Purification and properties of a germination-specific cortex-lytic enzyme from spores of *Bacillus megaterium* KM

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Two peptidoglycan-lytic enzyme activities were isolated from spores of *Bacillus megaterium* KM. Surface-bound lytic enzyme was extracted from dormant spores and hydrolysed a variety of peptidoglycan substrates including isolated spore cortex, but did not cause refractility changes in permeabilized spores. Germination-specific lytic enzyme activity appeared early in germination and had minimal activity on isolated peptidoglycan substrates, but caused refractility changes in permeabilized spores of several *Bacillus* species. The germination-specific lytic enzyme was shown to be a heat-sensitive 29 kDa protein with maximal activity at pH 6.5. It catalysed post-commitment muramic acid δ -lactam synthesis and displayed an inhibitor profile similar to that for post-commitment A_{600} loss. The relationship of the germination-specific enzyme to a recently proposed model of spore germination is discussed.

INTRODUCTION

The extreme dormancy of spores of *Bacillus megaterium* KM is rapidly and irreversibly broken by exposure to the spore-specific germinant L-alanine (Johnstone *et al.*, 1982a). This germination response is mediated by interaction of a receptor protein with L-alanine, which catalyses an irreversible commitment reaction (Stewart *et al.*, 1981). Biochemical analysis of early germination events has shown that commitment takes place in the absence of both germinant (Scott & Ellar, 1978a) and germinant-stimulated metabolism (Scott & Ellar, 1978b). The effect of specific inhibitors on the germination pathway has demonstrated that germinant-dependent proteolytic activity is essential for germination (Boschwitz *et al.*, 1983, 1985) and, in particular, constitutes part of the commitment reaction (Foster & Johnstone, 1986). It has recently been proposed that an Hg²⁺-sensitive cortex-lytic enzyme is activated by this proteinase, which catalyses all subsequent germination events (Foster & Johnstone, 1986).

Activation of a lytic enzyme as a primary event in spore germination was originally suggested by Powell & Strange (1956). The spore-specific cortical peptidoglycan has been strongly implicated in the maintenance of spore dormancy (Ellar, 1978) and is therefore a prime candidate as a substrate for such lytic activity. Although release of peptidoglycan fragments occurs late in germination (Hsieh & Vary, 1975), selective hydrolysis of cortical peptidoglycan is an early germination event (Johnstone & Ellar, 1982). A number of lytic enzymes have been isolated from spores, which can be divided into two classes (Brown, 1977). Firstly, core enzymes have been extracted from germinating spores which are able to cause germination-like changes in permeabilized spores (Gombas & Labbe, 1981; Ando & Tsuzuki, 1984). Secondly, surface-bound enzymes can be extracted from dormant spores which are unable to 'germinate' permeabilized spores, but are able to hydrolyse isolated spore cortex (Brown & Cuhel, 1975; Brown *et al.*, 1982).

The role of both types of enzymes in the germination process has not been established (Brown *et al.*, 1978; Gombas & Labbe, 1985).

The present work describes the isolation, properties and substrate specificities of core- and surface-bound cortex-lytic enzymes from spores of *B. megaterium* KM and the relation of these enzyme activities to known germination events.

MATERIALS AND METHODS

Spore preparation and germination

Spores of *Bacillus megaterium* KM (Stewart *et al.*, 1981) and *Bacillus cereus* T (Boschwitz *et al.*, 1983) were prepared as described previously (Stewart *et al.*, 1981) and stored at a concentration of 10 mg dry wt./ml in distilled water at -20°C . Spores of *Bacillus subtilis* 168 Sueoka (Leighton & Doi, 1971) were prepared as described by Watabe *et al.* (1981) and stored as described above.

Unless otherwise stated, spores of *B. megaterium* KM were permeabilized at 50 mg dry wt./ml before germination as previously described (Foster & Johnstone, 1986). Spores were subsequently heat-activated at 10–100 mg dry wt./ml at 70°C for 30 min and cooled on ice. Germination was initiated by the addition of a final concentration of 1 mM-L-alanine to a spore suspension of either 0.05 or 10 mg dry wt./ml in 50 mM-Tris/HCl, pH 7.5, containing 50 mM-KCl. Germination was measured over a 60 min period as described by Foster & Johnstone (1986) and the V_{max} of germination determined by the method of Stewart *et al.* (1981) and normalized by dividing by the initial A_{600} .

