Extraction of Spore-lytic Enzyme from *Clostridium perfringens* Spores

By DAVID E. GOMBAS AND RONALD G. LABBE*

Food Microbiology Laboratory, Department of Food Science and Nutrition, University of Massachusetts, Amherst, Massachusetts 01003, U.S.A.

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Various chemical reagents known to extract spore coat protein were used to extract spore-lytic enzyme (SLE) from intact and germinated spores of *Clostridium perfringens*. Of the reagents tested, 7.2 M-urea plus 10% (v/v) mercaptoethanol, pH 2.85, solubilized the most SLE activity per mg spores. The quantity of SLE extracted was dependent on the initial pH of the reagent, with a maximum between pH 2.7 and 3.0. Germinated spores yielded more SLE than non-germinated spores upon urea/mercaptoethanol extraction. SLE release during spore germination probably utilizes a trigger mechanism not satisfied by germination alone. Significant amounts of SLE were released during germination when spores were suspended in potassium chloride or a complex germinant mixture containing brain–heart infusion, yeast extract and chloramphenicol, but not during germination with sodium nitrite, which non-enzymically lysed the cortical peptidoglycan. Greater solubilization of SLE activity was obtained by urea/mercaptoethanol extraction of spores germinated with nitrite than of spores germinated with either potassium chloride or the complex germinant.

INTRODUCTION

Powell & Strange (1953) postulated the presence of an enzymic lytic system in bacterial spores and its role during germination. Since then, the spore-lytic enzymes of *Bacillus cereus* have been isolated and extensively studied by Strange & Dark (1957), Gould *et al.* (1966), Warth (1972) and Brown *et al.* (1975, 1977, 1978). In most cases, this lytic system was found to hydrolyse the peptidoglycan of the spore cortex, resulting in germination-like changes in the spore. Warth (1972) found that *B. cereus* spores contain four separate cortex-degrading enzymes. Brown *et al.* (1977) reported that cortex-lytic activities were localized in both the core and in the coat fraction, and these could be isolated separately by particular extraction procedures. Further evidence for the association of cortex-lytic (germination) enzyme with the coat fraction follows from the observation that, in several cases, treatment of dormant spores with reagents which remove spore coat have resulted in a reduced ability of those spores to germinate. This has occurred with, for example, urea/mercaptoethanol (UME) treatment of *Bacillus megaterium* (Vary, 1973), alkaline dithioerythritol/sodium dodecyl sulphate treatment of *B. cereus* (Aronson & Fitz-James, 1976), dithiothreitol/urea or alkaline treatment of *Clostridium bifermentans* (Wyatt & Waites, 1974), and alkaline treatment (Duncan *et al.*, 1972) or alkaline dithiothreitol treatment of *Clostridium perfringens* (Labbe *et al.*, 1978). These observations could be the result of the cortex-lytic enzymes being either solubilized and removed or simply inactivated by the treatments. Indeed, Brown *et al.* (1978) have extracted and purified a cortex-lytic enzyme from *B. cereus* spores using urea/mercaptoethanol and guanidine/mercaptoethanol. However, it is not clear whether this enzyme plays the only or a major role in germination

since they were unable to observe germination-like changes in sensitized spores following treatment with this enzyme.

On the other hand, very little has been published on the lytic systems of other bacterial spores. In particular, a similar cortex-lytic enzyme was only recently reported in *C. perfringens* by Ando (1979), who found that spores germinated in the presence of potassium chloride at pH 7.0 exuded an enzyme capable of hydrolysing cortical fragments and causing germination of alkali-treated (coat-altered) spores.

In this study, several of the methods known to extract bacterial spore coats were tested for their ability to extract active spore-lytic enzyme from *C. perfringens* spores.

METHODS

Organism. *Clostridium perfringens* NCTC 8798, Hobbs serotype 9 (H9) was originally obtained from C. Duncan, University of Wisconsin.

Media and sporulation. Spores of *C. perfringens* H9 were grown and cleaned as described by Franceschini & Labbe (1979). Final spore suspensions contained less than 1% vegetative cells, as determined by phase-contrast microscopy.

