A MANGANESE-STIMULATED ENDONUCLEASE FROM *BACILLUS SUBTILIS*

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Received October 9,1973

SUMMARY

An endonuclease activity has been identified in extracts of Bacillus subtilis. This activity is stimulated by Mn^{++} or Ca^{++} ions but not by Mg^{++} ions. The enzyme catalyzes the breakdown of native DNA of high molecular weight to fragments of molecular weights ranging from 3×10^6 to 20×10^6. A variety of DNA's from sources such as B. subtilis, Salmonella and T7 phage are attacked. About 61% of the activity of the cells is released into the medium during protoplast formation under conditions where 98% of the glucose 6-P dehydrogenase activity is retained by the cells.

INTRODUCTION

Several deoxyribonucleases have been identified in B. subtilis including a Ca^{++}-dependent exonuclease (1), an ATP-dependent nuclease (2, 3, 4, 5) and a heat-activated, Mg^{++}-dependent endonuclease (6). We now report the existence of a new endonuclease activity which can be detected in extracts of B. subtilis and which is distinguished from the others on the basis of its metal requirements and the size of its products. The reaction catalyzed by this activity may be similar to the one detected in whole cells of B. subtilis by Haseltine and Fox (7).

MATERIALS AND METHODS

Crystalline egg white lysozyme was purchased from Pentex Corporation; deoxyribonuclease I from Calbiochem; streptomycin sulfate from F. R. Squibb & Sons; Dextran T500 from Pharmacia; polyethylene glycol (Carbowax 6000) from Union Carbide Chemical Company; DEAE-cellulose DE-52 (Whatman microgranular) from Reeve-Angel; and bovine serum albumin from Armour Pharmaceutical Company. ^{3}H-Thymidine and ^{14}C-thymine were purchased from New England Nuclear Corp. B. subtilis strain BD170 (thr-5 trpC2), a derivative of strain 168, was used as the source of enzyme. Strain BD204 (hisB2 thy) was used for the isolation of radioactive DNA.

Preparation of DNA. ^{3}H-DNA was isolated as previously described from cultures of BD204 (8). ^{3}H-and ^{14}C-labeled T7 phage DNA was prepared by phenol extraction of whole phage after purification by CsCl density gradient centrifugation.

Sucrose gradients. All centrifugations were performed at 20° in a Spinco SW 50.1 rotor at 44,000 rpm. Centrifugation in neutral sucrose gradients was carried out in 5 to 20% linear gradients containing 0.1% sodium dodecyl sulfate, 0.05 M EDTA, 1 m M NaCl. Centrifugation in alkaline sucrose gradients was carried out in 5 to 20% linear gradients containing 0.9 M NaCl and 0.1 M NaOH. Molecular weights were determined by the method of Burgi and Hershey (9).

Enzyme assay. The endonuclease was assayed using ^{3}H-DNA from B. subtilis entrapped in beads of polyacrylamide according to the method of Melgar and Goldthwait (10). The incubation mixture contained 0.9 ml DNA-gel slurry (25 nmoles, S.A. = 1970-1990 cpm/nmole) in 0.05 M Tris, 0.01 M $MnCl_2$, pH 8, and enzyme in a final volume of 1.0 ml. The slurries were incubated with gentle shaking at 37° for 60 min. The reactions were stopped by the addition of 0.1 ml 0.5 M EDTA, pH 8.5. Aliquots of 0.3 ml were added to scintillation vials, diluted to 1.0 ml with water and counted in a liquid scintillation counter after the addition of 5.0 ml of Triton-toluene counting fluid (1 part Triton X-100 to 2 parts toluene, containing 5 gm of 2.5-diphenyl-oxazole [PPO] per liter of toluene). One unit of enzyme is defined as the amount needed to solubilize 1 nmole DNA (nucleotide equivalent) per 60 min. Protein was determined by the method of Warburg and Christian (11) and by the method of Lowry et al. (12) using a bovine serum albumin standard.

