Spore Lytic Enzyme Released from Clostridium perfringens Spores During Germination

YOSHIKAI ANDO
Hokkaido Institute of Public Health, Hokkaido, Sapporo, Japan

Received for publication 4 June 1979

The exudate of fully germinated spores of Clostridium perfringens was found to contain a large amount of a spore lytic enzyme which acted directly on alkali-treated spores of the organism to cause germination. Although no detectable amount of the enzyme was found in dormant spores during germination in a KCl medium, the enzyme was produced rapidly and released into the medium. The optimal conditions for enzyme activity were pH 6.0 and 45°C. Maximum activity occurred in the presence of various univalent cations at a concentration of 50 mM. The enzyme was readily inactivated by several sulphydryl reagents. A strong reducing condition was generated in the inorganic germination of the spores, a minimum Eh level of −350 mV being reached 30 min after initiation of germination. Furthermore, adenosine triphosphate-dependent pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) was identified in both dormant and germinated spores. The relationship between the release of active enzyme and the generation of reducing conditions during germination is discussed.

MATERIALS AND METHODS

Preparation of spores. Spores of C. perfringens type A NCTC 8238 were produced and cleaned as described previously (1). The cleaned spores were lyophilized and stored in a desiccator at room temperature.

Preparation of alkali-treated spores. Spores (1 mg/ml) were suspended in 0.1 N NaOH and incubated at 0°C for 30 min. The spores were centrifuged, washed four times with cold deionized water, and stored at 4°C.

Preparation of spore extracts. Spores (20 to 100 mg/ml) were suspended in 10 ml of water or 0.1 M potassium phosphate buffer (pH 7.0) and disrupted by shaking with glass beads (2 to 10 g; 0.10 to 0.11 mm in diameter) in a cell homogenizer (B. Braun Apparatebau, Melsungen, West Germany) in an oxygen-free N₂ atmosphere. The suspension was usually shaken for 10 2-min cycles while being cooled by bursts of liquid CO₂. Alternatively, spores (100 mg) were ruptured in the dry state by shaking with glass beads (0 mg) in a dental amalgamator (Shofu Dental Manufacturing Co. Ltd., Tokyo, Japan) for 20 30-s cycles at 4°C. The disrupted spores were extracted with 0.1 M potassium phosphate buffer (pH 7.0). The extracts were centrifuged at 12,000 × g for 20 min in the cold. The supernatant fluids were used as the crude enzymes. In the third method, extracts were prepared from intact spores by the method of Brown and Cubel (4) with minor modification. Spores (100 mg/ml) were suspended in 7.2 M urea containing 10% (vol/vol) 2-mercaptethanol (UME) at pH 3.0. After the mixture was incubated at room temperature for 2 h, it was centrifuged at 12,000 × g for 20 min. The supernatant
fluid was dialyzed against 50 mM potassium phosphate buffer (pH 6.0) at 4°C overnight. Usually precipitates appeared which were removed by centrifugation, and the supernatant fluid was used as the crude enzyme.

**Preparation of cortical fragments.** Cortical fragments of the spores were prepared from disrupted spores as described by Hashimoto et al. (14).

**Preparation of cell walls.** Cell walls were prepared from vegetative cells grown in fluid thioglycolate medium as described by Kawata and Takumi (18).

**Assay of spore enzymes.** The spore lytic enzyme was assayed by measuring the germination rate of the alkali-treated spores. The decrease in optical density at 540 nm of the spore suspensions was followed by the use of a Klett-Summerson photoelectric colorimeter. Germination was also confirmed by phase-contrast microscopy. The germination rate was estimated from the slope of plots of percent decrease in optical density versus time. One unit of lytic enzyme activity was defined as the amount of extract that caused a germination rate of 0.01% per min.

Pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) was assayed by measuring the formation of acetoxydramate from pyruvate and hydroxylamine (21). Protein was measured by the method of Lowry et al. (20).