Extraction and purification of lytic enzymes from spores of *B. megaterium* KM

Surface-bound cortex-lytic enzyme was extracted during the permeabilization procedure. The permeabilization supernatant was dialysed for 24 h against three

Abbreviations used: DPA, pyridine-2,6-dicarboxylic acid; PMSF, phenylmethanesulphonyl fluoride; PNAG, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide; Tos-Arg-OMe, *N*²-*p*-tosyl-L-arginine methyl ester ('TAME'); PAGE, polyacrylamide-gel electrophoresis.

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changes of 100 vol. of 50 mM-potassium phosphate buffer, pH 7.0, at 4 °C and assayed in this buffer. The surface-bound cortex-lytic enzyme was not purified further.

Germination-specific cortex-lytic enzyme activity was extracted from spores which had been germinated at 10 mg dry wt./ml in the presence of chloramphenicol (100 µg/ml) for 30 min at 30 °C. After addition of 10 mM-D-alanine (final concn.), the spores were centrifuged (10000 g, 30 s, 4 °C) and resuspended in 4 M-LiCl in 50 mM-Tris/HCl, pH 7.5, at 20 °C. After 2 min, the suspension was centrifuged (14000 g, 2 min, 4 °C) and the supernatant diluted into an equal volume of 50 mM-Tris/HCl, pH 7.5, at 4 °C and re-centrifuged (14000 g, 2 min, 4 °C). The resulting supernatant was sequentially dialysed at 4 °C for 24 h at each stage against 50 vol. of (i) 0.1 M-LiCl/50 mM-KCl/5 mM-EDTA/1 mM-2-mercaptoethanol/50 mM-Tris/HCl, pH 7.5, (ii) 0.5 M-KCl/5 mM-EDTA/1 mM-2-mercaptoethanol/50 mM-Tris/HCl, pH 7.5, and (iii) 0.1 M-KCl/5 mM-EDTA/1 mM-2-mercaptoethanol/10 mM-potassium phosphate buffer, pH 7.5. The dialysis residue was then centrifuged (20000 g, 45 min, 4 °C) and the pellet washed once in the final dialysis buffer by centrifugation (20000 g, 45 min, 4 °C) and resuspension. The pellet was redissolved in 0.1 M-KCl/0.4% (v/v) Triton X-100/2 mM-EDTA/1 mM-mercaptoethanol/0.2 M-potassium phosphate buffer, pH 7.5, and centrifuged (20000 g, 5 min, 4 °C) to remove all insoluble material. The partially purified enzyme was then applied to a column (45 cm × 1.5 cm) of Bio-Gel P-100, which was eluted with the resuspension buffer at 4 °C.

Germination-specific cortex-lytic enzyme was concentrated before analysis by SDS/PAGE by affinity purification on *B. megaterium* KM spore cortex isolated as previously described (Johnstone & Ellar, 1982). Spore cortex was added to enzyme fractions at a final A_{600} of 0.25, incubated for 30 min at 4 °C, centrifuged (15000 g, 2 min, 4 °C) and the cortex washed once in distilled water by centrifugation (15000 g, 2 min, 4 °C) and resuspension. This procedure removed more than 98% of the initial activity from the enzyme-containing fractions. The cortex was resuspended in gel sample buffer and analysed by SDS/PAGE on 12.5%-(w/v)-polyacrylamide slab gels (Laemmli, 1970), which were run at a constant current of 25 mA.

Assay for lytic enzyme activity

The surface-bound enzyme was assayed in dialysis buffer at 30 °C. The germination-specific enzyme was assayed in 0.1 M-KCl/0.4% (v/v) Triton X-100/2 mM-EDTA/1 mM-2-mercaptoethanol/0.1 M-potassium phosphate buffer, pH 6.5, at 30 °C. Permeabilized *B. megaterium* KM spores (Foster & Johnstone, 1986) were routinely used as the germination-specific enzyme substrate. Permeabilized *B. cereus* T and *B. subtilis* 168 spores were prepared by the UDS method of Brown *et al.* (1982). Isolated *Micrococcus lysodeikticus* cell walls and *B. megaterium* KM spore cortex were prepared as previously described (Johnstone & Ellar, 1982).

