Purification and some characteristics of a calcium-binding protein from *Bacillus cereus* spores

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A novel calcium-binding protein has been purified from the dormant spores of *Bacillus cereus* T. Purity of this protein was verified by SDS-PAGE and reversed-phase HPLC. Its calcium-binding ability was verified by a competitive calcium-binding assay using Chelex-100 resin and ⁴⁵Ca autoradiography. The protein is heat-stable and is retained by hydrophobic matrices (phenyl-Sepharose) in a calcium-dependent manner. SDS-PAGE and amino acid composition indicate the molecular mass of the protein to be 24 kDa.

Introduction

Large amounts of calcium are accumulated in bacterial spores during sporulation. Calcium also plays a role in germination. For example, *Bacillus cereus* spores and *Bacillus megaterium* spores with low contents of Ca²⁺ were unable to germinate in L-alanine and inosine unless Ca²⁺ was in the medium or a strong electrolyte was present (Rowley & Levinson, 1967; Sacks, 1972). More than 90% of the calcium which accumulates during sporulation is released during germination (Rode & Foster, 1966). CaCl₂ can activate *B. cereus* T spores (Douthit & Preston, 1985; Nakatani *et al.*, 1985) and calcium can cause germination if the spore coat is removed or weakened. Studies involving stepwise extraction of Ca²⁺ from labelled spores (Kamat *et al.*, 1985) indicate that part of the spore calcium may be involved in L-alanine-induced germination.

Calcium-binding proteins, which are frequently involved in calcium regulation in eukaryotic cells, have only recently been reported in bacteria, and have been purified only from vegetative cells of *Myxococcus xanthus* (Inouye *et al.*, 1981) and *Streptomyces erythreus* (Leadlay *et al.*, 1984). Until this report, dipicolinic acid was the only spore component with documented calcium-binding properties. This study reports a purification scheme for a calcium-binding protein from spores of *Bacillus cereus* T, and describes some characteristics of the protein and its amino acid composition.

Methods

Micro-organism. *Bacillus cereus* T, from the North Carolina State University culture collection, was sporulated at 30 °C on fortified nutrient agar, harvested, and washed with deionized water as detailed by Johnson *et al.* (1982). Clean spores were lyophilized and stored at 4 °C.

Crude extract. Dried spores were mixed with two volumes of 250–300 µm diameter glass beads and broken in a cell homogenizer (Braun, MSK) while cooling with liquid CO₂. Breaking required approximately six pulses of 1 min each. The breaking efficiency was checked microscopically and by colony counts. The broken powder was suspended in a minimal volume of extraction buffer [50 mM-Tris/HCl, pH 7.5, containing 75 mM-NaCl, 1 mM-EDTA, 1 mM-phenylmethylsulphonyl fluoride (PMSF), 1 mM-dithiothreitol (DTT), 1% (v/v) 2-propanol, 1 mM-benzamide, and 7.5 µg pepstatin A ml⁻¹].

Heat treatment. The crude slurry was heated at 90 °C for 2 min to denature the majority of unwanted spore macromolecules and clarified by centrifugation at 15000 g for 1 h at 4 °C. The pellet was resuspended in one-tenth of the original extraction buffer volume and centrifuged again. The supernatant fluids were combined and dialysed at 4 °C overnight against buffer A (50 mM-Tris/HCl, pH 7.5, with 5 mM-CaCl₂, 1 mM-PMSF, 1 mM-DTT, 1% 2-propanol, 1 mM-benzamidine, and 7.5 µg pepstatin A ml⁻¹).

Phenyl-Sepharose chromatography. The dialysed extract (about 600 ml from 28 g dried spores) was applied to a 1.5 × 30 cm phenyl-Sepharose column, pre-equilibrated with buffer A at a flow rate of 1 ml min⁻¹. After the sample was loaded, the column was washed until the A₂₈₀ reached the lowest stable background, then washed to the stable background again with buffer A containing 500 mM-NaCl. The calcium-binding protein, determined by the Chelex assay (see below), was eluted by washing with buffer A containing 7.5 mM-EGTA but without NaCl or CaCl₂. The peak fractions (about 24 ml) were pooled and extensively dialysed at 4 °C against buffer B (40 mM-Tris/HCl, pH 8.0).

