DYNAMIC INTERACTIONS BETWEEN CELL WALL POLYMERS, EXTRACELLULAR PROTEASES AND AUTOLYTIC ENZYMES [1]

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Received November 26, 1969

A sporulation mutant of Bacillus subtilis 168 contains three autolytic activities with pH optima at 5.5, 8.0 and 9.5. Reducing groups and N-terminal groups are liberated during autolysis at pH 5.5 and 8.0, but only N-terminal groups are liberated at pH 9.5. The enzyme activity is constant during growth, but the substrate is variable. Two of the enzymes (pH 5.5 and 8.0) are sensitive to proteases whereas one (pH 9.5) is not. The presence of multiple autolytic activities might explain why sporulation mutants lyse more rapidly than the parent strain.

Recently there has been a renewed interest in the physiological role of autolysins. Although these enzymes have been implicated in cell growth, division and transport of macromolecules (1) convincing evidence for these biological functions is not available. The study of autolysins has been hampered by the lability of the enzymes and the association of the enzymes with polymers of the cell wall. The observations that postexponential cultures of sporulation mutants lyse more rapidly than the parent suggested that they might contain other autolysins which had been heretofore unidentified. In fact, although the N-acyl muramyl L-alanine amidase was the dominant activity in the parent strain, Bacillus subtilis 168, we occasionally found multiple activities. Therefore, we decided to investigate the autolysin(s) associated with cell walls of sporulation mutants.

In this report we describe a sporulation mutant with multiple autolytic activities. Some of these activities are destroyed by proteases from the parent strain.

MATERIALS AND METHODS

Bacterial strains. A tryptophan requiring strain of Bacillus subtilis 168 (Marburg) and a sporulation mutant SR22, deficient in extracellular protease(2) were used. Growth

[1] Supported by grants E288F from the American Cancer Society and GM12956 from the National Institutes of Health

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conditions and preparation of cell walls were carried out as described previously (3).

Autolysin assay. The substrate was cell walls from B. subtilis 168 that had been treated with boiling 2% sodium dodecyl sulfate (4). The substrate (1.0 mg) was suspended in 0.9 ml of the appropriate buffer: 50 mM acetate (pH 5.5), Tris (pH 8.0) or glycine (pH 9.5). At time 0, 0.1 ml of crude enzyme was added and the mixture was incubated at 37 C. Lysis was measured at 600 nm with a Beckman DUR recording spectrophotometer. Activity was expressed as the change in optical density per minute.

Chemical assays. Reducing groups were determined by the method of Thompson and Shockman (5). Glucose was the standard. N-terminal groups were determined by the method of Ghuysen et al. (6) using alanine as the standard.

Enzymes. Trypsin (twice recrystallized, salt free) was obtained from Worthington Biochemical Corporation. Subtilisin was obtained from Nutritional Biochemicals Corporation. Crude protease from B. subtilis was provided by Dr. John Spizizen.

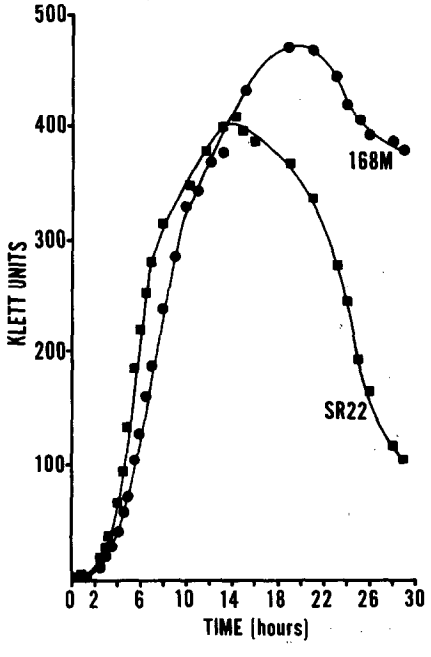

Figure 1. Growth of B. subtilis 168 trp-2 and a sporulation mutant (SR22) at 37C. Cultures were grown from small inocula (1% v/v) in minimal medium supplemented with 0.02% acid-hydrolyzed casein; 22 mM glucose; 5 mM $MgSO_4$ and 50 µg/ml L-tryptophan. The turbidity was measured with a Klett-Summerson colorimeter using filter #66.

RESULTS AND DISCUSSION

When cells were incubated in minimal medium at 37 C, the mutant, unlike the parent, lysed rapidly during postexponential growth (Fig. 1). Cell walls were prepared from postexponential cells of both strains by mechanical disintegration and differential centrifugation. The cell walls were suspended in 20 mM ammonium carbonate (pH 8.9) at 37 C. The rate of autolysis of the mutant was five-fold greater than that of the parent. Crude autolysates were tested for lysis of heat-inactivated cell walls from the parent as a function of pH. Three pH optima (5.5, 8.0 and 9.5) were recognized in the mutant whereas the optimal pH for autolysis of the parent strain was 9.5 as noted previously (7). Reducing groups and N-terminal groups were liberated during autolysis at pH 5.5 and 8.0, but only N-terminal groups were liberated at pH 9.5.