A unit of enzyme activity was defined as the amount of enzyme which caused a decrease in A_{600} of 0.001 unit/min of a substrate suspension of initial A_{600} of 0.08–0.10 unit at 30 °C. Partially purified germination-specific enzyme (redissolved precipitate) was used to study the properties of the enzyme.

Table 1. Substrate specificities of surface-bound and germination-specific lytic enzymes

Enzymes were extracted and assayed as described in the Materials and methods section. All substrates were sensitive to lysozyme (30 µg/ml), apart from non-permeabilized *B. megaterium* spores. The control substrates (expressed as 100% enzyme activity) were isolated *B. megaterium* KM spore cortex and permeabilized *B. megaterium* KM spores for the surface-bound and germination-specific lytic enzymes respectively. –, Not tested.

Substrate	Enzyme activity (% of control)	
	Surface-bound	Germination-specific
Non-permeabilized <i>B. megaterium</i> KM spores	< 1	< 1
Permeabilized <i>B. megaterium</i> KM spores	< 1	100
Permeabilized <i>B. cereus</i> T spores	–	167
Permeabilized <i>B. subtilis</i> 168 spores	–	33
<i>M. lysodeikticus</i> vegetative cells	< 1	< 1
<i>B. megaterium</i> KM vegetative cells	< 1	< 1
Isolated <i>B. megaterium</i> KM spore cortex	100	< 1
Isolated <i>M. lysodeikticus</i> cell walls	30	< 1

Effect of inhibitors on post-commitment A_{600} loss and germination-specific lytic-enzyme activity

Portions of enzyme were preincubated for 5 min at 30 °C with inhibitors before addition of substrate. EDTA and 2-mercaptoethanol were omitted from the assay buffer when HgCl_2 and ZnCl_2 were used. In order to determine the effects of inhibitors on post-commitment A_{600} loss, permeabilized spores were germinated for 20 min before addition of 10 mM (final concn.)-D-alanine. These spores were 95% committed and had lost less than 15% of their initial A_{600} . Inhibitors were then added and the A_{600} of the spore suspension recorded at 30 °C.

Mode of action of germination-specific lytic enzyme

Germination-specific lytic enzyme (18 units/mg dry wt. of substrate) was added to permeabilized spores (1 mg dry wt./ml) and the appearance of new cortical reducing groups measured by the method of Johnstone & Ellar (1982) after 15 min at 30 °C. The peptidoglycan was reduced with 0.1 M- NaBH_4 (60 Ci/mol) at 4 °C, and hydrolysed samples analysed by Dowex-50 chromatography (Ward, 1973).

Chemicals

D-Alanine, L-alanine, 2-mercaptoethanol, chloramphenicol, dithiothreitol, *Micrococcus lysodeikticus* cells, Triton X-100, Daltons MK VII L standards, Tos-Arg-OMe, PMSF, penicillin G, PNAG and Dowex-50 (X2, 200–400 mesh) were all obtained from Sigma. Analytical-grade HgCl_2 and ZnCl_2 were from BDH Biochemicals, and Bio-Gel P-100 (fine grade) from Bio-Rad Laboratories. NaBH_4 (5 Ci/mmol) was from Amersham

Table 2. Purification of the germination-specific lytic enzyme

Germination-specific lytic enzyme was purified from 1 g dry wt. of spores as described in the Materials and methods section. Protein concentrations were measured by the method of Lowry *et al.* (1951). In the final purification step, active enzyme could not be recovered and the yield represents the amount of enzyme activity removed from the supernatant by cortex treatment.

Procedure	Total enzyme activity (units)	Total protein (μ g)	Specific activity (units/ μ g of protein)	Yield (% total original activity)	Purification factor
1. 4 M-LiCl extract	441	6042	0.073	100	
2. Redissolved precipitate	433	20	1.36	98	18
3. Chromatography on Bio-Gel P-100	101	28	3.59	23	50
4. Enzyme bound to cortex	96	4	24.0	0 (22)	330

International, and all other chemicals were of analytical-reagent grade.