**Preparation of concentrated germination exudate.** Spores (20 mg/ml) were suspended in deionized water, heated at 75°C for 20 min, and then cooled. The heat-activated spores were germinated in 50 mM KCl and 50 mM potassium phosphate buffer (pH 7.0) at 40°C. After 90 min of incubation, germinated spores were removed by centrifugation. The resulting supernatant fluid was concentrated by ultrafiltration using a Diafilter G-01T (Bio-engineering Co. Ltd., Tokyo, Japan), to about one-tenth of the original volume and dialyzed against deionized water at 4°C overnight.

**Eh determination.** The Eh changes occurring during spore germination were determined as described previously (3).

**Chemicals.** The following chemicals were used: lysozyme, Worthington Biochemicals Co.; ATP and p-chloromercuribenzoic acid, Sigma Chemical Co.; N-ethylmaleimide, Tokyo Kasei Industrial Co.; 5,5'-dithio-bis-(2-nitrobenzoic acid), Wako Pure Chemical Co.; sodium pyruvate and coenzyme A, Boehringer Mannheim Co. All other chemicals used were reagent grade and were obtained from commercial sources.

**RESULTS**

**Lytic enzyme in dormant spores.** To determine whether dormant spores possess active lytic enzyme or not, unheated or heated spores were subjected to different extraction procedures as indicated in Table 1. When spores were disrupted in a potassium phosphate buffer, the enzyme activity was detected in the extracts made from heated spores, but not from unheated spores. It appeared that lytic enzyme might be present in somewhat inactive form in the dormant spores and become slightly activated during breakage in the buffer, presumably because part of the spores underwent germination before complete disruption. To test this, spores were first broken in water alone and then the mixture was made 0.1 M in potassium phosphate buffer (pH 7.0). As would be expected, there was no enzyme activity in the extracts made from either heated or unheated spores. Furthermore, with dry rupture of spores, essentially no enzyme activity was detected in the ruptured spores. It was also considered that the enzyme might have been bound firmly to some binding site(s) within the spore so that it was difficult to extract. However, attempts to solubilize active enzyme from disrupted spores with 1% sodium dodecyl sulfate, 0.1% Triton X-100, or 3 M KCl were unsuccessful. On the other hand, weak activity was observed in the UME extracts made from both heated and unheated spores.

**Lytic enzyme in germinating spores and their exudate.** Since dormant spores did not possess active enzyme, extracts were made from germinating spores at different times. Table 2 shows a net increase in enzyme activity in germinating spores, with the most rapid increase in the first 10 min of germination. There was a very slow increase in enzyme activity in germination exudate during the first 10 min, followed by a quite abrupt increase, thus showing a steady increase in total activity during the course of

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>Enzyme activity* (1/100 mg of spores in extracts from:</th>
<th>Heated spores</th>
<th>Unheated spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disruption in and extraction with phosphate</td>
<td>84</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Disruption in water and extraction with phosphate</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dry rupture and extraction with phosphate</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UME extracts from intact spores</td>
<td>44</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>
The enzyme activity was almost as effective as lysozyme in causing the decrease in turbidity of cortical fragments but it had no effect on cell walls (Fig. 2). In contrast, lysozyme could lyse cell walls slightly and initiation protein (autolysin) could lyse sporangial walls as well (6).

The spore lytic enzyme of B. cereus T has been reported to be a sulphhydryl (SH) enzyme because it was readily inactivated by SH group

Table 2. Activity of the spore lytic enzyme in germinated spores and their exudate in C. perfringens

