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Mung Bean Nuclease I. Terminally Directed Hydrolysis of Native DNA†

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ABSTRACT: Under conditions which favor the duplex structure of DNA, mung bean nuclease catalyzes a limited number of double-strand cleavages (probably less than 50) in the interior of native T_7 DNA. However, under conditions which are not as favorable to a tight helical structure, the large duplex polymers previously produced are completely degraded from their termini with a continuous accumulation of mono-, di-, and trinucleotides. The terminally directed activity is an intrinsic property of the enzyme molecule because (1) it is inactivated and reactivated in parallel with the single-strand activity and (2) the two activities coelectrophorese on analytical gels. Kinetic measurements indicate that the apparent K_m for the terminally directed hydrolysis of native DNA is relatively high. The pH optimum for both the hydrolysis of denatured DNA and the terminally directed hydrolysis of native DNA becomes more acidic with increasing salt concentration. The relative preference for single-stranded structures increases as the pH becomes more basic.

The preceding paper in this issue (Kowalski et al., 1976) describes the method of preparation and some of the properties of mung bean nuclease. Nucleases of this type, which were originally isolated and characterized as having a pronounced specificity for denatured as opposed to native DNA, have been used in a variety of experiments that require discrimination between the two forms of DNA. For example, two "single-strand specific" nucleases isolated from *Aspergillus oryzae* (S_1) and *Neurospora crassa* have found widespread application in annealing experiments where nonhybridized DNA is selectively hydrolyzed. Mung bean and S_1 nucleases have been used to specifically degrade the single-stranded ends of λ-phage DNA (Wu and Ghangas, 1975).

More recently, a great deal of interest has been generated in regards to the activity of these enzymes towards essentially native, bihelical DNA. The *N. crassa* endonuclease and the S_1 nuclease have been demonstrated to cleave (1) a specific region(s) in supercoiled DNA (Kato et al., 1973; Beard et al., 1973), (2) sites in bihelical DNA which are sensitive to partial denaturation (Landy et al., 1973), (3) across from single-strand interruptions (nicks) in a variety of DNA in the native form (Germond et al., 1973, 1974; Shishido and Ando, 1975), and (4) the non-hydrogen bonded loops of yeast $tRNA^{Phe}$ (Tenenhouse and Fraser, 1973). In addition, it has been shown that S_1 nuclease can hydrolyze the phosphodiester bonds involved in small mismatched regions (possibly at the level of a single base pair) produced by mutational alteration in the DNA duplex (Shenk et al., 1975). Kroeker and Fairley (1975) have shown that wheat seedling nuclease is capable of cleaving intact bihelical bacteriophage DNA to a limited extent producing double-stranded polymers, $2\text{-}3 \times 10^6$ daltons, which are resistant to further action by the enzyme. Mung bean nuclease has been used on naturally occurring native DNA as a structural probe of superhelical DNA (Wang, 1974) and as a structure–function probe of the binding of repressor protein to λ-phage DNA (Chan and Wells, 1974).

In this paper, we present evidence which demonstrates that under certain conditions native DNA is extensively hydrolyzed by mung bean nuclease. This is accomplished by two modes of hydrolysis. One mode is endonucleolytic and results in the formation of high-molecular-weight duplex fragments. The other mode of hydrolysis involves the complete degradation of these double-stranded fragments from both termini with the continuous accumulation of mono-, di-, and trinucleotides. A model for the mechanism by which mung bean nuclease cat-

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alyzes the hydrolysis of native DNA from the termini is presented and discussed.

Experimental Procedure

DNA. T_7 DNA was obtained by phenol extraction (Thomas and Abelson, 1966) of T_7 phage grown and purified by the method of Grossman (1968). T_7 [^{33}P]DNA was obtained from T_7 phage grown in a low phosphate medium containing 20 μCi of ^{33}P/mg of phosphorus. The original specific activity of the T_7 [^{33}P]DNA was 1.5×10^4 cpm/μg. By sedimentation analysis in alkaline sucrose gradients (5–20%), the T_7 [^{33}P]DNA was shown initially to be intact and estimated to contain fewer than 2 nicks/molecule after storage for 40 days at 4 °C; when stored frozen for 100 days at −20 °C, T_7 [^{33}P]DNA contained fewer than 1 nick/3 molecules. T_7 [^{33}P]DNA was not used after storage for these periods of time. Calf thymus DNA was purchased from Sigma (type V).