Preparation of germinated spores. Cleaned spores were suspended in 25 mM-sodium phosphate buffer, pH 6.0, and heated at 75 °C for 15 min. For spores germinated with nitrite (N-germinated spores), suspensions were brought to 0.1 M-sodium nitrite in the same buffer and a final spore concentration of about 5 mg (initial dry wt) ml⁻¹, and held at 45 °C for 45 min; at this time microscopic examination showed greater than 95% phase-dark spores. For 'non-germinated' spores, suspensions were treated similarly but the nitrite was omitted; less than 10% of these spores became phase-dark. For potassium chloride-germinated spores (K-germinated spores), sodium phosphate buffer and sodium nitrite were replaced by 50 mM-potassium phosphate buffer, pH 7.0, and 50 mM-potassium chloride. Spores germinated in a complex medium (CG-germinated spores) were prepared by incubation in a final concentration of 5.0% (w/v) brain-heart infusion plus 0.5% (w/v) yeast extract plus 30 µg chloramphenicol ml⁻¹. Greater than 95% of the spores germinated by these methods were sensitive to heating at 75 °C for 15 min.

Enzyme extraction. After germination, spores were pelleted by centrifugation at 3020 g for 10 min, washed once with cold sterile distilled water, and resuspended in one of the extraction reagents at 5.8 mg (initial dry wt) ml⁻¹. The 'acidic' extraction reagents included 7.2 M-urea (U), 3.6 M-guanidine.HCl (G), 10% (v/v) mercaptoethanol (ME), 50 mM-dithiothreitol (DTT), 7.2 M-urea plus 10% (v/v) mercaptoethanol (UME) and 3.6 M-guanidine.HCl plus 10% (v/v) mercaptoethanol (GME). The 'alkaline' reagents included UME, DTT and ME. Spores were extracted at 45 °C for 90–120 min, except for spores in reagents containing guanidine which were extracted for 60 min only (see below). After extraction the spores were removed by centrifugation at 3020 g for 10 min and the supernatant fluid was dialysed overnight against distilled water at 4 °C before assaying for enzyme activity and protein concentration. In some experiments enzyme activity was assayed during germination. In these cases, samples were taken at specified intervals, the spores were removed by centrifugation as above, and the supernatant was assayed directly without prior dialysis.

In some cases, spore pellets were re-extracted with acidic UME for 120 min, centrifuged as above, and the re-extracted material was dialysed overnight before assaying for activity and protein.

Enzyme assay. Spore-lytic enzyme (SLE) activity was assayed using spores stripped of their coat protein, as described by Franceschini & Labbe (1979). These were prepared by treating clean spores with 50 mM-dithiothreitol plus 1% (w/v) sodium dodecyl sulphate, pH 10.0, at 37 °C for 2 h followed by extensive washing with distilled water. Stripped spores were suspended in either 25 mM-sodium phosphate, 25 mM-Tris/maleate or 25 mM-maleate buffer, pH 6.0, to a turbidity of 0.4 to 0.5 at 600 nm. Assays were performed at 37 °C in a Beckman 25 spectrophotometer equipped with a kinetic system. Activity was determined from the linear decrease in turbidity. One unit (U) of activity was defined as a decrease in A_{600} of 0.01 min⁻¹.

In some experiments, cortical fragments, prepared from strain 8798 spores by the method of Hashimoto *et al.* (1972), were used in place of stripped spores, and the turbidity was monitored at 400 nm.

Protein assay. Protein was assayed by the method of Bradford (1976), using crystalline bovine serum albumin as a standard.

Chemicals. Urea, mercaptoethanol, dithiothreitol, chloramphenicol and bovine serum albumin were obtained from Sigma. Guanidine.HCl was from Eastman Kodak, sodium nitrite was from J. T. Baker Chemical Co. (Phillipsburg, N.J., U.S.A.), and potassium chloride and sodium thioglycollate were from Fisher Scientific (Fairlawn, N.J., U.S.A.). Brain-heart infusion and yeast extract were obtained from Difco. All chemicals were reagent grade.