<table>
<thead>
<tr>
<th>Time of germination (min)</th>
<th>Enzyme activity (U/10 mg of spores)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germinated spores</td>
</tr>
<tr>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>10</td>
<td>121</td>
</tr>
<tr>
<td>30</td>
<td>123</td>
</tr>
<tr>
<td>60</td>
<td>68</td>
</tr>
</tbody>
</table>

\[\text{a The enzyme activity was measured as described in footnote b, Table 1.}\]

\[\text{b Spores (20 mg/ml) were suspended in 10 ml of water, heated at 75°C for 20 min, and cooled. The heat-activated spores were germinated in the KCl medium (20 ml; 50 mM KCl and 50 mM potassium phosphate buffer, pH 7.0) at 40°C. Four samples were used simultaneously for each germination time. At the time indicated the sample was quickly chilled at 0°C to stop subsequent germination and centrifuged in the cold. The supernatant fluid was used as the germination exudate. The pelleted spores were washed once with cold 0.1 M potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and disrupted as described in the text.}\]

Some properties of the lytic enzyme. Since the lytic enzyme caused the decrease in optical density of alkali-treated spores, the mode of action might be similar to that of lysozyme or the initiation protein (8). Therefore, various agents affecting lytic enzyme activity were tested and compared to effects on lysozyme and initiation protein.

Among metal ions tested, univalent cations were all effective in stimulating the enzyme activity, whereas divalent cations were all inhibitory (Table 3). The enzyme was optimally active at 50 mM KCl and pH 6 (data not shown). The pH optimum was greatly different from the pH optima for lysozyme (10.0) and initiation protein (9.0) (8).

The enzyme activity was maximum at an incubation temperature of 45°C (Fig. 1). The optimal temperatures for lysozyme and initiation protein were 60 and 50°C, respectively (8). The decreased activity at 55°C might be due to some inactivation of the enzyme, because its activity was significantly reduced by heating the enzyme at 60°C for 5 min and was completely lost after 20 min.

The substrate of action of spore lytic enzyme is believed to be mucopeptide in the cortex (12, 22). The enzyme was almost as effective as lysozyme in causing the decrease in turbidity of cortical fragments but it had no effect on cell walls (Fig. 2). In contrast, lysozyme could lyse cell walls slightly and initiation protein (autolysin) could lyse sporangial walls as well (6).

The spore lytic enzyme of B. cereus T has been reported to be a sulphhydryl (SH) enzyme because it was readily inactivated by SH group.

Table 3. Effect of ions on activity of the spore lytic enzyme in C. perfringens

<table>
<thead>
<tr>
<th>Ion added</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.8</td>
<td>39.6</td>
</tr>
<tr>
<td>KCl</td>
<td>41.3</td>
<td>62.7</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>43.8</td>
<td>62.8</td>
</tr>
<tr>
<td>RbCl</td>
<td>41.7</td>
<td>61.6</td>
</tr>
<tr>
<td>CsCl</td>
<td>47.8</td>
<td>62.9</td>
</tr>
<tr>
<td>NaCl</td>
<td>38.2</td>
<td>61.1</td>
</tr>
<tr>
<td>LiCl</td>
<td>31.3</td>
<td>57.4</td>
</tr>
<tr>
<td>CaCl2</td>
<td>8.4</td>
<td>17.6</td>
</tr>
<tr>
<td>MgCl2</td>
<td>6.5</td>
<td>11.4</td>
</tr>
<tr>
<td>MnCl2</td>
<td>4.6</td>
<td>11.5</td>
</tr>
<tr>
<td>CoCl2</td>
<td>7.4</td>
<td>14.1</td>
</tr>
</tbody>
</table>

\[\text{a The enzyme activity was measured as described in the text. The reaction mixture contained enzyme preparation (174 U), one of the salts indicated, and alkali-treated spores. The reaction mixture was incubated at 40°C. OD, Optical density.}\]

\[\text{b The salt was used at 50 mM and the pH was not adjusted.}\]
The time potential curves of both heated and unheated spores along with optical density curves are shown in Fig. 3. With heated spores, the initial level of redox potential (Eh, +338 mV) rapidly fell to a minimum level of Eh −350 mV in about 30 min, after which it gradually rose to the same level as that of the final potential that could be attained by unheated spores, probably due to the “poising effect” (15) of the system.