Preparation of T_7 DNA Duplex Fragments. Because the large duplex DNA fragments produced by the limited endonucleolytic activity of mung bean nuclease on native T_7 DNA are resistant to further endonucleolytic cleavages by the enzyme (see Results), these polymers provide an ideal substrate for certain experiments involving the end-preferring activity of mung bean nuclease. The DNA duplex fragments were prepared by 18-h incubation at 25 °C of native T_7 DNA or T_7 [^{33}P]DNA (240 μg/ml) with mung bean nuclease (1 unit/ml) in 0.05 M sodium acetate, pH 5.0, containing 0.03 M NaCl, 0.01 mM zinc acetate, 1 mM cysteine, 0.001% Triton X-100. The DNA was precipitated with 2 volumes of ethanol at 4 °C and dissolved in 0.03 M NaCl.

Enzyme Assays. Hydrolysis of T_7 [^{33}P]DNA was monitored using the standard acid-soluble assay (Kowalski et al., 1976) and counting the resulting acid-soluble material in ACS scintillator (product of Amersham Searle). The enzyme reaction was quenched by adding an aliquot to 20 μg of calf thymus DNA at 4 °C, followed by the addition of an equal volume (<0.05 ml) of 0.02 M $La(NO_3)_3$ in 0.2 N HCl. After centrifugation (10 000 rpm, 10 min), the supernatant was added to 10 ml of ACS scintillation fluid and counted in a Packard Tri-carb scintillation counter (discriminator 0-1000, gain 9%).

In some cases, mung bean nuclease activity was determined by measuring the hyperchromicity produced by the hydrolysis of denatured and native DNA. Incubations of enzyme (<0.02 ml) with denatured calf thymus DNA (80 μg/ml) were done at 23 °C in 1.1 ml of sodium acetate buffer of various concentrations and pH values containing 0.01 mM zinc acetate and 1 mM cysteine. The reaction was monitored continuously at 260 nm on a Cary 14 recording spectrophotometer. The increase in absorbance over the first 1–1.5 min of the reaction was linear and was taken as the initial rate of hydrolysis. Incubation of enzyme (<0.02 ml) with native calf thymus DNA (90 μg/ml) was done at 23 °C in 2.5 ml of sodium acetate buffer (of various concentrations and pH values) containing 0.01 mM zinc acetate, 1 mM cysteine, and 0.001% Triton X-100. The reaction was monitored at 260 nm on a Gilford spectrophotometer at regular intervals over 1–4 h, depending on the rate. An initial rapid increase in absorbance (<2% of the total hyperchromicity), which occurred during the first 10–40 min, was subtracted, since it presumably represented hydrolysis of a small amount of single-stranded material. The subsequent slow increase in absorbance was linear up to 50% of the total hyperchromicity and was taken as the initial rate of hydrolysis of native DNA. Both native and denatured DNA were at concentrations sufficient to saturate the enzyme in 0.05 M sodium acetate (pH 5.0).

The standard assay for mung bean nuclease is described in the preceding paper of this issue (Kowalski et al., 1976).

General Methods. Qualitative analysis of the size distribution of DNA hydrolysis products was performed as described by Birnboim (1966) using a Bio-Gel P-60 column (1.2 × 20 cm). The method of Tomlinson and Tener (1963) was used to determine the chain length of short products produced by mung bean nuclease digestion of T_7 DNA duplex fragments.

Gel electrophoresis was performed as described in the preceding paper of this issue (Kowalski et al., 1976). Sedimentation analysis of T_7 DNA in neutral and alkaline sucrose gradients was done as previously described (Kroeker and Fairley, 1975).

Mung bean nuclease was prepared as described in the preceding paper of this issue (Kowalski et al., 1976).

