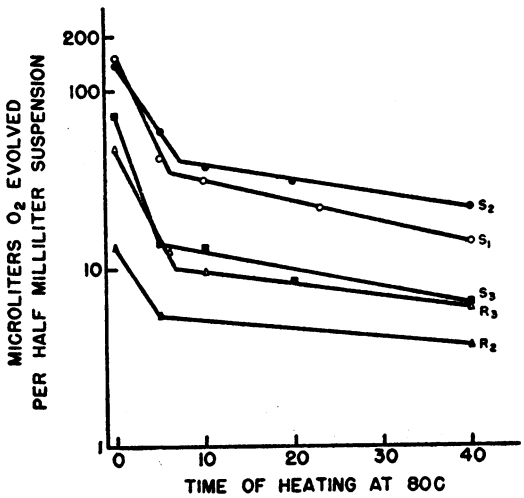

Figure 3. Thermal inactivation curves of spore extracts centrifuged at various speeds.

S_1 = Supernate from 20,000 G, 25 minutes;
S_2 = Supernate from 80,000 G, 1 hour;
R_2 = Residue from 80,000 G, 1 hour;
S_3 = Supernate from 140,000 G, 4 hours;
R_3 = Residue from 140,000 G, 4 hours.

TABLE 2

Enzymatic activity per mg of intact spores, of disrupted spores, and of spores heated to 80 C for 5 minutes before grinding

	μL/MG/23 MIN	
	Total activity	Per cent destroyed in 5 min at 80 C
Intact spores	70	0
Disrupted spores		
not pre-heated	97	36
pre-heated	66	0

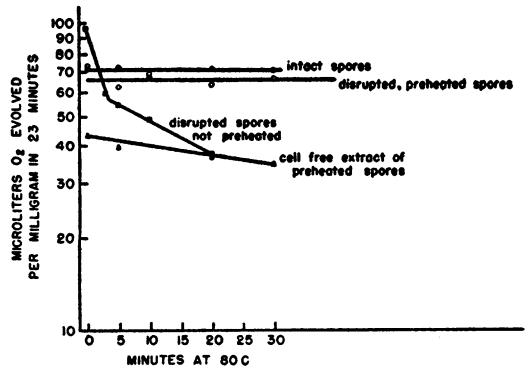

Figure 4. Thermal inactivation at 80 C of catalase activity of intact spores and disrupted spores.

enzyme with particles of large size, as appeared to be the case with the alanine racemase.

The behavior toward heat of the ground spore extract, as compared to that of intact spores, suggests that the intact spore might contain intracellular enzyme which is heat sensitive and which is not measurable until the spore is cracked. If this is the case, then preheating the spore to a temperature known to destroy the sensitive enzyme (80 C, 5 minutes) before grinding should release only heat resistant enzyme, and the total activity should be less than spores not so heated before grinding. Also, unheated spores, when mechanically disrupted, should have more activity per mg dry weight than the intact spores.

The data obtained support such a hypothesis. Table 2 shows that the total activity per mg of a suspension of ground pre-heated spores is the same as the activity per mg of intact spores. However, as shown in figure 4, a cell-free extract of pre-heated spores is somewhat more heat sensitive than is a suspension of intact spores. It can be seen in figure 4 also that when spores are not heated to destroy intracellular enzyme prior to grinding, the total activity per mg is greater than with intact spores, and a considerable part of the activity can be destroyed by heating to 80 C for 5 minutes.

An attempt was made to destroy the surface catalase of spores by shaking one gram of spores in 30 ml H_2O_2 (3 per cent) for 24 hours. The spores then were washed free of peroxide and tested for catalase activity, both as intact spores and after grinding. It was found that this peroxide treatment destroyed about 90 per cent of the activity of both intact and disrupted spores. This was an unexpected result in view of the fact that incubation in the Warburg apparatus resulted in maximum evolution of gas in about 20 minutes. However, the manometric experiments were not carried out for as long a period as the experiment described here.

In order to see whether other aerobic spores also contained catalase, a supply of dry clean *Bacillus globigii* spores was obtained from Dr. Harlyn Halvorson. This preparation failed to show oxygen evolution when mixed with H_2O_2 and observed microscopically. When the spores were tested manometrically, it was found that only 8 μL of gas were evolved per mg in 23 minutes, indicating that these spores had been washed essentially free of catalase. However, after these spores had been disrupted by the

method described and again tested manometrically, it was found that the activity had increased fivefold.

SUMMARY

The presence of measurable enzymatic activity in preparations of bacterial spores is often laid to contamination by vegetative cells or debris. Such has been the case with catalase, a criterion of cleanliness of a spore preparation being the absence of catalase activity when observed microscopically.

We have shown that a suspension of *Bacillus terminalis* (*Bacillus cereus*) which appears clean microscopically exhibits such catalase activity, and that this activity is completely resistant to a time and temperature that will destroy the catalase activity of vegetative cells of the same organism.

It has been found also that this high degree of heat resistance only obtains if intact spores are tested. Grinding the spores mechanically increases the activity per mg, and it is found that now the catalase activity follows two different thermal inactivation curves.

These two fractions, differing in heat resistance, cannot be separated by centrifugation, indicating that their resistance is not merely a function of the size of the particle.

Spores which were heated to 80 C for 5 minutes to destroy the sensitive fraction, exhibited, when mechanically disrupted, the same specific activity as did intact spores which had not been ground.

The data suggest that the heat sensitive enzyme is contained inside the spores and is not measurable until the spore is disrupted. This hypothesis is strengthened by the observation that a fivefold increase in catalase activity was obtained by grinding very clean *Bacillus globigii* spores.

REFERENCES

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