RESULTS

Effect of permeabilization on *B. megaterium* KM spore germination

In order to study the effects of metabolic inhibitors on germination, spores must first be permeabilized to allow entry of inhibitors through the spore coats, which normally act as a permeability barrier to many molecules (Aronson & Fitz-James, 1976). Effective permeabilization procedures therefore disrupt the spore coats sufficiently to allow access of large molecules to the spore cortex without grossly affecting the germination response. The extent of permeabilization is determined by the ability of lysozyme (14.3 kDa) to cause loss of spore refractility by cortex hydrolysis (Brown *et al.*, 1982). The permeabilization procedure used in the present study resulted in spores which lost more than 80% of their initial A_{600} after exposure to lysozyme at 30 μ g/ml, but which germinated to the same extent as untreated spores (results not shown). The maximum rate of permeabilized spore germination was 50% of that of untreated spores, and permeabilization resulted in a decrease in the K_m for L-alanine-induced germination from 17.8 to 7.1 μ M. Such a decrease in K_m has been observed as a result of other permeabilization procedures (Somerville *et al.*, 1970) and is probably the result of an increased free concentration of L-alanine due to the removal of non-specific binding sites rather than due to an increased affinity of a receptor for L-alanine.

Extraction of lytic enzyme activities from dormant and germinating spores

Two peptidoglycan lytic enzymes were found associated with spores. The first, referred to as the 'surface-bound enzyme', was extracted during the permeabilization treatment of dormant spores. Since permeabilized spores germinated almost normally, it was concluded that this enzyme was irrelevant to the germination process and was not studied further. The second peptidoglycan lytic enzyme, referred to as the 'germination-specific enzyme', was only extracted from germinating spores by LiCl. The germination-specific lytic enzyme could be distinguished from the surface-

Fig. 1. Time course of extraction of germination-specific lytic enzyme during germination

○, Percentage loss in A_{600} at the times indicated after addition of 1 mM-L-alanine (final concn.); △, percentage commitment at times indicated, measured as described by Stewart *et al.* (1981); ●, percentage of maximum germination-specific lytic-enzyme activity (0.17 unit/mg dry wt. of spores) at times indicated. Germination-specific lytic enzyme activity was assayed as described in the Materials and methods section.

bound lytic enzyme by its substrate specificity (Table 1). It was selected for further study on account of its ability to cause loss of refractility of permeabilized spores and its inability to hydrolyse other peptidoglycan substrates.

Purification of the germination-specific cortex-lytic enzyme

The steps in the purification procedure and the yields obtained for the germination-specific cortex-lytic enzyme are shown in Table 2. Maximal enzyme activity was extracted 30 min after addition of L-alanine, when the greatest rate of A_{600} loss is observed in this system (Fig. 1). No active enzyme could be extracted from dormant spores, and the amount of extractable activity fell rapidly as germination progressed. No enzyme activity could be detected in the supernatant resulting from centrifugation of germinating spore suspensions (results not shown).

The germination-specific lytic enzyme was relatively

Fig. 2. SDS/PAGE showing purification of the germination-specific lytic enzyme

Germination-specific lytic enzyme was purified as described in Table 2 and run on a 12.5%-(w/v)-polyacrylamide slab gel as described in the Materials and methods section. Tracks: (a) M_r markers of sizes indicated by the open arrows ($\Rightarrow$): bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20000) and α -lactalbumin (14200); (b), trichloroacetic acid precipitate of crude extract from 40 mg dry wt. of spores; (c) and (d) redissolved precipitate from 80 mg dry wt. of spores. (c) Represents the trichloroacetic acid precipitate of the supernatant remaining after treatment of redissolved precipitate with cortex. (d) Represents protein bound to cortex from redissolved precipitate; (e)–(h), cortex affinity-purified proteins from consecutive fractions eluted from the Bio-Gel P-100 column to which redissolved precipitate from 680 mg dry wt. of spores was applied. Tracks (e), (f), (g) and (h) had protein containing 2, 31, 52 and 5 units of enzyme activity respectively applied. The solid arrow ($\blacktriangleright$) indicates the position of the 29 kDa germination-specific lytic enzyme.