Results

DNase Activity as a Function of pH and Ionic Strength. The activity of the enzyme on denatured calf thymus DNA as a function of pH and concentration of sodium acetate is shown in Figure 1A. Increasing concentrations of sodium acetate cause the pH profiles to shift in the acidic direction. The optimum pH values are 5.3 at 0.025 M sodium acetate, 5.0 at 0.05 M sodium acetate, and 4.7 at 0.10 M sodium acetate. At 0.2 M sodium acetate, the enzyme activity is appreciably reduced throughout the pH range studied. Also shown in Figure 1A (dashed line) for comparison is the low, but measurable rate of hydrolysis of native calf thymus DNA by the enzyme at 0.025 M sodium acetate. This curve is expanded and presented with other data on native DNA in Figure 1B. Similar to the profiles for denatured DNA, the activity–pH profiles for native DNA shift towards acidic pH as the sodium acetate concentration is increased. At a pH optimum for hydrolysis of denatured DNA, the rate of hydrolysis (in terms of the hyperchromicity assay) of denatured DNA is greater than 1000 times that for native DNA. As the pH is lowered, however, the rates for denatured and native DNA converge. At 0.025 M sodium acetate, pH 4.1, the ratio of the rates is only 3 (denatured/native).

Limited Endonucleolytic Cleavage of Native T_7 DNA. Although mung bean nuclease exhibits a vast preference for single-stranded DNA, when appreciable amounts of enzyme are incubated with native T_7 DNA under appropriate conditions (e.g., 0.1 M salt, pH 5.0, 22 °C) a limited number of sites in the DNA are cleaved. Table I shows a time course for such a reaction. As seen from the table, the weight-average molecular weight of the products, as determined from neutral sucrose gradient analysis, approaches a limit of approximately 1×10^6 after 28 h of incubation. The enzyme activity present after 28 h of incubation (as determined by the standard assay with denatured DNA) was 100% of the starting activity. Sucrose gradient analysis of a control reaction run without enzyme showed that after 28 h less than 0.2% of the DNA was acid soluble and that the bulk of the T_7 DNA was intact. Alkaline sucrose gradient analysis of the products formed after 28 h of incubation with the enzyme yielded a molecular weight of 0.5×10^6, indicating that the 28-h fragments contained few, if any, single-strand interruptions. During the time course, a small amount of acid-soluble products accumulated (<3%). At 28 h, the reaction conditions were modified by diluting the reaction mixture threefold with water (lowering salt concentration) and by raising the temperature to 30 °C. Under these conditions, which are less favorable to the structural stability

of the DNA duplex, a rapid increase in the production of acid-soluble products occurred (in spite of a threefold dilution of the enzyme), as shown in the table. This rapid rate of acid-soluble product formation was accompanied by only a slight decrease in the weight-average molecular weight of the large DNA fragments, indicating that these cleavages occur predominantly at the ends of the DNA. This effect can also be seen from the sucrose gradient profiles plotted in Figure 2. Panel C shows the sedimentation profile of the products present after 28 h of incubation. After an additional 80 min of incubation under the modified conditions, the major products sedimented only slightly slower, while the amount of small products increased (panel D).

Hydrolysis at the Ends of Native DNA

Size Distribution of the Products Resulting from the Hydrolysis of Native DNA. The preceding experiment, in which native T_7 DNA was incubated with mung bean nuclease, suggests that most of the phosphodiester bonds are cleaved near the ends of the DNA duplex, producing small products following an initial limited endonucleolytic action by the enzyme. In order to further characterize this apparent end-preferring mode of hydrolysis of native DNA, the size distribution of products produced during intermediate levels of hydrolysis of native calf thymus DNA was investigated using the method of Birnboim (1966). The profile of products eluting from the molecular sieving column shown in Figure 3A is consistent with an end-preferring mode of hydrolysis, i.e., the reduction in the amount of large DNA is accompanied by an increase in the amount of small products without the appearance of products of an intermediate size. The three profiles shown in Figure 3A represent reactions of native calf thymus DNA with mung bean nuclease which had reached 0, 25, and 61% acid-soluble products; other stages of the reaction gave similar profiles. In contrast, the hydrolysis of denatured calf thymus DNA proceeds in a predominantly endonucleolytic fashion s illustrated in Figure 3B. Thus, when the reaction was terminated at the 21 and 36% level of acid-soluble products, a wide distribution of intermediate sized products was evident.