**Table 4. Inactivation of the spore lytic enzyme by oxidation or treatment with thiolglycolate reagents**

<table>
<thead>
<tr>
<th>Treatment of enzyme* (conc., mM)</th>
<th>Enzyme activity (% decrease in OD at 60 min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>64.3</td>
</tr>
<tr>
<td>Ferricyanide (10)</td>
<td>6.9</td>
</tr>
<tr>
<td>HgCl₂ (0.1)</td>
<td>0.0</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate (0.2)</td>
<td>30.0</td>
</tr>
<tr>
<td>N-Ethylmaleimide (50)</td>
<td>17.0</td>
</tr>
<tr>
<td>5,5’-Dithio-bis-(2-nitrobenzo-</td>
<td>28.6</td>
</tr>
<tr>
<td>za) (5)</td>
<td></td>
</tr>
<tr>
<td>Monoiodoacetate (50)</td>
<td>32.1</td>
</tr>
</tbody>
</table>

* Chemicals were present during assay. Sodium thioglycollate was used at a final concentration of 0.1% (wt/vol).

The enzyme activity was measured as described in the text. The reaction mixture contained enzyme preparation (163 U), one of the chemicals indicated with or without sodium thioglycollate, 50 mM sodium and potassium phosphate buffer (pH 6.0), and alkali-treated spores. The mixture was incubated at 40°C. OD, Optical density.

---

**FIG. 2. Lysis of spore cortical fragments and cell walls of C. perfringens by lytic enzymes.** Lysozyme was used at 100 µg/ml in 50 mM sodium and potassium phosphate buffer (pH 7.8), and the spore lytic enzyme (SLE) was used at 92 U in 50 mM sodium and potassium phosphate buffer (pH 6.0). The reaction mixture contained enzyme solution and cortical fragments or cell walls to give an initial optical density (OD; 580 nm) of approximately 0.4. The mixture was incubated at 40°C. Symbols: 0, cell walls; •, cortical fragments.

**FIG. 3. Changes in redox potential during germination of C. perfringens spores.** Spore suspension (15 mg/0.5 ml) was introduced (at the arrow point) to the KCl medium (14.5 ml) which had been bubbled with oxygen-free N₂. The changes in Eh and optical density (OD) were simultaneously measured by the use of a specially designed Klett tube as described previously (3). Symbols: 0, heated spores; •, unheated spores.
The sudden decrease in Eh coincided with the rapid decrease in optical density in the early germination. Microscopic examination showed that almost all the spores had become phase dark at the time when Eh reached the minimum level. On the other hand, unheated spores produced a much weaker reducing condition, a final Eh potential of +30 mV being reached more slowly about 80 min after inoculation.

Since the electrode potential developed in bacterial cultures reflects the state of the dynamic equilibrium of the culture between the metabolic activity of the cellular enzyme and the constituents of the medium (15), some anaerobic metabolism via spore enzymes could be involved in the development of reducing power during germination. The most likely candidate for the metabolism capable of producing such a low Eh might be pyruvate-dependent phosphoroclastic reaction since pyruvate proved to be a prime end product from endogeneous 3-phosphoglyceric acid in the early germination (2).

Table 5 shows that incubation of spore extracts with pyruvate under the conditions described for the assay of pyruvate:ferredoxin oxidoreductase resulted in a significant rate of acetohydroxamate formation. The omission of ATP reduced the rate about one-third. ATP presumably acted as an activator of the reaction as was reported with the same enzyme in a blue-green alga (cyanobacterium) (19).

**DISCUSSION**

Thus far, *B. cereus* spores have been the only source of spore lytic enzyme that can be extracted as a solubilized enzyme (11), although the existence of autolytic enzyme systems has been reported with some other *Bacillus* spores (16, 22). Gibbs (9) recently reported the presence of a lytic enzyme similar to *B. cereus* spore enzyme in the extracts of *C. bifermentans* spores. More recently, Brown et al. (5) have reported the existence of two kinds of lytic enzymes, “surface” and “core” enzymes, each having some different properties and location, in *B. cereus* T spores.

