Purification and Biosynthesis of a Spore Coat Protein in *Bacillus megaterium* QM B1551

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A spore coat protein of *Bacillus megaterium* QM B1551 was purified from an SDS–DTT extract of lysozyme-digested spores using Sephadex, DEAE-cellulose chromatography and polyacrylamide disc gel electrophoresis. The molecular weight of the protein was approximately 12000. It constituted about 13% of spore dry weight and was rich in lysine, aspartic acid, glycine and alanine residues. The sum of these four amino acids constituted 53.9% of the total amino acids. Small amounts of phosphorus and sugar were detected, but no amino sugar. The protein was synthesized in the cytosol of sporulating mother cells at t_3 , and accumulated on the forespores.

INTRODUCTION

The bacterial spore coat, the outermost spore structure, is a spore-specific constituent and makes the first contact with elements in the environment, such as germinants, germicides, UV light or heat. It has been suggested that the spore coat may play an important role in germination, dormancy or resistance.

Many investigators (Aronson & Fitz-James, 1976; Goldman & Tipper, 1978; Mitani & Kadota, 1976; Munoz *et al.*, 1978; Uchida & Kadota, 1976; Watabe *et al.*, 1975) have reported that spore-specific components extracted from spore coats with SDS and DTT consist mainly of a protein with a molecular weight of approximately 10000. The spore coat protein comprises 25–80% (w/w) of the mature spore and is one of the few sporulation-specific antigens described to date. Characterization of the spore coat protein is a necessary prerequisite for determining its contribution to dormancy.

The purpose of this investigation was to isolate and identify the spore coat protein of *B. megaterium* QM B1551 and to determine the time of its synthesis in sporulating cells.

METHODS

Strain and preparation of spores. *Bacillus megaterium* QM B1551 was cultivated in Schaeffer's medium with shaking at 37 °C for 48 h. Cells were collected by centrifugation and washed repeatedly with water. Vegetative cells were removed completely by this procedure so that more than 99% of the remaining structures were spores, which were stored lyophilized.

Spore fractionation and purification of spore coat protein. Spores were suspended to give 5 mg dry wt ml⁻¹ in 100 mM-sodium borate, pH 10.0, containing 0.1 M-NaCl, 0.5% (w/v) SDS and 0.03 M-DTT. The mixture was incubated at 37 °C for 2 h and centrifuged at 12000 g for 20 min. The supernatant solution (Sup-1) was filtered through a membrane filter (pore size 0.45 µm). Treated spores (S-1) were suspended to give 1 mg dry wt ml⁻¹ in Tris/HCl, pH 8.0, treated with lysozyme (100 µg ml⁻¹) at 37 °C for 60 min, and centrifuged (Sup-2). The pelleted material was washed with water and lyophilized (S-2). The lyophilized sediment was extracted with

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Fig. 1. Procedure for the fractionation of spores.

100 mM-sodium borate, pH 10.0, containing 0.5% SDS, 0.03 M-DTT and 0.1 M-NaCl at 37 °C for 2 h, then centrifuged. The supernatant solution was filtered through a membrane filter (Sup-3). The procedure is summarized in Fig. 1.

The supernatant fractions were used for the purification of the spore coat protein by means of Sephadex G-200, Sephadex G-100 and DEAE-cellulose column chromatography with 100 mM-sodium borate, pH 10.0, containing 0.1% SDS, each used according to the manufacturer's instructions. The material was stored in 100 mM-sodium borate, pH 10.0, containing 0.1% SDS and then further purified by disc gel electrophoresis (see below).

Preparation of forespore and mother cell cytoplasm. This was done according to the method of Fujita *et al.* (1977).

Electron microscopy. Thin sections were prepared as described previously (Nishihara *et al.*, 1980) and observed with an electron microscope (JEOL, JEM-120, 80 kV).

Disc gel electrophoresis. For PAGE, 10% polyacrylamide disc gels containing 1.0% SDS were used. Samples were incubated in solution A (2% SDS, 2% (v/v) β -mercaptoethanol, 2% (v/v) phenol red, 8 M-urea in 100 mM-borate buffer, pH 10.0) at 45 °C for 60 min, and 20 μ l of the mixture were applied to disc gels. Electrophoresis was carried out at 5 mA per tube for 3 h at pH 10.0, and gels were stained with Coomassie blue (Weber & Osborn, 1969).

Preparation of anti-spore coat protein serum. Antibodies were prepared using 2.5 kg white female rabbits and the following schedule. Antigen (spore coat protein) (1.5 mg) was mixed with an equal volume of Freund's complete adjuvant and injected into the footpad of the rabbit at zero time. Three weeks following initial injection, the rabbit was injected with a further 0.5 mg of a similar antigen preparation. One week later the final (0.5 mg) injection was given. The rabbit was bled from the peripheral vein of the ear on consecutive days one week following the final injection.