Fig. 3. Purification of germination-specific lytic enzyme by Bio-Gel P-100 column chromatography

Redissolved precipitate from 680 mg dry wt. of spores was applied to a Bio-Gel P-100 column as described in the Materials and methods section with a flow rate of 2.3 ml/h and 0.9 ml fractions were collected. $\circ$, A_{280} ; $\bullet$, units of germination-specific lytic-enzyme activity/ml of fraction.

unstable in LiCl concentrations approaching 4 M; for this reason the LiCl extract was immediately diluted to decrease the LiCl concentration. Subsequently, multiple dialysis steps were necessary to prevent the formation of an insoluble precipitate of lithium phosphate (Herbold & Glaser, 1975). At least 98% of the enzyme activity was recovered from the precipitate produced in the dialysis procedure (Table 2). The redissolved precipitate (Fig. 2, tracks c and d) showed a much simpler polypeptide

profile as compared with that of the LiCl extract (Fig. 2, track b) when analysed by SDS/PAGE.

In common with many other autolytic enzymes (Gombas & Labbe, 1985), the enzyme was retarded on an M_r exclusion column of Bio-Gel P-100. Most of the protein was eluted at the void volume (V_0), but the enzyme was eluted at $1.9\text{--}2.4 \times V_0$ (Fig. 3). The high affinity of the enzyme for isolated *B. megaterium* KM spore cortex was employed to concentrate the enzyme for analysis by SDS/PAGE. Under the conditions used (30 min at 4 °C), more than 98% of the enzyme activity was absorbed on to cortex from the redissolved precipitate (Fig. 2, track c). The resulting supernatant (Fig. 2, track d) contained a distinctive polypeptide profile. This affinity-purification procedure could only be used as a final purification step, since active enzyme could not be re-extracted from spore cortex by a variety of treatments, including 5 M-LiCl, extremes of pH and non-ionic detergents. The M_r of the germination-specific enzyme as judged by SDS/PAGE of Bio-Gel column fractions (Fig. 2, tracks e–h) was 29000.

Effect of temperature, trypsin and pH on enzyme activity

Incubation of redissolved precipitate with trypsin (10 μ g/ml, 30 min, 30 °C) or heating at 100 °C for 15 min completely destroyed all enzyme activity. The isolated enzyme was relatively heat-sensitive (Fig. 4) and was completely inactivated after incubation at 50 °C for 30 min. Incubation of the enzyme at 0–4 °C for 24 h resulted in a loss of less than 5% of initial enzyme activity.

The pH profile of enzyme activity was determined by using permeabilized spores as a substrate in the buffer

Fig. 4. Temperature-sensitivity of the germination-specific lytic enzyme

Redissolved precipitate was incubated for 30 min at the temperature indicated before assaying for germination-specific lytic enzyme activity as described in the Materials and methods section.

systems of Stewart *et al.* (1981). The enzyme had a sharp optimum at pH 6.5 (Fig. 5), which falls within the broad optimum pH range for both the commitment and overall germination reactions (Stewart *et al.*, 1981).

Effect of inhibitors on enzyme activity and on post-commitment A_{600} loss by permeabilized spores

In order to establish a relationship between the germination-specific lytic enzyme on permeabilized spores and post-commitment A_{600} loss during normal germination of permeabilized spores, the effect of a range of inhibitors on these two processes was examined (Table 3). D-Alanine, a competitive inhibitor of the normal germination-triggering mechanism, had no effect on the germination-specific lytic-enzyme activity. This therefore demonstrates that the germination-specific enzyme interacts at a post-commitment site in the germination pathway. There is a high level of correlation between the effects of all the inhibitors tested on post-commitment A_{600} loss and on germination-specific lytic-enzyme activity. The decreased levels of inhibition of 1 mM-HgCl₂ and PNAG on post-commitment A_{600} loss may be due to the presence of non-specific binding sites in intact permeabilized spores. Inhibition of germination-specific lytic enzyme activity by PNAG and Tos-Arg-OMe may be due to structural homology between spore cortex peptidoglycan and these inhibitors.