Although activity of lytic enzyme was barely detectable in the dormant spores of *C. perfringens* by the extraction procedures (except UME extract), a large amount of it was present in the exudate of fully germinated spore of the organism. The lytic enzyme could germinate alkali-treated spores of the organism. In this respect, the enzyme is similar to lysozyme or the initiation protein (8). However, the enzyme is quite different from the latter two enzymes in pH and temperature optima, as well as substrate specificities. The enzyme was optimally active in the presence of K*+* and other univalent cations at a concentration of 50 mM. This finding is compatible with the fact that ionic germination of the spores occurred optimally when the germination mixture contained KCl at this ionic strength (1). This implies the involvement of the spore lytic enzyme in the ionic germination of the spores.

In contrast to *B. cereus* spores, *C. perfringens* spores did not possess any detectable amount of active enzyme unless they were germinated. One possibility is that the enzyme may be released from its binding site(s) within the spore by an increased ionic strength as a result of the accumulation of K*+* during germination. This hypothesis seems unlikely, since no lytic enzyme activity was detected in the spore extracts even when disrupted spores were extracted with a much higher molarity of KCl (3 M) or other membrane-destructive agents. Another more likely possibility is that the enzyme was present in dormant spores in an inactive form and subsequently was activated in situ upon germination. The *C. perfringens* enzyme, like *B. cereus* enzyme (11), was found to be an SH enzyme, because its activity for the germinability of alkali-treated spores depended on its SH groups. These enzymes were readily inactivated by oxidation or treatment with SH group reagents. Therefore, it is reasonable to consider that the enzyme must be present in an inactive oxidized (—SS—) form in dormant spores and it will be altered in some way to become an active reduced (—SH) form during germination.

The activity of pyruvate:ferredoxin oxidoreductase in the spore extracts was significantly stimulated by ATP. Previous studies showed that there was a sharp increase in spore ATP

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>Sp act* in the extracts† from:</th>
<th>Dormant spores</th>
<th>Germinated spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete*</td>
<td></td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>ATP omitted</td>
<td></td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>ATP, CoA, and pyruvate omit-</td>
<td>tetrathionate</td>
<td>0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Expressed as nanomoles of acetohydroxamate formed per minute per milligram of protein.
† Spores were germinated for 60 min as described in footnote b. Table 2. The spore extracts were prepared as described in the text.
‡ The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM sodium pyruvate, 0.1 mM coenzyme A (CoA), 25 mM 2-mercaptoethanol, 20 mM ATP, 400 mM hydroxylammoniumchloride, and spore extract (10 to 15 mg of protein) in a final volume of 2.5 ml. The mixture was incubated at 40°C.
level derived from endogenous 3-phosphoglyceric acid in the early germination of *C. perfringens* spores (2). Therefore, germinating spores of the organism are to give rise to the most intensive reducing power, even corresponding to the potential of the hydrogen electrode, by anaerobic metabolism via pyruvate:ferredoxin oxidoreductase.

It is conceivable that one of the early events in initiation of germination of *C. perfringens* spores is the uptake of K⁺ by either passive or active transport, followed by the endogenous metabolism which causes the generation of strong reducing conditions within the spore, which in turn allow spore lytic enzyme to be functional. Once the enzyme is activated, it causes extensive degradation of the cortex, resulting in subsequent germination as judged by the loss of absorbency or refractility. Accordingly, the activation of the enzyme does not appear to be the prime event in initiation of spore germination in the organism. This assumption is in accord with the hypothesis that peptidoglycan hydrolysis is a relatively late event occurring during germination of certain *Bacillus* spores (13, 17).

Further studies are in progress to determine the properties of more purified enzyme preparations.

ACKNOWLEDGMENTS

I thank James C. Vary for valuable advice and critical reading of the manuscript.

REFERENCES CITED