Determination of spore coat antigen by immunodiffusion assay. Antispore coat protein serum was mixed with 2% (w/v) agar containing 20 mM-Tris/HCl, pH 7.9, 20 mM-NaCl and 2 mM-EDTA. The mixture was poured into a test tube (50 $\times$ 3 mm) to a height of 25 mm. A sample (3 μ l) containing spore coat antigen was poured into the test tube, which was then incubated at 25 °C for 24 h. The distance from the top of the agar/antigen mixture to the precipitation band was measured. The amount of spore coat antigen in a sample was estimated from a calibration curve using variable amounts of spore coat protein. The distance was linear with respect to the logarithm of concentration of spore coat protein (0.5 to 1.0 μ g in 3 μ l).

Analysis. Amino acid composition was determined with an amino acid analyser (JEOL, JLC-5AH) after hydrolysis of samples with 6 M-HCl at 105 °C for 20 h in a sealed tube. The amount of half-cystine was determined after oxidation (Moor, 1963). Phosphorus, sugar and amino sugar were determined by the methods of Chen *et al.* (1956), Dubois *et al.* (1956), and Randle & Morgan (1966), respectively. Protein was determined by the Lowry method.

Fig. 2. Thin-sectioned spores and spore fractions. (a) Intact spores; (b) SDS/DTT-treated spores (fraction S-1); (c) fraction S-2. The bar markers represent 1 μm .

RESULTS

Components extracted with SDS, DTT and NaCl

Spores were successively treated with SDS/DTT, lysozyme, and again with SDS/DTT, as described in Methods. SDS/DTT-treated spores are sensitive to lysozyme (Vary, 1973). Electron microscopic examination of fraction S-2 (Fig. 2c) and the distribution of spore coat antigen in each fraction (Table 3; Dormant spores) suggested that Sup-3 contained extracted spore coat protein. The substances present in this second SDS/DTT extract (Sup-3) represented 20% of spore dry weight (Table 1), of which 85.5% was protein. Electrophoresis showed that Sup-3 was heterogeneous, with several major bands of protein. The predominant band had an R_F of 0.75. This portion of the gel was extracted with borate buffer and its protein content was determined. The extracted spore coat protein represented 75% of the protein content of the SDS/DTT extract (Sup-3), and corresponded to 13% of the protein present in whole spores.

Table 1. *Relative dry weight and protein content of spore fractions*

Spores were fractionated as described in Methods. Values in parentheses represent protein as a percentage of dry wt.

Fraction	Percentage of dry wt
Intact spore	100
Sup-1	17.0 (75.8)
S-1	83.0
S-2	30.8
Sup-3	20.0 (85.5)
Residue	10.8

Fig. 3. Sephadex G-200 column chromatography of Sup-3. Sup-3 was applied to a column (2.1 × 82 cm) of Sephadex G-200; 3.1 ml fractions were collected. Fractions A and B are indicated. Arrow, Blue dextran; half arrow, cytochrome *c*.

Purification of the spore coat protein

When Sup-3 was fractionated by column chromatography on Sephadex G-200 (2.1 × 82 cm), using 100 mM-borate buffer, pH 10.0, containing 0.1% SDS, two peaks (fractions A and B) were obtained (Fig. 3). Fraction B was concentrated, applied to a Sephadex G-100 column (1.6 × 100 cm) and eluted as a single peak of protein with 100 mM-borate buffer, pH 10.0, containing 0.1% SDS. Appropriate fractions were pooled, applied to a DEAE-cellulose column (1.8 × 1.4 cm) and eluted with 100 mM-Tris/HCl, pH 7.6, containing 0.1% SDS with a linear gradient of 0–1.0-M-NaCl. Only a single peak, eluting at approximately 0.4 M-NaCl, was detected by the measurement of A_{280} . The peak fractions were collected and dialysed against 100 mM-borate buffer, pH 10.0, containing 0.1% SDS, and subjected to SDS-PAGE. The section of the gel corresponding to the major Coomassie blue staining band was cut out and extracted with 100 mM-borate buffer, pH 10.0. This extracted spore coat protein showed only a single band in SDS-PAGE, which had an apparent molecular weight of 12 000 when compared with standard proteins.

Amino acid composition of the spore coat protein

The amino acid composition of the spore coat protein is given in Table 2. The protein contains large amounts of lysine, aspartic acid, glycine and alanine, with these four amino acids constituting 53.9% of the total amino acids. The protein contains little methionine or histidine. The phosphorus and sugar contents of the protein were 0.71 and 1.36 $\mu\text{mol mg}^{-1}$, respectively, but amino sugar was not detected.

Table 2. *Amino acid composition of the spore coat protein and Sup-3*

Values for amino acids are molar percentages and for phosphorus, sugar and hexosamine, $\mu\text{g (mg protein)}^{-1}$.