Table 1. *Extraction of spore-lytic enzyme from non-germinated and nitrite-germinated spores of C. perfringens*

Clean spores were heated (75 °C, 15 min), then either incubated in sodium phosphate buffer (non-germinated spores) or germinated in sodium nitrite (N-germinated spores) as described in Methods. Spores were washed once in cold distilled water before extraction. Extracted spores were removed by centrifugation and the extracts were dialysed overnight before assaying for protein and SLE activity. Reagents: U, 7.2 M-urea; G, 3.6 M-guanidine.HCl; ME, 10% (v/v) mercaptoethanol; DTT, 50 mM-dithiothreitol; UME, 7.2 M-urea plus 10% (v/v) mercaptoethanol; GME, 3.6 M-guanidine.HCl plus 10% (v/v) mercaptoethanol.

Extraction procedure		Non-germinated spores			N-germinated spores		
Reagent	pH	Protein (mg ml ⁻¹)	Activity (U ml ⁻¹)	Specific activity (U mg ⁻¹)	Protein (mg ml ⁻¹)	Activity (U ml ⁻¹)	Specific activity (U mg ⁻¹)
NaPO ₄	6.0	0.05	0.1	NM			
NaNO ₂	6.0				0.05	0.2	NM
UME	8.3	0.12	0.4	2.9	0.28	0.1	NM
DTT	8.3	0.30	0.6	1.8	0.32	0.1	NM
U	3.0	0.15	1.7	11	0.17	2.8	16
G	3.0	0.05	0.4	NM	0.05	0.1	NM
ME	3.0	0.47	140	290	0.15	0.1	NM
UME	3.0	1.00	170	170	1.10	320	290
GME	3.0	0.06	0.9	15	0.67	68	100
DTT	3.0	0.16	4.3	27	0.19	0.1	NM

NM, Not measurable.

RESULTS AND DISCUSSION

Extraction of spore-lytic enzyme (SLE)

Non-germinated spores and nitrite-germinated spores (N-germinated spores) were extracted with sodium phosphate, sodium nitrite and reagents known to extract spore coat protein (Table 1). Detectable SLE activity was found in all extracts of non-germinated spores, with acidic urea/mercaptoethanol (UME) and mercaptoethanol (ME) extractions yielding the highest specific activity (170 and 290 U mg⁻¹, respectively). Extraction of non-germinated spores with dithiothreitol/sodium dodecyl sulphate, pH 10.0, did not yield detectable activity (data not shown).

Extraction of N-germinated spores failed to yield significant enzyme activity except with acidic UME and guanidine/mercaptoethanol (GME). The specific activity obtained using acidic UME (290 U mg⁻¹) was comparable with that obtained when non-germinated spores were extracted with acidic ME, but the SLE activity extracted from the N-germinated spores was more than twice that obtained from the non-germinated spores (320 versus 140 U ml⁻¹). No enzyme activity was detected in the supernatant fluid of heated (75 °C, 15 min), intact spores or N-germinated spores.

The surprisingly high SLE activity obtained from N-germinated spores prompted an investigation into the extraction of spores germinated by means other than nitrite. These germinants were KCl (Ando, 1979) and a 'complex germinant' (CG). Like nitrite, both of these germinants led to rapid germination without observable outgrowth. However, unlike the supernatant fluid from N-germinated or non-germinated spores, the germination exudates with these germinants had significant levels of SLE activity (94 and 11 U ml⁻¹ for CG- and K-germinated spores, respectively; Table 2). Spores germinated in these media yielded significant SLE activity only when extracted with acidic ME, U and UME. The latter extracted the highest concentration of SLE regardless of the method of germination. As in the case of N-germinated spores, extraction of CG- or K-germinated spores with alkaline reagents did not yield significant levels of active SLE, nor did extraction with DTT, pH 3.0.

Table 2. *Extraction of spore-lytic enzyme from CG- and K-germinated spores of C. perfringens*

Clean spores were heated (75 °C, 15 min) and germinated in either complex germinant (CG) or potassium chloride (K) as described in Methods. Extraction conditions and reagents were as in Table 1. Four K-germinated spore pellets were collected after extraction and each was re-extracted in UME, pH 3.0, for 120 min. Both the re-extracted material and the initial extracts were treated identically.

