DEGRADATION OF DUPLEX DNA BY S_1 NUCLEASE FROM ASPERGILLUS

Tom St. John, Jerry D. Johnson,[1] and James Bonner[2]

Division of Biology, California Institute of Technology
Pasadena, California 91109

Received January 14,1974

Summary: A nuclease from Aspergillus which selectively degrades single-stranded polynucleotides, S_1, has been purified by DEAE-cellulose and Sephadex G-100 chromatography. The purified enzyme is still capable of degrading both ØX174 RF and native calf thymus DNA. The hydrolysis of double-stranded DNA appears to be endonucleolytic as is single-strand cleavage.

Enzymes which preferentially degrade single-stranded DNA (S_1 nucleases) have recently been employed as tools in nucleic acid reassociation experiments (1, 2, 3). One such enzyme has been prepared from Aspergillus oryzae. It is inexpensive, can be extensively purified by DEAE-cellulose chromatography (1), and retains activity for several months if stored in liquid N_2 (4). These properties would seem to make it the enzyme of choice for routine use in hybridization experiments. We have analyzed the substrate specificity of this enzyme with regard to single- and double-stranded polynucleotides to determine whether the extent of hydrolysis with double-stranded material is sufficient to introduce spurious results in hybridization experiments.

METHODS

S_1 nuclease was isolated from commercial Takadiastase powder by DEAE-cellulose chromatography as described by Sutton (1971). Activity assays included 0.03 M $NaC_2H_3O_2$ (pH 4.5), 0.01 M NaCl, 0.03 mM Zn_2SO_4, 40 µg/ml native or heat-denatured DNA, and 5 µg/ml of enzyme protein. Reaction mixtures were incubated at 37°C. One-ml aliquots were withdrawn and hydrolysis terminated by addition of 1/10 volume of 77% trichloroacetic acid followed by 50 µg

[1]Present address: Department of Chemistry, University of Colorado, Colorado Springs, Colorado 80907.

[2]To whom correspondence should be addressed.

carrier protein, bovine serum albumin. The mixture was stored on ice for 15-30 min. For assays of UV absorbance, the precipitate was removed by centrifugation and supernatant monitored at 260 and 320 nm. Appropriate blanks were prepared from duplicate reaction mixtures lacking only DNA. The fraction of input DNA digested by the enzyme is determined by treating an aliquot of the reaction mixture for 30 min at 100°C in 0.5 M perchloric acid (PCA). The amount of absorbance rendered acid-soluble by the PCA treatment is taken as 100%. For radioactivity determinations, the trichloroacetic acid precipitate was trapped on a nitrocellulose filter, oven dried, and counted in a liquid scintillation spectrometer. To assay double-strand degradation, ØX174 RF DNA (gift of Lloyd Smith) was incubated in a standard reaction mixture, spread onto grids from a basic protein film in formamide (5), and examined in the electron microscope. Analytical ultracentrifugation was done in a Spinco Model E equipped with an ultraviolet scanner.

RESULTS

The activity of S_1 nuclease recovered from DEAE-cellulose chromatography (DEAE-enzyme) was assayed with native (DS) and heat-denatured (SS) calf thymus DNA (Table 1). The enzyme is clearly much more active with denatured DNA but does solubilize some native DNA. Its activity on ØX174 RF DNA confirms that the enzyme is indeed capable of degrading intact duplex. Molecular length measurements of linear ØX174 DNA fragments found in these reaction mixtures shows that S_1 continues to degrade double-stranded DNA after circle opening.

Electrophoretic analysis of the DEAE-enzyme fraction on SDS-containing polyacrylamide gels indicated the presence of a heterogeneous mixture of protein components (Fig. 1). In an attempt to further purify the S_1 fraction and, if possible, eliminate activity with double-stranded DNA, the DEAE-enzyme was chromatographed on a 2.5 x 80 cm column of Sephadex G-100 equilibrated with assay buffer plus 10% (v/v) glycerol. Three major peaks of protein were eluted; S_1 activity coincided with a protein peak eluted at about 0.8 column volumes (Fig. 2). SDS-acrylamide gel analysis of this fraction showed a single major

TABLE I

Hydrolysis of native (DS) and heat-denatured (SS) calf thymus DNA and double-stranded ØX174 DNA by S_1 nuclease. Reaction conditions and assay methods are as described in METHODS.

Incubation time, minutes	% DNA hydrolyzed					
	DEAE-Enzyme			G-100-Enzyme		
	SS	DS	*ØX174 RF	SS	DS	*ØX174 RF
2	35	1.5	N.D.	42	-0.9	39
5	68	3.5	50	69	0.1	72
10	81	2.0	71	81	-3.5	87
20	90	3.0	88	87	0.9	96
40	94	3.0	N.D.	90	-2.3	98

*% of circles converted to linears; preparation contained 4.5% linears at t=0; 5.7% linears after 20 min incubation without S_1.

N.D. = not determined.

component, molecular weight ca. 52,000 daltons. This represents >90% of the protein. Minor bands with molecular weights of 23-30,000 daltons were also visible (Fig. 1). Activity assays of the G-100 fraction (G-100-enzyme) are presented in Table I. The apparent activity of G-100-enzyme with native calf thymus DNA was reduced below levels of detection. However, no reduction in the rate of ØX174 RF cleavage could be observed. Relative to hydrolysis of single-stranded calf thymus DNA, the rate of ØX174 RF circle opening actually increased somewhat.