The small products produced by the terminally-directed hydrolysis of native DNA by mung bean nuclease were further characterized by separating the products according to their chain length on a DEAE[1]-cellulose column by the method of Tomlinson and Tenner (1963). The column was calibrated with mono-, di-, and trinucleotides bearing 5′-phosphoryl groups. The duplex T_7 DNA fragments (produced as described under Experimental Procedures) were digested with mung bean nuclease until 6.1% were in the acid-soluble form and placed on a DEAE-cellulose column. Of the three peaks observed, 55% of the eluting DNA appeared in the mononucleotide peak, 39% in the dinucleotide peak, and only 4% was found as trinucleotides. It was concluded that the products of the terminally directed hydrolysis of native DNA possess 5′-phosphoryl groups because: (1) greater than 99% of acid-soluble activity applied to the column was recovered in products which coeluted with 5′-terminated standards and (2) the ω-monophosphatase activity of mung bean nuclease would have dephosphorylated 3′-phosphoryl-terminated products. The endonucleolytic activity of mung bean nuclease on denatured DNA also results in the production of 5′-phosphoryl-terminated products (Mikulski and Laskowski, 1970).

Rates of Hydrolysis of Native T_7 DNA. Figure 4 shows a time course for the release of acid-soluble products from native

[1] Abbreviation used: DEAE, diethylaminoethyl.

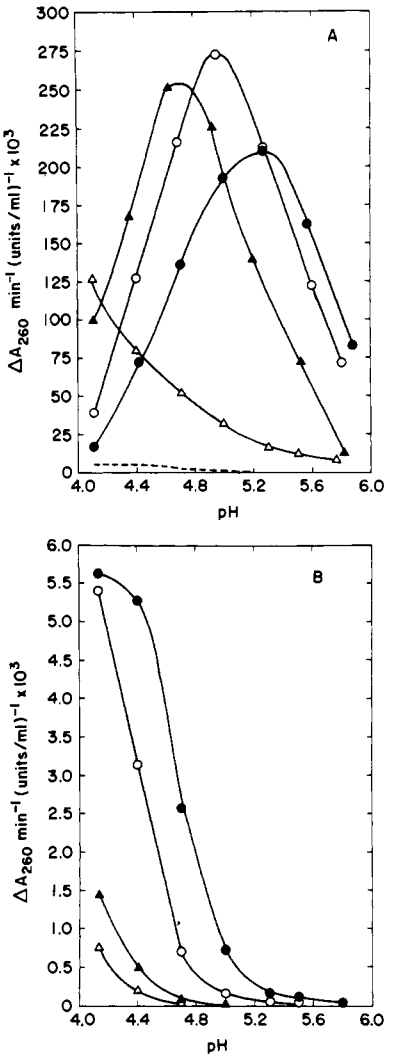

FIGURE 1: Effect of pH and ionic strength on the rates of hydrolysis of denatured DNA (A) and native DNA (B) by mung bean nuclease. Shown for comparison in A (dashed line) is the rate of hydrolysis of native DNA in 0.025 M sodium acetate. The concentrations of sodium acetate are: (●) 0.025 M; (○) 0.05 M; (▲) 0.10 M; (△) 0.20 M. Sodium acetate solutions were titrated to the indicated pH with acetic acid. Initial rates, determined at 23 °C by the hyperchromicity assay (see Experimental Procedures) are normalized for the concentration of enzyme activity (units/ml) which was determined by the acid-soluble assay against denatured DNA. Notice that the ordinates of A and B differ by a factor of 50.