Mode of action of the germination-specific lytic enzyme

Since the germination-specific cortex-lytic enzyme was only able to hydrolyse peptidoglycan in permeabilized spores, its effect on spore peptidoglycan structure was

Fig. 5. Effect of pH on germination-specific lytic-enzyme activity

Redissolved precipitate was incubated for 5 min at 30 °C at the pH indicated before addition of substrate. ▲, 50 mM-potassium acetate buffer/150 mM-KCl; ○, 50 mM-potassium phosphate buffer/150 mM-KCl; ●, 50 mM-Tris/HCl/200 mM-KCl.

Table 3. Effect of inhibitors on post-commitment A_{600} loss and on germination-specific lytic enzyme activity

The effect of inhibitors on post-commitment A_{600} loss of L-alanine-induced germination of permeabilized *B. megaterium* KM spores and on germination-specific lytic-enzyme activity on permeabilized *B. megaterium* KM spores was determined as described in the Materials and methods section.

Inhibitor	Inhibition of A_{600} loss (%)	
	Post-commitment A_{600} loss	Germination-specific lytic enzyme activity
D-Alanine (10 mM)	< 1	< 1
PMSF (3.3 mM)	18	15
EDTA (5 mM)	< 1	< 1
HgCl ₂ (1 mM)	75	98
ZnCl ₂ (1 mM)	91	95
Tos-Arg-OMe (50 mM)	98	97
PNAG (5 mM)	50	86
Penicillin G (100 µg/ml)	< 1	< 1

determined. After treatment with germination-specific enzyme, permeabilized spore cortex was reduced with NaB³H₄ and a 59% increase in new reducing termini was detected when compared with the untreated control. Less than 5% of the initial A_{600} of the spore suspension was lost during the 15 min time period. After separation by Dowex-50 chromatography, the most significant increase in peak area was due to production of new muramic acid δ -lactam residues in the spore cortical peptidoglycan (Fig. 6). Such an increase in cortical muramic acid

Fig. 6. Mode of action of the germination-specific lytic enzyme

Cortical reducing termini were determined 15 min after addition of germination-specific lytic enzyme in permeabilized spores of *B. megaterium* KM as described in the Materials and methods section. After hydrolysis, reducing termini were fractionated on a column (18 cm $\times$ 1.2 cm) of Dowex 50 (X2, 400 mesh) equilibrated with 0.1 M-pyridine/acetate buffer, pH 2.80, with a flow rate of 30 ml/h (Johnstone & Ellar, 1982). Fractions (2.0 ml) were eluted with the same buffer (80 ml total) and then with 0.133 M-pyridine/acetate buffer, pH 3.85 (80 ml total). The hydrolysed cortex from 4 mg dry wt. of spores was applied to the column for each run. ○, Permeabilized heat-shocked spores incubated for 15 min at 30 °C in the absence of germination-specific lytic enzyme; ●, permeabilized heat-shocked spores incubated for 15 min at 30 °C with germination-specific lytic enzyme. Peaks: 1, muramitol; 2, glucosaminol; 3, reduced muramic acid δ -lactam.

δ -lactam residues has been observed early during germination of *B. megaterium* KM spores and arises because of uncross-linking of the radially expanded cortex (Johnstone & Ellar, 1982).

DISCUSSION

Hydrolysis of cortical peptidoglycan has been implicated as a crucial stage in the germination pathway of spores of *Bacillus megaterium* KM (Johnstone & Ellar, 1982; Foster & Johnstone, 1986). The mechanism whereby cortex hydrolysis is initiated is therefore central to an understanding of the germination pathway. The lytic-enzyme activity extracted during permeabilization of spores of *B. megaterium* KM is probably equivalent to the surface-bound enzyme characterized from spores of *Bacillus cereus* T (Brown & Cuhel, 1975). These two enzymes have similar substrate specificities in that they both degrade isolated spore cortex, but are unable to cause refractility changes in permeabilized spores (Brown *et al.*, 1982; Table 1). These enzymes, which are considered to be irrelevant to the germination process, probably become attached to the spore surface when the mother cell lyses during Stage VII of sporulation.

