# A third DNA-dependent ATPase from *Bacillus cereus* free of ATP-dependent DNase activity

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The purification of ATP-dependent DNase from *Bacillus cereus* led to the isolation and characterization of a third DNA-dependent ATPase. The enzyme called ATPase III has been purified free of nuclease activity. None of the expected ATPases proved to be identical with ATP-dependent DNase–DNA-dependent ATPase. Separation of ATPase I, II and III and a DNase specific for single-stranded DNA from the same source excludes the possibility of ATP-dependent DNase being the action of a single enzyme molecule.

*Recombination* *Unwinding* *DNA dependence* *ATPase* *ATP dependence* *DNase*

## 1. INTRODUCTION

Enzymes which degrade ATP to ADP and $P_i$ using DNA as cofactor have been detected in both prokaryotic [1–3] and eukaryotic cells [4,5]. Several publications deal with enzymatic reactions implied in replication and recombination of the genetic material in which DNA-dependent hydrolysis of ATP occurs [1,2,3,6].

ATP-dependent DNase, the so-called 'recombination enzyme' of bacteria [7], was the first DNA-dependent ATPase described in several microorganisms [7–18]. However, the uncoupling of DNase and ATPase activities of ATP-dependent DNase [19–21] involves the possibility of separation of ATPase and DNase as different entities. We reported that purified DNA-dependent ATPase I and ATPase II from *B. cereus* did not contain DNase activities [22,23]. This paper aims to determine whether the third DNA-dependent ATPase of *Bacillus cereus* can be separated from its DNase activity.

Here we describe the partial purification and characterization of DNA-dependent ATPase III, compare the 3 ATPases from *B. cereus* and refer to their possible roles in the ATP-dependent DNase activity.

## 2. MATERIALS AND METHODS

The following buffers were used: (A) 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 mM 2-mercaptoethanol; (B) buffer (A) plus 30% (v/v) glycerol; (SSC) 150 mM NaCl, 15 mM sodium citrate (pH 7.0).

[$\gamma$-$^{32}$P]ATP (200 Ci/nmol) was prepared according to [24]. Protein was determined by the method in [25], using crystalline bovine serum albumin (Serva) as standard. ATP was coupled to AH-Sepharose 4B (Pharmacia) as in [26]. [$^3$H]DNA ($5 \times 10^3$ cpm/nmol nucleotide) was isolated from *B. cereus 130 thy*$^-$ as in [27]. T7 DNA was prepared according to [28]. Heat denaturation of DNA was carried out at 100°C for 10 min in SSC buffer followed by rapid chilling in ice. DNA-dependent ATPase assay and ATP-dependent DNase assay on native and on denatured DNAs were done as in [23].

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## 3. RESULTS

### 3.1. *Purification of DNA-dependent ATPase III from* B. cereus

The initial steps of purification of DNA-dependent ATPase I, II and III of *B. cereus* NRRL B-569 were essentially the same [23]. The separation of these enzymes on DEAE-cellulose column (2.5 $cm^2$ × 4 cm) is shown in fig.1. Further purification and characterization of ATPase I and II has been reported [22,23]. After the first DEAE-cellulose chromatography DNA-dependent ATPase III represented a small portion of the total activity of this group of enzymes. Peak fractions of ATPase III (75–110) shown in fig.1 were combined and subjected to a second DEAE-cellulose column (0.6 $cm^2$ × 10 cm) equilibrated with buffer B. The loaded column was first washed with 200 ml 0.25 M KCl and with 150 ml 0.3 M KCl in buffer B to remove the rest of ATPase I and II which were found to elute at 0.18 and 0.24 M KCl, respectively [23]. Proteins bound to the column were eluted stepwise (10 ml each) from 0.35–0.7 M KCl in buffer B (fig.2). We could not detect any ATP-dependent DNase activity on native or on denatured DNA after this step of purification. ATP-independent DNase activity specific for denatured DNA was eluted at 0.45 M KCl. Fractions containing ATPase activities (eluted between 0.4–0.6 M) were pooled, dialyzed against buffer B and were applied to a phospho-cellulose column (0.75 $cm^2$ × 6 cm) pre-equilibrated with buffer B.