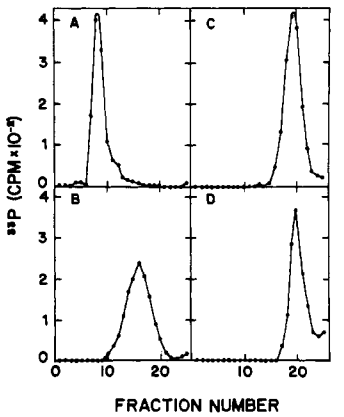

FIGURE 2: Neutral sucrose gradient sedimentation profiles of native T_7 DNA and hydrolysis products produced by the action of mung bean nuclease. Experimental conditions are given in Table I and the gradient profiles shown are from the following time points: (A) 0 time; (B) 12 h; (C) 28 h; (D) 28 h + 80 min.

TABLE I: Time Course for the Early Phase of Native T_7 DNA Hydrolysis by Mung Bean Nuclease.[a]

	Original Reaction Conditions						Modified Reaction Conditions		
Time (h)	0	6	12	22	25	28	28.33	28.83	29.33
% acid soluble	<0.2	<0.2	0.9	1.8	2.1		2.9	5.1	7.9
9.9									
Mol wt ($\times 10^{-6}$)	26	5.8	4.2	1.8	1.5	1.4	1.0	0.9	0.8

[a] The original reaction conditions were 0.11 ml of T_7 [^{33}P]DNA (480 μg/ml, 800 cpm/μg in 0.01 M KCl, 0.01 M Tris-HCl, pH 7.1) and 0.11 ml of 0.1 M sodium acetate, pH 5.0, 0.08 M NaCl, 0.02 mM zinc acetate, 2 mM 2-mercaptoethanol, 0.002% Triton X-100. The reaction was initiated with 0.9 units of mung bean nuclease (in 1.8 μl containing 50% glycerol) and run at 22 °C. At 28 h, the reaction conditions were modified by diluting threefold with water and raising the temperature to 30 °C. An aliquot (0.01 ml) was removed at the times indicated and the percentage of acid-soluble DNA was determined as described under Experimental Procedures. Also, at the times indicated, 0.01 ml was removed and diluted to 0.1 ml to attain a final Tris-HCl concentration of 0.02 M, pH 8.1. Neutral sucrose gradient sedimentation was performed with these samples and molecular weight were calculated as previously described (Kroeker and Fairley, 1975). Some of the profiles resulting from sedimentation in a sucrose gradient are shown in Figure 2.

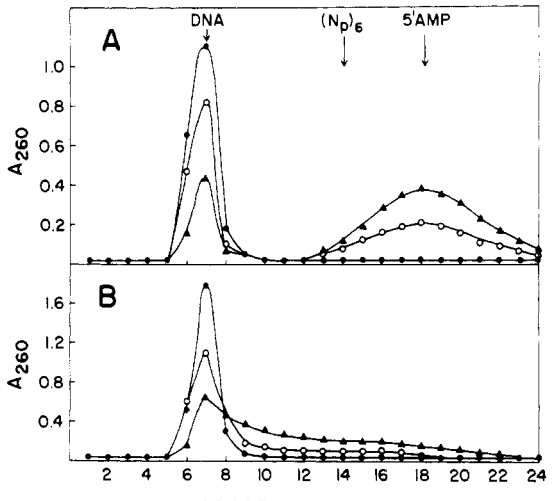

FIGURE 3: Size distribution of the products at various stages in the digestion of native (A) and denatured (B) calf thymus DNA by mung bean nuclease. Reaction mixtures (at 37 °C) consisted of 0.25 mg/ml of DNA, 0.05 M sodium acetate (pH 5.0), 0.01 mM zinc acetate, 1.0 mM 2-mercaptoethanol, and 0.001% Triton X-100. The reactions with native and denatured DNA contained 1 and 0.065 units/ml of mung bean nuclease, respectively. Aliquots (0.5 ml) were removed from the reaction containing native DNA at 0 (●), 3 (○), and 6 h (▲) and terminated by adding 0.1 ml of 1 M Tris-HCl, pH 8. Likewise, aliquots were removed from the reaction containing denatured DNA at 0 (●), 4 (○), and 6 min (▲). The samples were then placed on a Bio-Gel P-60 column (1.1 × 20 cm) which was equilibrated with 0.1 M Tris-HCl, pH 8. The flow rate was maintained at 1 ml/min and 1.0-ml fractions were collected. The positions of DNA markers used to calibrate the column are indicated by arrows.