In contrast, we consider that the germination-specific lytic enzyme plays an essential role in mediating the L-alanine-dependent germination response of spores of *B. megaterium* KM. The germination-specific lytic

enzyme could not be extracted from dormant spores and is activated during the germination process (Fig. 1). Since germination occurs normally in the absence of protein synthesis *de novo* (Keynan, 1978), this enzyme must be present in an inactive form in the dormant spore. The loss of extractable activity later during germination may be due to the temperature-sensitivity of the activated enzyme (Fig. 4) or due to loss of enzyme bound to released peptidoglycan fragments. The inactive dormant-spore enzyme must, however, remain resistant to the heat-shock treatment (70 °C for 30 min) to which spores are exposed before germination. The lack of intrinsic heat-resistance of spore enzymes has been shown previously (Gombas & Labbe, 1985), although the mechanism whereby heat-resistance is conferred *in vivo* is unknown.

The isolated germination-specific lytic enzyme displays several other characteristics that suggest its involvement in the germination response. These include a very high affinity for spore peptidoglycan, a pH optimum within the overall pH range for the commitment and germination reactions (Fig. 5), an inhibitor profile similar to that observed for post-commitment A_{600} loss (Table 3) and the ability to catalyse formation of muramic acid δ -lactam (Fig. 6), which is a post-commitment event (Foster & Johnstone, 1986). The high specificity of the enzyme for intact spore cortex may reflect the need for a stressed peptidoglycan for enzyme activity. Such a requirement has been postulated for other bacterial autolysins (Koch, 1985). It may also account for the lack of degradation of the primordial cell wall during germination (Cleveland & Gilvarg, 1975). These results, together with its observed $HgCl_2$ -sensitivity, suggest that the germination-specific lytic enzyme represents the $HgCl_2$ -sensitive site II in the recently proposed model of the germination pathway (Foster & Johnstone, 1986). Thus this enzyme may be responsible for selective cortex hydrolysis, and consequently regulates A_{600} loss during germination. The loss of heat-resistance observed early in the germination sequence (Foster & Johnstone, 1986) is therefore probably due to conversion of the inactive enzyme into its heat-sensitive active form as an early germination event. The ability of the germination-specific lytic enzyme to germinate spores of other species, together with conservation of cortex peptidoglycan structure in all species so far examined (Warth, 1978), suggests that this enzyme may be an essential element in other *Bacillus* spore-germination mechanisms.

It is noteworthy that the germination-specific lytic enzyme is extremely sensitive to Zn^{2+} and that Zn^{2+} release is an early, but post-commitment, event during *B. megaterium* KM spore germination (Johnstone *et al.*, 1982a; Foster & Johnstone, 1986). Whether Zn^{2+} plays a role in regulating germination-specific lytic enzyme activity during germination remains to be established.

L-Alanine-dependent proteolytic cleavage has been proposed as the mechanism whereby the germination-specific enzyme is activated (Foster & Johnstone, 1986). Activation of a spore-lytic enzyme by release from an inactive bound form was proposed for spores of *B. cereus* T (Gould *et al.*, 1966). Subsequently an enhancing factor was extracted from spores, which increased lytic enzyme activity, possibly by proteolytic activation (Mencher & Blankenship, 1971). In contrast, lytic enzyme activation during germination of *Clostridium perfringens* has been shown to be an energy-dependent process (Ando &

Tsuzuki, 1984). Regulation of enzyme activity by proteolytic cleavage has been demonstrated for both spore and autolytic enzymes. The spore-specific proteinase responsible for hydrolysis of spore low- M_r acid-soluble core proteins is activated early in germination by proteolytic cleavage (Loshon *et al.*, 1982). The proteinase involved is, however, not essential for the normal germination response (Postemsky *et al.*, 1978). The autolytic muramidase of *Streptococcus faecium* is also proteolytically activated (Pooley & Shockman, 1969; Kawamura & Shockman, 1983).

Identification of the germination-specific cortex-lytic enzyme as a 29 kDa protein will allow antibodies to be raised against the purified protein. These antibodies may be used to demonstrate proteolytic cleavage of the enzyme during germination. In addition, immunocytochemical techniques may permit one to show where the enzyme is located within the spore. The relative immobility to macromolecules in the dormant spore (Stewart *et al.*, 1980; Johnstone *et al.*, 1982b) suggests that the lytic enzyme and the germinant receptor will be located within the same spore compartment.

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