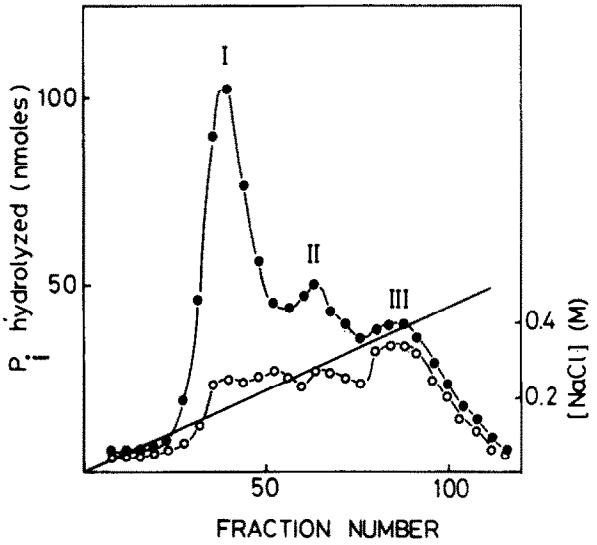


Fig.1. DEAE-cellulose chromatography of DNA-dependent ATPases from *B. cereus*. ATPase activities were measured in the presence (●) and in the absence (○) of T7 DNA. NaCl concentration gradient (——) chromatography was carried out with the 50% ammonium sulphate precipitate of *B. cereus* cell extract (fraction III) [23] on DEAE-cellulose column (2.5 $cm^2$ × 14) equilibrated with buffer B. The column was washed with 50 ml buffer B and elution was done with a linear salt gradient of 0–0.45 M NaCl in buffer B (300 ml total vol.).

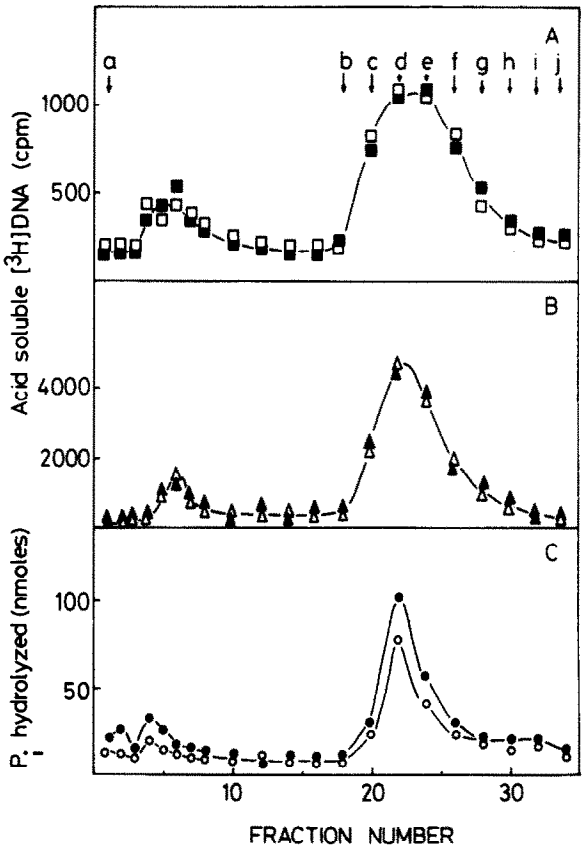


Fig.2. Second DEAE-cellulose chromatography of ATPase III from *B. cereus*. (A) DNase activity on native [$^3$H]DNA in the presence (■—■) and in the absence (□—□) of ATP. (B) DNase activity on denatured [$^3$H]DNA in the presence (▲—▲) and in the absence (△—△) of ATP. (C) ATPase activity in the presence (●—●) and in the absence (○—○) of T7 DNA. The concentration changes are indicated by the arrows. Buffer B containing: (a) 0.30; (b) 0.35; (c) 0.40; (d) 0.45; (e) 0.50; (f) 0.60; (g) 0.70; (h) 0.80; (i) 0.90; (j) 1.0 M KCl.

The DNA-dependent ATPase activity did not increase significantly after this chromatography. ATPase III and DNase specific for single-stranded DNA were not retained by the phospho-cellulose column. The fraction that passed through the column was used directly for a third DEAE-cellulose column (0.75 $cm^2$ × 6 cm) (fig.3). The higher resolution of a linear gradient elution from 0.2–0.6 M KCl in buffer B (200 ml total) resulted in the partial separation of ATPase III from DNase specific for single-stranded DNA.