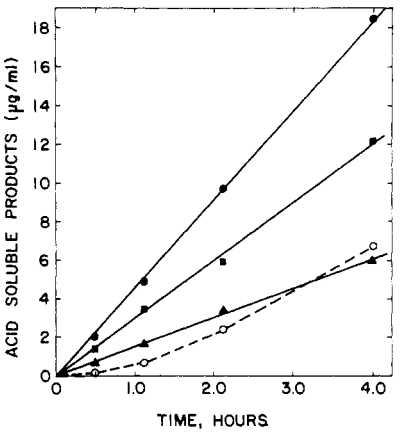

FIGURE 4: Time course for the liberation of acid-soluble products from native T_7 [^{33}P]DNA. Reactions (0.3 ml) were conducted at 30 °C and contained 8–24 μg/ml of T_7 [^{33}P]DNA fragments, 0.015 M NaCl, 0.02 M sodium acetate (pH 5.0), 0.01 mM zinc acetate, 1.0 mM 2-mercaptoethanol, 0.001% Triton X-100, 3.3 units/ml of mung bean nuclease. The assay procedure is described under Experimental Procedures. (●) 24 μg of DNA/ml; (■) 16 μg of DNA/ml; (▲) 8 μg of DNA/ml; (—) native T_7 [^{33}P]DNA fragments (see Experimental Procedures); (- - -) intact native T_7 [^{33}P]DNA (16 μg/ml).

T_7 DNA (dashed line). The rate of products released increases with time. At 0.5 h, only 0.9% of the DNA is acid soluble, whereas at 4 h, 42% of the DNA is acid-soluble. The figure also shows the time course of reactions in which native T_7 DNA fragments (prepared as described under Experimental Procedures) were used as substrates. As can be seen from Figure 4, the hydrolysis of these substrates proceeds with a linear time course during the 4 h of incubation. After this period of time, 75% of the substrates has been converted to the acid-soluble form. When the concentrations of the native duplex fragments were 8, 16, and 24 μg/ml, the rates observed were 1.6, 3.1, and 4.6 μg of acid soluble DNA ml^{-1} h^{-1}, respectively. Since the rate of hydrolysis is directly proportional to the substrate concentration, these concentrations of substrate appear to be well below the apparent K_m for this reaction. The rate of products released from intact T_7 DNA (16 μg/ml), following an initial lag period, approaches the same rate as the reaction containing the duplex fragment substrates of the same concentration. This behavior for intact duplex T_7 DNA is consistent with a predominantly end-preferring mode of hydrolysis by mung bean nuclease following an initial small number of endonucleolytic cleavages.

Physical Association of the End-Preferring Activity with Mung Bean Nuclease. The question naturally arises as to whether the terminally directed activity on native DNA is an intrinsic property of the mung bean nuclease or a trace contaminating activity that copurifies with mung bean nuclease. Two types of experiments were designed to resolve these possibilities. In the first experiment, 80% of the mung bean nuclease activity (assayed on denatured DNA) was inactivated by dialyzing the enzyme against 0.05 M sodium acetate, pH 5.0. This inactivated enzyme hydrolyzed native T_7 DNA fragments at a rate 20% of that observed for the fully active enzyme. However, upon addition of 0.1 mM zinc acetate, 1.0 mM cysteine, and 0.001% Triton X-100 (all in final concentration) to the reaction mixture, the rate approached that of the fully active enzyme within 20 min. This inactivation and reactivation of the end-preferring activity of mung bean nuclease on native DNA parallels that on denatured DNA (Kowalski et al., 1976). In the second experiment, purified mung bean nuclease was electrophoresed in analytical polyacrylamide gels. The activity on denatured DNA, as well as

on native T_7 DNA fragments, was measured after eluting segments sliced from the gel. Similar electrophoresis patterns were obtained for both activities.