Component	Sup-3	Spore coat protein
Lys	10.4	12.3
His	1.7	1.1
Arg	8.8	5.3
Asp	14.8	15.6
Thr	4.9	4.9
Ser	6.0	7.6
Glu	6.2	4.5
Pro	Trace	Trace
Gly	11.7	12.5
Ala	10.7	13.5
Val	3.5	4.4
Met	2.4	0.9
Ile	4.5	5.0
Leu	7.4	7.9
Phe	2.1	2.1
Tyr	9.6	2.6
Cys (half)	—	2.6
Phosphorus	3.5	2.2
Sugar	5.7	24.6
Hexosamine	22.6	0.0

Fig. 4. Immune double diffusion of vegetative cell cytoplasm and purified spore coat protein. A, anti-spore coat serum; B, purified spore coat protein; C, vegetative cell cytoplasm (numbers indicate dilution ratio).

Antigenic localization of the spore coat protein within the spore

When purified spore coat protein was reacted with spore coat protein antiserum, only a single precipitation band was observed, suggesting homogeneity (Fig. 4). Each fraction obtained from spores was reacted with spore coat protein anti-serum. Three of the fractions yielded precipitate bands, showing that each contained spore coat antigen (Table 3). Quantitative determination of spore coat antigen in each fraction showed that Sup-3 contained 10.6% of the spore coat antigen of the whole spore, while Sup-1 and Sup-2 each contained only 1.9% of the spore coat antigen of the whole spore (Table 3).

Table 3. *Amounts of spore coat antigen in each spore and forespore fraction*

Cell type	Fraction	Spore coat antigen
		(percentage of spore dry wt)
t_6 (forespores)	Sup-1	13.5
	Sup-2	10.5
	Sup-3	0.4
t_9	Sup-1	9.8
	Sup-2	5.7
	Sup-3	8.9
Dormant spores	Sup-1	1.9
	Sup-2	1.9
	Sup-3	10.6

Fig. 5. Occurrence of the spore coat antigen in the mother cell fraction of the sporulating cell (percentage of spore dry wt) (●). —, Turbidity; ----, frequency of refractile bodies. Sample preparation and assay are described in Methods.

Spore coat antigen in sporulating cells and forespores

Cells were cultivated in double-strength Schaeffer's medium and harvested at intervals. Mother cell cytoplasm and forespores were prepared in the presence of the protease inhibitor phenylmethylsulphonylfluoride (1 mM), as described by Fujita *et al.* (1977). Sup-1 and Sup-3 were prepared from forespores by the same procedure as used for spores. The distribution of the spore coat antigen was determined using immunodiffusion (Fig. 5). Cytosolic spore coat antigen was maximal at t_3 and then decreased. Before t_3 , no forespores were detected; they began to appear at t_3 . In forespores at t_6 , the spore coat antigen was mostly present in Sup-1 and Sup-2 (Table 3), but as maturation progressed, the spore coat antigen transferred from Sup-1 to Sup-3.

DISCUSSION

The purification of spore coat proteins from *B. megaterium* QM B1551 has not previously been reported. We describe here the purification of the major protein of the spore coat from SDS/DTT extracts of lysozyme-digested spores. The origin of the protein was determined to be the spore coat both by electron microscopy and by the distribution of the spore coat antigen in each fraction of spores.

The protein, of approximate molecular weight 12000, constitutes about 13% of the dry weight of the spore and is rich in lysine, aspartic acid, glycine and alanine residues. Proteins of

molecular weight 8000–12000 were extracted earlier from spores of *B. subtilis* and *B. cereus* (Aronson & Fitz-James, 1976; Pandey & Aronson, 1979). Mitani & Kadota (1976) and Uchida & Kadota (1976) independently observed that *B. subtilis* spores contain a protein with a molecular weight of 10000 as the main component of the spore coat, and Watabe *et al.* (1975) reported that *B. thiaminolyticus* spores contain a protein of molecular weight 10000. In *B. cereus* (Aronson, 1981), the major structural protein of the spore coat is a precursor of 65000, which is synthesized in the sporulating cells. About 40% of this precursor is converted to spore coat monomer of molecular weight 13000.

In *B. subtilis* (Munoz *et al.*, 1978), a precursor spore coat protein with a molecular weight of 25000 is made initially at t_1 of the sporulation cycle and is converted to the mature spore coat protein with a molecular weight of 18500 at t_2 – t_3 . Jenkinson *et al.* (1981) reported that in *B. subtilis*, an alkali-soluble spore coat protein with a molecular weight of 12000 is synthesized at an early stage of sporulation (t_2), and a 36000 molecular weight protein is synthesized at a very late stage (t_6). In our study, the synthesis of a spore coat protein began at t_1 in the cytoplasm of sporulating cells. The amount of the protein increased in the mother cell cytoplasm until t_3 and then decreased. This might indicate that the newly synthesized spore coat protein was deposited in the forespores and then translocated to the inner part of the spore coat. Our result does not indicate the presence of a precursor of the spore coat protein, since only one antigenic species was ever observed (Table 3). These facts might indicate processing of the spore coat protein following synthesis, leading to accumulation and maturation in the forespore.

The synthesis of the proteins in sporulating cells requires clarification. Further investigations are clearly warranted.

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