The increase in DNA-dependent ATPase activity was detected in this purification step. ATPase fractions [15–32] were combined and subjected to ATP–Sepharose affinity chromatography. The column (0.75 $cm^2$ × 2 cm) was equilibrated with buffer B. Bound ATPase III was eluted stepwise with increasing concentrations of KCL in buffer B (fig.4). DNA-dependent ATPase III activity eluted between 0.35–0.7 M KCl. Active fractions showed some DNA-independent ATPase activity, but did not contain DNase activity.

### 3.2. *Assay conditions*

Optimal conditions for ATPase III activity were tested. We found that enzyme has a broad pH optimum (7.5–9.0). The enzyme requires bivalent cations for its catalytic activity with an optimum at 1–2 mM, $Mn^{2+}$ and $Mg^{2+}$ being more effective than $Co^{2+}$ and $Ca^{2+}$.

### 3.3. *Requirement of DNA*

The cofactor specificity of ATPase III was tested using native and denatured T7 DNA. Denatured DNA proved to be the preferred cofactor; however, considerable activity (~30%) was detected in the presence of double-stranded DNA. The half-saturation value for denatured T7 DNA was 1.5 μg/ml.

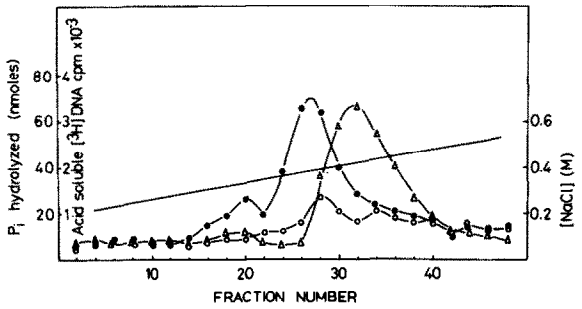


Fig.3. Third DEAE-cellulose chromatography of DNA-dependent ATPase III from *B. cereus*. ATPase activities were measured in the presence (●——●) and in the absence of T7 DNA. (——) NaCl concentration gradient. DNase activity on denatured [$^3$H]DNA from *B. cereus* (Δ——Δ).

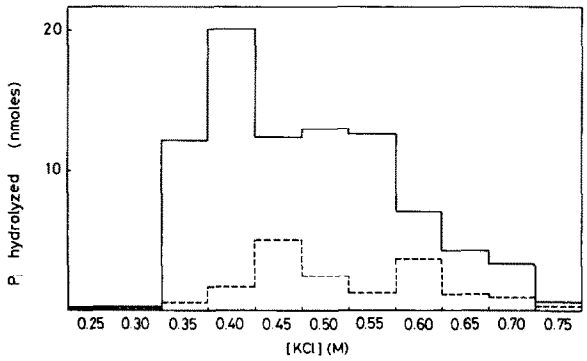


Fig.4. Affinity chromatography of DNA-dependent ATPase III on ATP–Sephadex. The pooled fractions of the third DEAE-cellulose chromatography were dialysed against buffer B and were adsorbed to the column of ATP–Sepharose, which was then washed successively with increasing concentrations of KCl. The elution profiles are shown for ATPase activity in the presence (——) and in the absence (– – –) of T7 DNA.

### 3.4. *Requirement of ATP*

DNA-dependent ATPase activity was measured at different concentrations of ATP and a regular saturation curve was obtained. The apparent $K_m$ value calculated from a double reciprocal plot was 0.4 mM.

### 3.5. *Molecular mass*

The $M_r$-values of ATPase I and II from *B. cereus* are close to each other under denaturing conditions on SDS–acrylamide gel (table 1). Sodium dodecyl sulfate gel electrophoresis [29] was unsuccessful in the case of ATPase III since the miniscule amount of purified protein was undetectable. Gel filtration on Sephadex G-200 column (2 $cm^2$ × 55 cm) showed that active fractions were excluded from the gel, the molecular weight of the native enzyme being >200 kDa.

### 3.6. *Comparison of ATPase I, II and III*

There is a difference between the susceptibility of ATPases towards different inhibitors (table 1).