Discussion

The mung bean nuclease, like the S_1 (Vogt, 1973), P_1 (Fujimoto et al., 1974), and wheat seedling nucleases (Hanson and Fairley, 1969), has an acidic pH optimum with denatured DNA as the substrate. The observation that the enzyme activity on native DNA falls off more rapidly with increasing pH than the activity on denatured DNA has practical consequences concerning the use of mung bean nuclease, in particular, and perhaps single-strand specific nucleases, in general, for the selective degradation of single-stranded DNA in the presence of native DNA. In order to amplify the activity on single- over double-stranded DNA, reactions should be carried out at 0.3–0.5 pH units above the pH optimum for a given salt concentration. This pH is a compromise between the desired specificity and the amount of enzyme activity. Preliminary results using the acid-soluble assay with native and denatured T_7 [^{33}P]DNA as substrates indicate that the preference of mung bean nuclease for single- over double-stranded DNA may be exaggerated further at even higher pH values. Conditions are being sought in which the activity responsible for the production of large amounts of acid-soluble material from native DNA can be suppressed without affecting the other activities.

The limited endonucleolytic activity of mung bean nuclease on native T_7 DNA suggests the presence of a small number of specific, susceptible sites on the T_7 genome to the action of the enzyme. This observation has been made for a variety of DNA that were cleaved to a limited extent by another "single-strand specific" enzyme, wheat seedling nuclease (Kroeker and Fairley, 1975). The physical and chemical nature of these sites, as well as the mechanism in which they are recognized and cleaved by mung bean nuclease, are currently being investigated. In this work, the endonuclease-resistant T_7 duplex fragments provide a convenient substrate to study the end-preferring mode of hydrolysis of native DNA.

Taken together, the results presented here suggest that the terminally directed activity of mung bean nuclease on native DNA is simply a manifestation of the single-strand specific endonuclease activity at the loose or partially denatured ends of native DNA. This denaturation need not be extensive, since small single-stranded areas formed even transiently at the ends of the duplex could be rapidly and efficiently removed by the enzyme. Continuous repetition of this process of local unwinding or loosening of the ends of the duplex and subsequent digestion by the single-strand specific endonuclease would give the appearance of end-preferring activity. The ease of denaturation of native DNA from the ends relative to internal portions is well documented. In duplex DNA of molecular weight around several hundred thousand, unwinding occurs almost exclusively from the ends (Crothers et al., 1965). A possible intermediate form of the substrate for the terminally directed hydrolysis of native DNA could entail the transient formation of protruding single-stranded ends. That such an intermediate form could be readily degraded by mung bean nuclease has been demonstrated by Wu and Ghangas (1975) using λ DNA which is known to have protruding single-stranded ends. In addition, the possibility still remains that the mung bean enzyme initially produces products from the ends of native DNA which are larger than trinucleotides but are rapidly degraded to the tri- to mononucleotides observed at various stages of the reaction.

The rate of hydrolysis of native DNA relative to denatured DNA by the mung bean enzyme is low presumably, since, in the case of the end-preferring activity, the concentration of the true substrate (partially denatured ends of DNA) is only a fraction of the total substrate concentration. Conditions that favor loosening or partial unwinding from the ends of the duplex (low pH, low salt, high temperature) increase the rate of digestion by increasing the concentration of the true substrate (Figure 1, 0.025 M sodium acetate, pH 4.1).

Since hydrolysis from the ends of the duplex DNA by the single-strand specific nuclease from mung beans is primarily determined by conditions which affect the structure of the substrate, the end-preferring activity observed here is likely to be attributable to all single-strand specific endonucleases. In fact, one preparation of S_1 nuclease has been suspected of being contaminated with an exonuclease-like activity on native DNA (Shishido and Ando, 1975). Another preparation of S_1 nuclease has been described as having a "nibbling effect" on native DNA (Shenk et al., 1975). Detailed knowledge of reaction conditions affecting the ability of the single-strand specific nucleases to recognize differences in the ordered structure of DNA should permit more advantageous usage o these enzymes in experiments involving native DNA.

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