Extraction procedure		CG-germinated spores			K-germinated spores			Re-extracted K-germinated spores		
		Protein (mg ml ⁻¹)	Activity (U ml ⁻¹)	Specific activity (U mg ⁻¹)	Protein (mg ml ⁻¹)	Activity (U ml ⁻¹)	Specific activity (U mg ⁻¹)	Protein (mg ml ⁻¹)	Activity (U ml ⁻¹)	Specific activity (U mg ⁻¹)
CG	7.0	0.08	94	1200						
K	7.0				NM	11	NM			
ME	8.5	0.38	0.2	<1	0.41	0.2	0.5			
UME	8.5	0.63	<0.1	<1	0.98	<0.1	<1	0.08	47	600
DTT	8.5	0.49	1.0	2	0.68	0.5	1			
U	3.0	0.06	22	370	0.10	58	580			
G	3.0	0.12	2.0	17	0.11	NT	NM			
ME	3.0	0.13	72	550	0.10	130	1300	0.11	300	2700
UME	3.0	0.23	110	470	0.16	300	1900	0.41	68	170
GME	3.0	0.11	1.0	9	0.09	0.5	6	0.32	200	630
DTT	3.0	NM	0.2	NM	0.04	0.9	20			

NM, Not measurable; NT, not tested.

Alkaline UME treatment of K-germinated spores produced about fivefold more protein than acidic UME treatment (0.98 versus 0.16 mg ml⁻¹). When both such treated spores were re-extracted with acidic UME, those spores which had originally been extracted with acidic UME continued to release both protein and active SLE, although at a much lower total activity than obtained during the first extraction (68 versus 300 U ml⁻¹). Those spores which had previously been extracted with alkaline UME did not release much more protein upon re-extraction (0.08 versus 0.98 mg ml⁻¹) but did release more enzyme (47 versus <0.1 U ml⁻¹), i.e. about the same as that released by re-extraction of spores originally extracted with acidic UME. If alkaline UME had been unable to extract SLE specifically, then high levels of SLE might have been expected upon re-extraction with acidic UME. As this was not the case, it is likely that, although effective in solubilizing spore proteins, alkali solubilizes SLE in an irreversibly inactive form. Acidic UME re-extraction of spores previously extracted with acidic ME and GME indicated that a substantial amount of active enzyme remained in the spore after the initial extraction with these reagents. Consequently, as with non-germinated or N-germinated spores, acidic UME was the best of the reagents tested for extraction of active SLE. SLE extracted by UME hydrolysed isolated cortical fragments of strain 8798 (data not shown).

Brown *et al.* (1978) reported that extraction of *B. cereus* with acidic UME produced inconsistent levels of SLE, while GME worked well. We found the contrary to be true for *C. perfringens*. Acidic UME extraction of N-germinated spores yielded a continuous increase in active SLE solubilized during 120 min, while GME extracts, first assayed at 60 min, showed a 40% loss of SLE activity by 120 min (data not shown). Unlike UME extractions, GME extractions were very dependent on the ratio of reagent to spores, losing activity more rapidly at greater dilutions. For example, N-germinated spores (7×10^9) extracted in 8.0 ml GME for 60 min yielded only 56% of the active SLE obtained when the same number of spores were extracted in 4.0 ml GME. Further, SLE activity in the more dilute system decreased 80% by 120 min.

The loss of activity upon prolonged extraction of spores in GME was not surprising, since this compound is an active protein denaturant. Urea acts in the same way yet, for some

reason, did not irreversibly inactivate SLE. These reagents probably act by peeling away layers of spore coat after disulphide cross-links, which secure the coat structure, are broken by mercaptoethanol. It is not known if SLE remained active during extraction, or was denatured by UME and renatured during dialysis. UME extracts assayed before dialysis did not display any activity, but since SLE activity is sharply inhibited by high ionic strength (D. E. Gombas & R. G. Labbe, unpublished data) it was not possible to distinguish between these possibilities.