Table 1

Comparison of properties of DNA-dependent ATPases from *B. cereus*

| Characteristics | ATPase | | |
|---|---|---|---|
| | I | II | III |
| $M_r$-value | 71000 | 82000 | >200000 |
| pH optimum | 7.0–9.5 | 7.5–9.5 | 7.5–9.0 |
| $K_m$ for ATP | 0.38 | 0.20 | 0.40 |
| $K_m$ for denatured T7 DNA ($\mu$M) | 0.9 | 0.3 | 4.5 |
| Bivalent cation optimum (mM) | 5–10 $Ca^{2+}$ > $Mg^{2+}$ | 0.5–1 $Mg^{2+}$ > $Ca^{2+}$ | 1–2 $Mn^{2+}$ > $Mg^{2+}$ |
| ATP-independent DNase activity | None | None | None |
| ATP-dependent DNase activity | None | None | None |
| DNA unwinding activity | Yes | Yes | Not detected |
| Inhibitor present | | | |
| none | 100% | 100% | 100% |
| actinomycin D (100 $\mu$g/ml) | 35% | 65% | 60% |
| ethidium bromide (100 $\mu$g/ml) | 8% | 13% | 15% |
| monovalent cations | | | |
| NaCl (200 mM) | 39% | 86% | 95% |
| KCl (200 mM) | 85% | 73% | 100% |
| *N*-ethylmaleimide (50 mM) | 55% | 71% | 89% |

ATPase I is more sensitive to actinomycin D, to *N*-ethylmaleimide and to high concentration of NaCl than ATPase II and III. There is no difference in their susceptibility towards ethidium bromide.

The main characteristics of ATPase I, II and III of *B. cereus* are summarized in table 1. The pH optima of all 3 enzymes are nearly the same, and none of the enzymes has DNase activity. Single-stranded DNA is the preferred cofactor for the ATPases. ATPase I has an absolute requirement for single-stranded DNA, whereas ATPase II and III also showed some activity with double-stranded T7 DNA. The bivalent cation requirement for ATPase I is roughly an order of magnitude higher than those for ATPase II and III. $Ca^{2+}$ is the most effective for ATPase I, $Mg^{2+}$ for ATPase II and $Mn^{2+}$ for ATPase III among the bivalent cations tested. The $K_m$ values for ATP are 0.2–0.4 mM.

## 4. DISCUSSION

We reported earlier the presence of a DNA-dependent ATPase–ATP-dependent DNase in *B. cereus* [16]. Enzymes with similar activities have been described in several microorganisms [12,13,15,17]. We found that the degrees of purification of DNase and ATPase were remarkably different, furthermore the hydrolysis of ATP under defined conditions (pH 7.0, 1 mM ATP, 40 mM $MgCl_2$) occurred without concomitant phosphodiesterase bond cleavage [30]. These data gave us the idea to separate ATPase from DNase on DEAE-cellulose columns using high chromatographic resolution.

During the purification of ATP-dependent DNase, 3 DNA-dependent ATPases of *B. cereus* could be separated (ATPase I, II and III), two of which were characterized earlier. The purified ATPase I and II did not contain DNase activity [22,23]. However, the possibility that ATPase III is identical with the ATP-dependent DNase still existed. Partial purification of ATPase III revealed that after affinity chromatography on ATP–Sepharose, the enzyme was free of DNase activity. The fact that none of the 3 DNA-dependent ATPases from *B. cereus* possess ATP-dependent or ATP-independent DNase activity, and that DNase specific for single-stranded DNA, originally though to be the ATP-dependent DNase [22], could be freed of ATPase activity (fig.3), exclude the possibility that ATP-dependent DNase activity is due to the action of a single enzyme species.

The inactivation of ATP-dependent DNase during DEAE-cellulose chromatography was observed by several authors [10,15,17,20,31,32]. The extent of inactivation differed, depending on the chromatographic resolutions used. There was no strict correlation between the number of ATP molecules degraded and the number of DNA phosphodiester bonds cleaved during the action of ATP-dependent DNase [33]. This fact as well as the chromatographic inactivation of ATP-dependent DNase can be explained simply by the chromatographic separation of ATPase and

DNase. On the basis of the properties of separated ATPases (I, II and III), and DNase specific for single-stranded DNA we consider ATP-dependent DNA degradation as consecutive reactions of unwinding ATPases tracking along the DNA, transiently unwinding the duplex as they move, followed by the nucleolytic action of a single-strand-specific DNase.

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