RESULTS

Preparation of enzyme. Unless otherwise indicated, all procedures were carried out at 0-4°. Cells of B. subtilis BD170 were grown in VY broth (4) at 37° with vigorous aeration to late log phase, harvested, washed with 0.01 M Tris·HCl, pH 8.0, and stored frozen. The cell-free extract was prepared by thawing 22.5 gm frozen cells (wet weight) with 95 ml 0.01 M Tris·HCl, 1 m M dithiothreitol, pH 8.0, (Buffer A) containing 48 mg of lysozyme followed by incubation at 37° with gentle shaking for 30 min. The high viscosity of this suspension was reduced by sonic oscillation in a Branson Sonifier with two 30-second pulses. The suspension was then centrifuged for 10 min in a Sorvall RC-2B centrifuge at 35,000 x g (extract, 105.5 ml). A freshly prepared solution of 5% streptomycin sulfate (22.2 ml) was added with stirring to the extract. After 10 min the suspension was centrifuged and the supernatant fluid was recovered (streptomycin fraction, 118 ml). The streptomycin fraction was stored frozen in 2 ml aliquots, since it was not stable to repeated freezing and thawing. To aliquots of the streptomycin fraction (2 ml) were added 0.23 ml of 20% (w/w) Dextran 500 and 0.62 ml of 30% (w/w) polyethylene glycol. NaCl (0.525 gm) was added and the mixture stirred for 30 min. After standing for 30 min more, the mixture was centrifuged at 10,000 x g for 10 min. Gly-

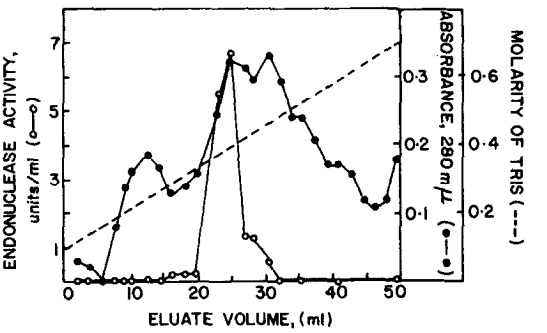

FIG. 1. Chromatography of the streptomycin fraction on DEAE-cellulose after phase partition. Elution was achieved with a linear gradient of Tris. HCl buffer ranging from 0.1 to 0.7 M at pH 7.6. The eluate fractions were assayed for protein and endonuclease activity as described in Methods. Eluate volumes from 19.8 to 28.6 ml were pooled to yield the DEAE fraction.

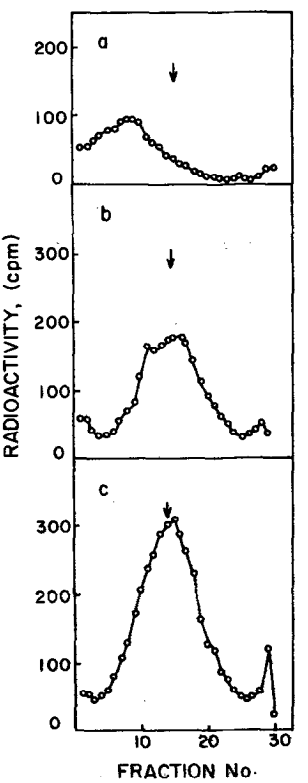

FIG. 2. Detection of endonuclease activity in crude extracts of B.subtilis. Crude extracts were prepared as described in Methods. Reaction mixtures (0.8 ml) contained 0.01 M Tris·HCl, pH 7.6, 1 m M dithiothreitol, 0.2 μg ^{3}H-DNA from B. subtilis (1.4×10^{5} cpm/μg) and 0.2 ml extract and were incubated at 37°. The reactions were stopped by the addition of 0.2 ml 0.25 M EDTA, pH 8.5. Samples of 0.1 ml were layered on neutral 5-20% sucrose gradients (5.0 ml) and centrifuged as described in Methods for 110 min. The direction of sedimentation was from right to left. The position of a marker of ^{14}C-DNA from T7 phage is shown by the arrow.

(a) No treatment of the ^{3}H-DNA; (b) Incubation for 30 min at 37°; (c) Incubation for 60 min at 37°.