Effect of pH on extraction of SLE

It was apparent that, of the methods tested, acidic UME yielded the highest activity of SLE. This was true for both non-germinated and germinated spores. UME has been used by a number of researchers for extracting spore coats, and the importance of pH was demonstrated by Gould & Hitchins (1963). We therefore examined the effect of the initial pH on extraction of protein and enzyme activity from N-germinated spores. The pH of UME was found to be critical in the extraction of SLE. The highest SLE activities were extracted at pH values of 2.7 and 3.0 (data not shown). At pH values below 2.3 or above 3.5, both extractable activity and protein dropped off dramatically (10% and <0.5% of optimum activity, respectively). Gould & Hitchins (1963) have reported this sharp pH range for extraction of spore coat material in *B. cereus*. They suggested that it might be because spores are reported to have a net zero charge around pH 3, and that this could influence the accessibility or reactivity of spore coat disulphide bonds. Brown *et al.* (1977) have suggested that this pH range is effective because mercaptoethanol is most effective as a reducing agent around pH 3. A starting pH of 2.85 was selected for all further studies.

Influence of the sulphydryl reagent on extraction of SLE

Urea (7.2 M) was included with 1.3 M-DTT or 1.3 M-sodium thioglycollate or 2.6 M (20%) ME, and each was compared with 7.2 M-urea plus 1.3 M (10%) ME (UME) for their ability to extract protein and SLE from N-germinated spores. Increasing the concentration of ME did not significantly affect the amount of protein or SLE extracted (data not shown). Similar levels of protein and enzyme activity were obtained when urea plus DTT was used in place of UME.

The importance of the sulphydryl reagent is not yet clear. Although acidic ME alone was capable of extracting significant amounts of SLE, DTT was not. When coupled with urea, both sulphydryl reagents responded synergistically, and with equal efficiency. However, urea plus thioglycollate, pH 2.85, was incapable of extracting active SLE, or significant amounts of protein.

Release of SLE during germination

As previously mentioned, after germination with KCl or CG, a significant level of SLE activity was detected in the exudate. Figure 1 shows the kinetics of release of this activity from spores germinated with KCl, NaNO₂ or CG. Microscopic examination at each sampling time showed that greater than 95% of KCl- and NaNO₂-treated spores had germinated within 30 and 45 min, respectively. However, only 60 U ml⁻¹ was released by germination with KCl and none with NaNO₂. No SLE activity was detected following incubation of heat-shocked or unheated spores (non-germinated) in sodium phosphate buffer (data not shown). Although the initial rate of SLE release and phase-darkening in CG occurred more slowly than in KCl, the final activity approached 40 times that of the K-germinated spores. The presence of chloramphenicol did not significantly affect the levels of SLE released by spores germinated in CG, but did prevent spore outgrowth (data not shown). As with UME extracts, the KCl exudate and the CG exudate were active in hydrolysing cortical fragments. These preparations were not characterized further.

Fig. 1. Release of SLE in exudate during germination of *C. perfringens* spores. Clean spores were heated (75 °C, 15 min) and germinated at 45 °C with sodium nitrite (○), potassium chloride (●) or complex germinant (□) as described in Methods. At intervals, samples were centrifuged and the supernatants were assayed for SLE activity without prior dialysis. Arrows indicate the times at which at least 95 % of spores were phase-dark.

Since SLE, present in the germination exudates of K- and CG-germinated spores, was absent from the supernatant of N-germinated spores, some relevant properties of NaNO_2 were explored. It was possible that NaNO_2 might inactivate SLE during germination. However, SLE did not lose any activity upon holding for up to 1 h in NaNO_2 , pH 6.0, at concentrations as high as 160 mM (final nitrite concentration during SLE assay was 32 mM). Ando (personal communication) has suggested that nitrite will non-enzymically hydrolyse spore peptidoglycan at pH 4.0. We tested this in our system by incubating cortical fragments in the presence of increasing concentrations of NaNO_2 , pH 6.0, and found that there was a significant increase in the rate of turbidity decrease with NaNO_2 at concentrations above 50 mM. In assay spore suspensions with 200 mM- NaNO_2 , turbidity decreased at a rate of $7.4 \times 10^{-3} A_{600} \text{ units min}^{-1}$; this was 16 times faster than in the absence of NaNO_2 . When incubated in 200 mM- KNO_2 , cortical fragments and assay spores both showed similar rates of decrease in turbidity to those observed with NaNO_2 , but neither cortical fragments nor assay spores showed a significant decrease in turbidity when incubated in 200 mM NaCl or KCl (data not shown). Further, NaNO_2 had a 10-fold higher spore-lytic activity at pH 6.0 than at pH 8.0 (data not shown), indicating the protonated form of nitrite was the active agent. Our results therefore support Ando's findings, i.e. that nitrite, or nitrous acid, will non-enzymically lyse spore cortex, and thereby cause spores to germinate.