Assuming momentarily that these activities represented three distinct enzymes, we wanted to determine if there was a difference in the order of appearance of these enzymes during growth. Cell walls were prepared from 30 liter cultures harvested at various points

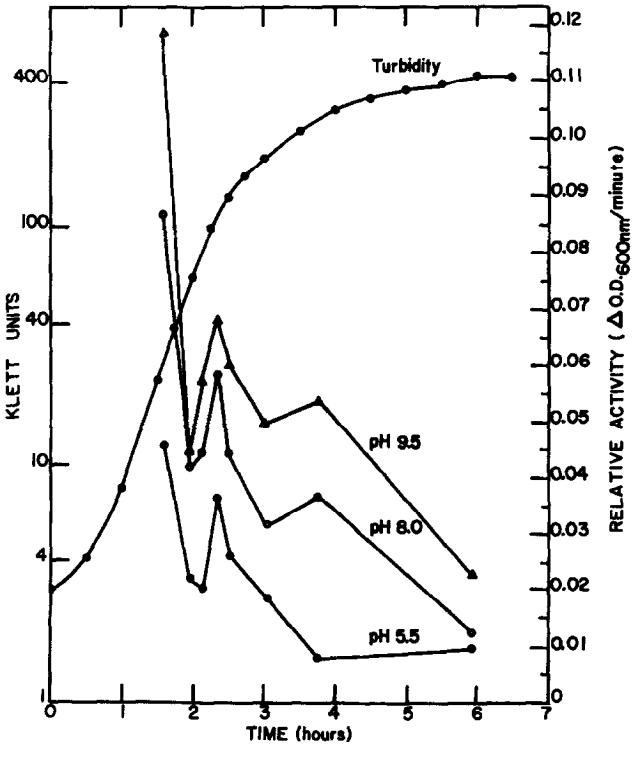

Figure 2. Variation in autolytic activities during growth of a sporulation mutant of B. subtilis. Cell walls were prepared from 30 liter cultures each corresponding to a distinct stage of the growth cycle. The rate of lysis of heat-inactivated cell walls is shown for each preparation at three pH values.

in the growth cycle. The rate of autolysis for each of the samples was determined at the three pH optima. The relative activities were similar throughout growth (Fig. 2). However, two peaks of activity were observed suggesting a difference in the amount of enzyme or changes in the substrate. To distinguish between these possibilities two experiments were designed. The results are summarized briefly as follows. When crude autolysates from all stages of growth were tested against substrate from one stage of growth (50 Klett units) the activities were essentially constant. Conversely, crude autolysate from one stage of growth (100 Klett units) was more lytic on substrate from exponential cells (50 Klett units) than that from postexponential cells (274 Klett units). Thus, the apparent peaks in enzyme activity were due to differences in substrate, not the concentration of enzyme in the cell wall. In the parent strain, multiple activities were observed only during early exponential growth. However, this strain was similar to the mutant in that substrate from exponential cells was hydrolyzed more rapidly than substrate from postexponential cells.

The presence of multiple activities in the protease negative mutant compared to a single activity in the parent strain suggested that these autolysins might differ in their sensitivity to proteases. Therefore, samples of crude autolysate were incubated with crude protease from Bacillus subtilis in 10 mM Tris buffer, (pH 8.0) at 37 C. Aliquots were removed at intervals and tested for autolytic activity. The pH 5.5 enzyme activity was decreased rapidly by this treatment. The pH 8.0 enzyme was more stable but less so than the pH 9.5 enzyme (table 1). In addition, trypsin and subtilisin rapidly inactivated the pH 5.5 enzyme but were less effective against the pH 8.0 and 9.5 enzymes.

TABLE 1. Effect of B. subtilis crude protease on autolysin activity. Crude autolysin was incubated with protease in 10 mM Tris buffer (pH 8.0) at 37 C. At various intervals after the addition of protease, aliquots were removed and assayed for lysis of heat-inactivated cell walls.

Time (min)	Rate (Δ O.D.$_{600nm}$/ min x 10^3) pH 5.5	pH 8.0	pH 9.5
0	105	170	145
5	70	170	145
10	60	170	145
15	50	150	140

As growth ceases in bacilli which are capable of sporulation, one of the earlier enzymatic changes is the appearance of extracellular proteases (2,8). Our data show that these proteases can inactivate autolysins which are capable of initiating cell death. For example, in the absence of cellular growth the balance between synthesis and focal degradation is disrupted so that lysis is favored. Therefore, sporulation mutants which lack proteases might undergo rapid lysis under these conditions. Previously, we have presented data which demonstrate that the pH 9.5 enzyme (N-acyl muramyl-L-alanine amidase) is tightly bound to teichoic acid (3,9). This binding could account for its remarkable stability to proteases and other treatments, and conceivably ensure its function in postexponential events. Alternatively, this association could be merely ionic. Preliminary experiments have demonstrated that the enzymes can be separated from teichoic acid in some asporogenic mutants, whereas the wild type complex is refractory to the same techniques. Studies are in progress to determine the exact role of the interaction between the wall polymers and the enzyme(s).

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