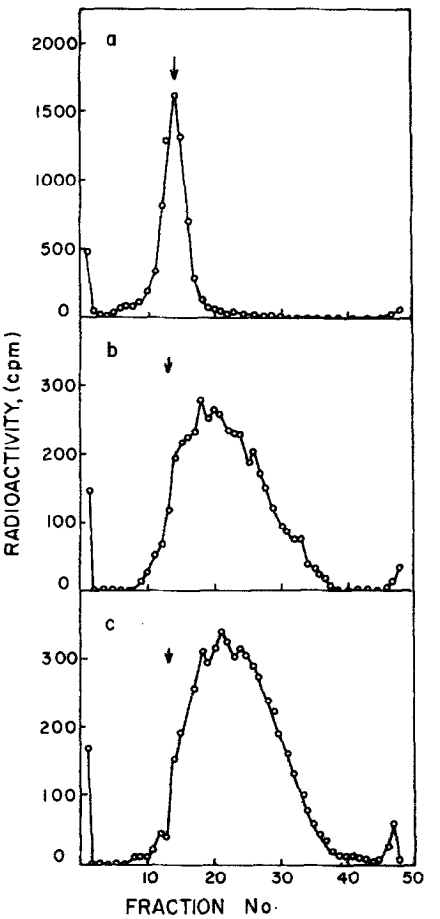

FIG. 3. Sucrose gradient analysis of T7 phage DNA after degradation with the B. subtilis endonuclease. Reaction mixtures (0.45 ml) contained 0.05 M Tris·HCl, pH 8, 0.01 M $MnCl_2$, 2.1 µg ^{3}H-DNA from T7 phage (4.5×10^4 cpm/µg) and 0.1 ml DEAE fraction. Incubations were carried out at 37°. Reactions were stopped by the addition of 0.5 ml 0.5 M EDTA, pH 8.5. Samples of 0.05 ml were added to an equal amount of 0.05 M Tris, 0.05 M EDTA, pH 8, layered on a 5-20% neutral sucrose gradient (5.0 ml) and centrifuged as described in Methods for 2.5 hr. The direction of sedimentation was from right to left. The position of a marker of ^{14}C-DNA from T7 phage is shown by the arrow.

(a) No treatment of the ^{3}H-DNA; (b) Incubation for 60 min; (c) Incubation for 60 min followed by the addition of fresh enzyme, 50 µl, (DEAE fraction) followed by renewed incubation for 30 min more.

cerol was added to the upper phase to a final concentration of 25% (w/v). The upper phase was dialyzed against two changes of 750 ml Buffer A containing 25% (w/v) glycerol (Buffer B). This dialysate was applied to a column of DEAE-cellulose (0.6 cm^2 X 6 cm) which had previously been treated with 0.1 M EDTA, pH 8.5, washed extensively with deionized water and then equilibrated with Buffer B. The column was eluted with 50 ml of a linear 0.1 to 0.7 M Tris·HCl gradient in Buffer B. Fractions of 1.8 ml each were collected and

TABLE I. Requirements for Endonuclease Activity

Additions	nmoles/hr[a]
none	0.4
0.01 M $MgCl_2$	0.3
0.01 M $MnCl_2$[b]	1.2
0.02 M $CaCl_2$	1.0
0.01 M $MnCl_2$ + .25 m M ATP	1.05
1 m M EDTA	0.0

[a]The assay conditions were as described in Methods, except that metal cofactors were omitted or added as indicated above. A dialyzed crude extract was employed for these studies.

[b]Metals were used at concentrations shown to be their optimum.

the peak fractions pooled (DEAE fraction, 8.2 ml). The activity eluted as a single peak from 0.35 - 0.45 M Tris (Fig. 1). The specific activity of the initial extract was 3 units/mg with 4.8 units/ml. The DEAE fraction retained 60-70% of the total activity and had a specific activity of 21-22 units/mg with 6.9 units/ml.

Requirements for the reaction. Endonuclease activity was stimulated by the addition of $MnCl_2$ and $CaCl_2$ but not by the addition of $MgCl_2$ to the reaction mixture (Table I). The addition of EDTA completely abolished the activity of the enzyme. ATP did not stimulate the reaction.

Nature of the product. When crude extracts of B. subtilis were incubated with transforming DNA of B. subtilis, the median molecular weight of the product as shown by neutral sucrose gradient dropped from 70 X 10^6 to 20 X 10^6 (Fig. 2). It was assumed in these calculations that the product was double-stranded. The single-strand molecular weights of the product as measured in alkaline sucrose gradients decreased from 14 X 10^6 to 7 X 10^6. These extracts were also active against native DNA isolated from T7 phage and Salmonella typhimurium.