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DEAE-cellulose chromatography. The dialysed fraction was loaded onto a DEAE-cellulose column (0.9 × 30 cm) at a flow rate of 0.8 ml min⁻¹. The column was washed with buffer B, then the protein was eluted with a gradient of NaCl (0–0.5 M) in the same buffer. Fractions (3.2 ml) were collected and analysed for calcium-binding activity by the Chelex assay.

Hydroxylapatite chromatography. Peak fractions (about 9 ml) from DEAE-cellulose chromatography were pooled, dialysed against buffer C (1 mM-sodium phosphate, pH 6.8), and applied to a hydroxylapatite column (0.9 × 15 cm) previously equilibrated with buffer C at a flow rate of 0.8 ml min⁻¹. The column was washed with two bed volumes of buffer C and eluted with a sodium phosphate gradient (1–200 mM). Fractions (2.4 ml) were collected and analysed for calcium-binding activity by the Chelex assay. The calcium-binding fraction was concentrated and extensively washed (dialysed) with 40 mM-Tris/HCl buffer, pH 7.4, using centrifugal concentration tubes (Centricon or Centrprep, MW cutoff 10000; Amicon). After dialysis, 1 mM (final concentration) CaCl₂ was added to stabilize the proteins which were not used immediately in the calcium-binding assays for 4 °C storage.

Chelex competitive assay. Calcium-binding activity was routinely checked by the Chelex-100 resin competitive assay (Tokuda *et al.*, 1987). A slurry of Chelex-100 resin (100–200 mesh, Bio-Rad) was washed extensively with 1 M-HCl, and 40 mM-Tris/HCl, pH 7.4, then resuspended in 40 mM-Tris/HCl, pH 7.4, at a resin-to-buffer ratio of 1/10. While rapidly stirring, 25 µl of resin was pipetted into a final reaction mixture volume of 1 ml which contained 10⁶ c.p.m. (0.1 µM) ⁴⁵CaCl₂ (50 µCi µl⁻¹, 1.85 MBq µl⁻¹; New England Nuclear) and variable concentrations of test substance. After 30 min of strong agitation at room temperature, the mixture was centrifuged at 3500 g for 5 min. A 0.1 ml aliquot of supernatant fluid from each reaction mixture was added to 5 ml of Scinti Verse II (Fisher) and subjected to liquid scintillation spectrometry with a Beckman LS 3801 instrument. Under this assay condition, a linear curve of c.p.m. from 6000 to 82000 was obtained by using 0.17–17 µg purified bovine brain calmodulin (Sigma), while only negligible background counting was given by bovine serum albumin.

Gel electrophoresis. Both denaturing (SDS) and non-denaturing PAGE were carried out on a 15% (w/v) resolving gel with the discontinuous system described by Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250 (Sigma) or silver stain (Bio-Rad) depending on the protein concentration.

⁴⁵Ca autoradiography. Calcium-binding ability was verified by autoradiography based on the methods of Maruyama *et al.* (1984). After non-denaturing electrophoresis, protein was electrophoretically transferred from the gel to nitrocellulose paper (0.1 µm, Micron Separation Inc.) at a constant current of 200 mA for 1 h at 4 °C according to the method of Towbin *et al.* (1979). After electrophoretic transfer, the membrane was washed with 10 mM-imidazole/HCl, pH 6.6, containing 60 mM-KCl and 5 mM-MgCl₂ and incubated at room temperature for 10 min in the same buffer containing 2.5 µM-⁴⁵CaCl₂. Membranes were washed for 5 min in 50% (v/v) ethanol, air-dried for 3 h, and exposed to X-ray film (X-Omat AR, Eastman Kodak) for 24 h.

Protein elution. The calcium-binding protein which was localized using the autoradiogram was excised from the membrane and dissolved in a minimum volume of 25% (v/v) acetonitrile in 0.1 M-ammonium acetate, pH 8.9, for 4 h at 37 °C. Elution typically required about 0.5 ml of solvent per cm² of membrane (Montelaro, 1987).