With this system, it is clear that germination, by itself, is necessary but not sufficient for release of SLE from the spore. The latter requires a release mechanism which seems to be satisfied by CG, only partially satisfied by KCl and not satisfied by NaNO_2 at all.

Germination with CG most closely approaches germination of spores in typical *C. perfringens* growth media. Therefore, gradual release of SLE during germination would be expected to aid in the germination of nearby spores which are deficient in this enzyme, or injured, e.g. by heat, but permeable enough to accept the enzyme. This could explain the presence of 'secondary' or satellite colonies which frequently appear on plates of heat-injured *C. perfringens* spores after prolonged incubation (Cassier & Ryter, 1971).

Kinetics of release of SLE by UME

As shown in Tables 1 and 2, the highest SLE extraction was obtained by extracting spores using acidic UME. However, it was not directly clear whether these conditions affected the

Fig. 2. Kinetics of release of SLE during UME extraction of *C. perfringens* spores. Clean spores were heated (75 °C, 15 min) and either 'non-germinated' (○), or germinated with sodium nitrite (●), potassium chloride (□) or complex germinant (■), as described in Methods, until at least 95 % of spores were phase-dark. Spores were washed once in cold distilled water, and extracted with UME, pH 2.85, at 45 °C. Samples were dialysed overnight before assaying for protein (a), SLE activity (b), or specific SLE activity (c).

quantity of active SLE extractable by UME. The kinetics of release of protein and SLE from non-germinated and N-, K- and CG-germinated spores are shown in Fig. 2. Germination was required for maximum protein extraction (Fig. 2a), but the germinant used did not significantly affect either the rate or extent of protein extraction. However, the germinant used did significantly affect the total activity extracted. The greatest amount of SLE, expressed as U ml⁻¹ or U mg⁻¹, which could be extracted with UME was obtained from spores germinated with nitrite. K- and CG-germinated spores yielded less activity (Fig. 2b, c). While UME was capable of extracting protein from the non-germinated spores at about half the rate of germinated spores, extraction of active SLE was even more retarded, resulting in a maximum specific activity only 10% of that obtained from N-germinated spores in 2 h.

Both Gould *et al.* (1966) and Ando (1979) have suggested that germination is a necessary step for maximum release of SLE from spores. Results presented here support this observation, as extraction of non-germinated spores yielded far less protein and active enzyme. The reason for this is not known. Although Gould *et al.* (1966) could not obtain evidence that this is due to activation of zymogens, they suggested it might be due to release of the enzyme from a bound form. Release of SLE in the presence of chloramphenicol suggests that *de novo* synthesis during germination is not responsible for the disparity in active enzyme levels. This is further supported by the results of Cleveland & Gilvarg (1975), who found that spores of *B. megaterium* lys mutants were equally capable of germination in the presence or absence of exogenous lysine. Further, UME extraction of vegetative cells prepared in Fluid Thioglycollate Medium, a glucose-rich asporogenic growth medium, did not yield detectable activity (data not shown), indicating that extractable SLE is not a product of vegetative cell metabolism.

It is not yet possible to say that the SLE released from the spore during germination is the same protein as the SLE extracted by UME. Indeed, Warth (1972) identified four separate cortex-degrading activities in spores of *B. cereus*. However, it is interesting to note that, although the differences are not stoichiometric, the germinated spores which exude the least amount of SLE, i.e. N-germinated spores, yield the greatest concentration of SLE upon UME extraction, and that the converse is also true.

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