When samples of the DEAE fraction were incubated with native T7 phage DNA, the median molecular weight of the DNA dropped from 25 X 10^6 to 13 X 10^6 in 60 min (Fig. 3). The product was heterodisperse, however, with molecular weights ranging from 21 X 10^6 to 3 X 10^6. Addition of fresh enzyme at the end of 60 min incubation followed by renewed incubation for 30 min yielded a product with a median molecular weight of 12 X 10^6. Following this incubation there was some gain in products of lighter weight and some loss of the larger fragments, but

TABLE II. Distribution of Endonuclease Activity in B. subtilis

Fraction[a]	Per cent activity	
	Endonuclease[b]	Glucose 6-P dehydrogenase[c]
Protoplast supernatant fraction	61	1.8
Protoplast lysate	35	97.9
Membrane fraction	4	0.3

[a]Cells of BD170 were grown to a concentration of 1 X 10^9 per ml in 50 ml VY broth. The cells were centrifuged, washed and resuspended in 25 ml of 0.6 M sucrose containing 0.05 M Tris·HCl, 0.005 M $MgCl_2$, 0.001 M KCl, pH 8.0, and 12.5 mg lysozyme. This mixture was incubated at 37°. Protoplast formation was followed by phase contrast microscopy and was complete in 30 min. The suspension was centrifuged at 10,000 X g for 15 min and the supernatant solution carefully decanted (protoplast supernatant fraction). The pellet was washed once in the sucrose-Tris buffer, centrifuged and resuspended in 2.5 ml of Buffer A. The pellet was dispersed with a Dounce homogenizer, centrifuged at 35,000 X g for 10 min and the supernatant fraction decanted (protoplast lysate). The pellet, after washing with Buffer A, was suspended in 0.5 ml of this same buffer (membrane fraction).

[b]Endonuclease assays were performed as described in Methods.

[c]Glucose 6-P dehydrogenase was used as a marker for the presence of cytoplasmic enzymes and was assayed by a published procedure (13).

the range of molecular weights was unchanged by the additional incubation. When Mg^{++} was substituted for Mn^{++}, no cleavage of T7 phage DNA was seen by sucrose gradient analysis.

Localization of the endonuclease activity. Protoplasts of B. subtilis were prepared, lysed and each fraction assayed for Mn^{++}-stimulated nuclease activity and for glucose 6-P dehydrogenase activity (Table II). Although the protoplast lysate contained nearly 98% of the glucose 6-P dehydrogenase activity, it contained only 35% of the nuclease activity. Conversely, the protoplast supernatant fraction contained only 2% of the glucose 6-P dehydrogenase activity, but had 61% of the nuclease activity.

DISCUSSION

A Mn^{++}-stimulated endonuclease has been identified in extracts of B. subtilis. The products of the enzymatic reaction, even after prolonged incubation, are of rather large molecular weight, in excess of 3 X 10^6. These features lead us to conclude that this endonuclease has not previously been identified in extracts of B. subtilis. The heat-stable endonuclease of B. subtilis isolated by McCarthy and Nester (6) is stimulated by Mg^{++} and yields a product of approximately 1 X 10^5 daltons.

Haseltine and Fox (7) have reported that intact cells of B. subtilis are capable of cleaving B. subtilis DNA by introducing double-strand breaks into the molecules to yield a product of 10 X 10^6 daltons. It is possible that the Mn^{++}-stimulated endonuclease may be responsible for this activity.

Two biological processes known to occur in B. subtilis would appear to require the function of an endonuclease able to generate double-stranded fragments of approximately 10 X 10^6 daltons from B. subtilis DNA: the production of intermediates in transformation (14, 15), and the production of bacterial DNA fragments during PBSX phage induction (16).

Under conditions of protoplast formation in which 98% of glucose 6-P dehydrogenase activity was retained by the protoplast, 61% of the nuclease activity appeared in the protoplast supernatant fraction. Material released in this fraction is often associated with the periplasmic space of the cell (17). It is of interest that the production of the double-stranded fragments in transformation occurs at the cell surface (8).

The activity of the enzyme is at least predominantly endonucleolytic. After 90 min incubation, only 1% of the radioactivity is non-sedimentable by sucrose gradient centrifugation. The analysis of alkaline sucrose gradients shows that the products of digestion do not seem to be heavily nicked. This argues against a mechanism in which double-strand scissions are produced by an accumulation of randomly placed, single-strand breaks.

ACKNOWLEDGMENTS

We would like to acknowledge valuable discussions with I. Smith, L. Mindich and R. Davidoff-Abelson, and the expert secretarial assistance of A. Howard.

This investigation was supported by National Science Foundation grant No. GB-18146 and Public Health Service grant No. AI-10311 from the National Institute of Allergy and Infectious Diseases, and Career Development Award 1-K4-GM8837 from the National Institutes of Health, awarded to D.D.

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