Reversed-phase chromatography. A 2.1 mm × 3 cm HPLC butyl column (Brownlee Labs) was used for desalting. The elution gradient was 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile.

Amino acid analysis. Amino acid composition was determined by the Protein Sequencing Facility at North Carolina State University, using the method of Bidlingmeyer *et al.* (1984). Salt-free samples from HPLC reversed-phase chromatography were lyophilized and hydrolysed by gas-phase HCl hydrolysis in a 150 °C oven for 1 h. After hydrolysis, phenylthiocarbamyl derivatives of the amino acids were formed and analysed by reversed-phase HPLC on a Pico-Tag amino acid analysis column. Molecular mass was determined from the mol % tyrosine.

Results and Discussion

Purification of spore calcium-binding protein

Purification of a novel calcium-binding protein from endospores was carried out using freeze-dried *B. cereus* T spores. Lyophilization of the spores enhanced the extraction of calcium-binding protein, avoided formation of sticky bubbles and need for anti-foam, and reduced preparation volumes. Lyophilization also might decrease losses due to protease activity. Disruption of wet spores during purification of small acid-soluble proteins has been shown to allow significant proteolysis (Johnson & Tipper, 1981; Setlow, 1985). Lyophilized spores were broken and heat treated as described in Methods. Heat treatment was the first step of purification. About 80% of the calcium-binding activity, as determined by Chelex assay, remained after heat treatment. The heat-treated crude extract was loaded onto a phenyl-Sepharose column. Calcium-binding protein was eluted from phenyl-Sepharose with few contaminants in about the first 1.5 bed volumes by washing with buffer A containing 7.5 mM-EGTA and no salt (Fig. 1). This fraction was further purified using DEAE-cellulose chromatography, where a major peak of calcium-binding ability eluted between 0.35 and 0.4 M-NaCl. The protein preparation eluted from the DEAE-cellulose column was further chromatographed through a hydroxylapatite column. A single peak of calcium-binding activity was resolved between 90 and 120 mM-phosphate. This fraction contained a homogeneous protein of molecular mass approximately 24 kDa as determined by SDS-PAGE (Fig. 2). The purity of this calcium-binding protein was also verified by reversed-phase HPLC. A single peak eluted at a position corresponding to approximately 61% acetonitrile (Fig. 3). Calcium-binding ability was routinely determined qualitatively during purification by the Chelex competitive assay. Due to the lack of a reliable quantitative activity assay for this novel protein, no precise purification data are available. Approximately 10 µg purified calcium-binding protein was obtained from 100 g wet weight of spores and the yield was about 1%. The detectable calcium-binding capacity of purified calcium-binding protein decreased during storage at 4 °C,

Fig. 1. Elution profile of phenyl Sepharose chromatography. Heat-treated crude preparation was loaded onto a phenyl Sepharose column and 4 ml fractions were collected and assayed for absorbance at 280 nm (○) and calcium-binding ability (●). The inset shows a silver-stained SDS-PAGE gel (15% acrylamide) of fractions 38 and 39 eluted from the affinity column; the apparent molecular mass is indicated on the left.

Fig. 2. SDS-PAGE of the calcium-binding protein of *B. cereus* T spores. Electrophoresis was carried out on a slab gel of 15% polyacrylamide. Lane A contains molecular mass markers, from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α -lactalbumin. Lane B contains calcium-binding protein (single band denoted by an arrow).

Fig. 3. Elution profile of HPLC reverse-phase chromatography. A 2.1 mm $\times$ 3 cm HPLC butyl column was used for the desalting of calcium-binding protein which eluted from nitrocellulose membranes. The elution gradient (---) was 0–100% acetonitrile. —, A_{280} .

especially in the absence of calcium (data not shown). Instability of purified mammalian duodenal calcium-binding protein and porcine intestinal calcium-binding protein in the absence of calcium during storage has been reported (Randolph & Strothkamp, 1988).

Calcium-binding ability

Calcium-binding ability of the purified protein was verified directly by ^{45}Ca autoradiography in the presence of excess amounts of other cations (Mg^{2+} and K^{+}) (Fig. 4). The autoradiogram, using protein transferred from a non-denaturing polyacrylamide gel, indicated that one protein with specific calcium-binding ability was present. Migration on the non-denaturing gel appeared close to the calmodulin control. Autoradiography using protein transferred from denaturing (SDS) acrylamide gels was unsuccessful using the *B. cereus* spore calcium-binding protein although it was successful using bovine brain calmodulin (Sigma) as control (data not shown).

Fig. 4. Autoradiogram showing ^{45}Ca binding to the calcium-binding protein of the *B. cereus* T. Lane A, bovine brain calmodulin (10 µg); lane B, bovine serum albumin (40 µg); lane C, spore calcium-binding protein (approx 1.5 µg).

Amino acid composition and analysis

The amino acid composition of the spore calcium-binding protein is shown in Table 1. From the amino acid composition, the molecular mass was determined to be 24 kDa and the protein is composed of 222 amino acid residues. The molecular mass is consistent with that estimated from SDS-PAGE (Fig. 2). The amino acid composition provides information for comparative studies with calmodulin from bovine brain and protein S from *Myxococcus xanthus*. Protein S is structurally similar to bovine brain calmodulin, and the calcium-binding domains of these two proteins have a high level of homology (Inouye *et al.*, 1981). None of these three proteins contains Cys residues. The unique trimethyllysine residue present in calmodulin was not determined in the *B. cereus* spore calcium-binding protein. The spore calcium-binding protein has an average hydrophobicity ($H\phi_{ave}$) of 0.90 kcal (3.77 kJ) per residue and a discriminant function (Z) of 0 according to the calculation methods described by Bigelow (1967) and Barrantes (1975), respectively. Bovine brain calmodulin has similar values of 0.88 kcal (3.68 kJ) per residue and 0.02, respectively. Proteins with similar values have been described as soluble non-membrane proteins (Barrantes,

Table 1. Comparison of amino acid compositions of the calcium-binding protein of *B. cereus* T spores, bovine brain calmodulin and protein S from *Myxococcus xanthus*

Amino acid	Calmodulin*		Protein S†		Spore calcium-binding protein‡	
	No. of residues	Mol %	No. of residues	Mol %	No. of residues	Mol %
Asx	23	15.5	34	16.3	24	10.8
Thr	12	8.1	9	4.3	5	2.3
Ser	4	2.7	14	6.7	10	4.5
Glx	27	18.2	24	11.5	43	19.4
Pro	2	1.4	15	7.2	4	1.8
Gly	11	7.4	16	7.7	22	9.9
Ala	11	7.4	16	7.7	23	10.4
Val	7	4.7	18	8.6	18	8.1
Met	9	6.1	1	0.5	1	0.5
Ile	8	5.4	13	6.2	6	2.7
Leu	9	6.1	15	7.2	25	11.3
Tyr	2	1.4	7	3.3	1	0.5
Phe	8	5.4	10	4.8	10	4.5
His	1	0.7	0	0.0	6	2.7
Lys	7	4.7	8	3.8	15	6.8
Arg	6	4.1	7	3.3	9	4.1
TML§	1	0.7	—	—	—	—
Trp	0	0.0	1	0.5	—	—
Cys	0	0.0	0	0.0	0	0.0
Total	148		209		222	

* Watterson *et al.* (1980). † Inouye *et al.* (1981). ‡ This study.
§ TML, trimethyllysine. —, Not determined.

1975). The majority of the calmodulin from many cells appears to be readily soluble (Burgess *et al.*, 1983). However, consistent with it being assembled on the cell surface, protein S has higher values of 1.08 kcal (4.52 kJ) per residue and 0.25, respectively. Generally, proteins which are related to each other have similar hydrophobicity index values (Bigelow, 1967).

The small amount of purified calcium-binding protein obtained has prevented further characterization at this time. Conclusive evidence concerning the function of this calcium-binding protein in spores of *B. cereus* T is not yet available. However, since calcium plays a vital role in bacterial spores, the finding of calcium-binding protein may help us to discover more about the regulation and role of calcium in bacterial sporulation and germination.

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