The question of whether the double-strand activity is endo- or exonucleolytic was approached by measuring release of acid-soluble products from native calf thymus DNA, $\bar{M}_w$ 5-10 x 10^6 (Fig. 3). After 30 min incubation essentially no acid-soluble products were found. Further incubation resulted in a moderately rapid release of acid-soluble products. Supplementing the reaction mixture with a second aliquot of S_1 nuclease after 30 min increases the rate of this

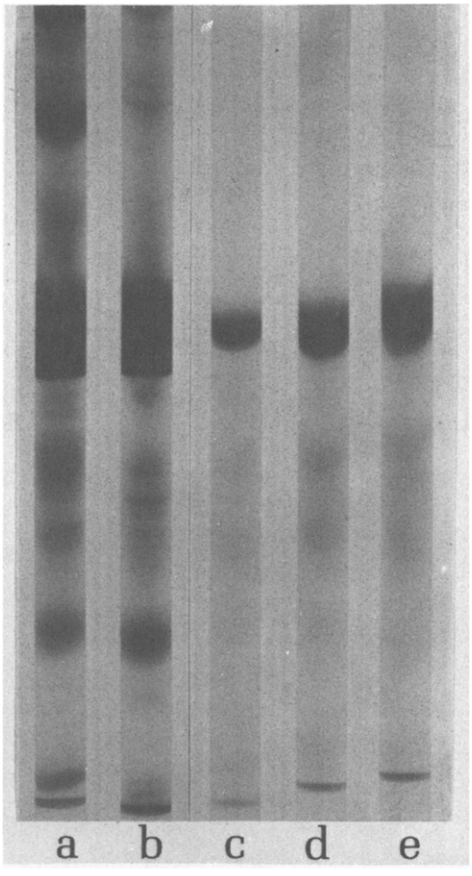

Figure 1. SDS-acrylamide gel analysis (6) of S_1 preparations. (a) 50 µg crude powder prior to DEAE-cellulose chromatography; (b) 50 µg DEAE-enzyme; (c) 10 µg G-100-enzyme; (d) 20 µg G-100-enzyme; (e) 40 µg G-100-enzyme.

digestion. Addition of single-stranded calf thymus DNA after 30 min of incubation shows the initial S_1 activity has not been inactivated. Addition of double-stranded DNA has no effect. The observed lag in appearance of acid-soluble products from high molecular weight DNA is characteristic of endonuclease activity (7). The action of S_1 nuclease on linear ØX174 RF DNA fragments also suggests that the activity of this enzyme on duplex DNA is endonucleolytic (Table II). The measured decrease in length of ØX174 RF DNA subsequent to ring opening follows that predicted by equations which describe random endonucleolytic cleavage (8). A mean length for linear fragments can be calculated

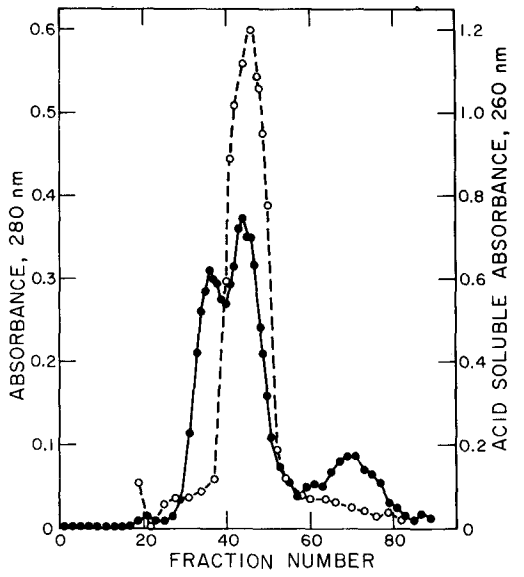

Figure 2. G-100 chromatography of DEAE-enzyme as described in text; —●—●—, absorbance at 280 nm; ---o---o---, TCA-soluble absorbance at 260 nm from assays using 10-µl aliquots of eluant incubated 10 min with denatured calf thymus DNA as substrate in the standard assay (see METHODS).

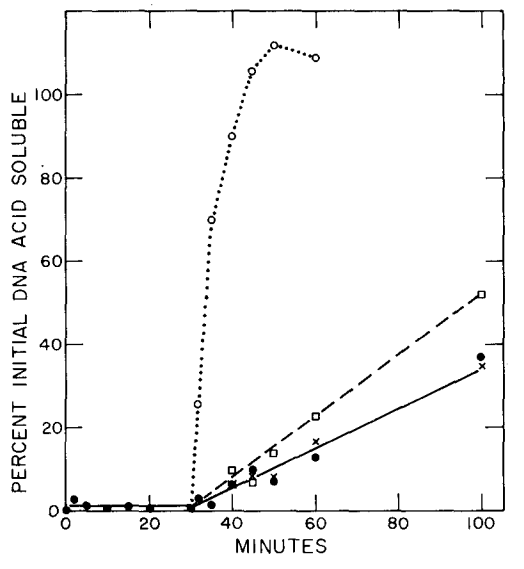

Figure 3. Action of S_1 on native calf thymus DNA. A standard reaction mixture (see METHODS) containing double-stranded DNA of M_w 5-10 x 10^6 daltons was incubated for 30 minutes then split into 4 equal portions. Aliquots then received (a) no additions, —●—●—; (b) an equivalent of heat-denatured DNA, ...o...o...; (c) an equivalent of untreated DNA, ---x---x---; or (d) an equivalent of enzyme, ---□---□---.

TABLE II

Digestion of linear ØX174 RF DNA fragments by S_1 nuclease

Incubation time, minutes	Mean Fragment Length	
	Measured	Calculated
0	100* (39)	100**
1	86.6 (21)	87.8
3	74.3 (22)	81.6
5	62.8 (54)	71.1
10	44.6 (23)	57.2
20	50.8 (33)	45.1
40	41.7 (61)	33.9

* Length of closed circular ØX174 RF DNA is taken as 100. Numbers in parentheses are the number of DNA fragments measured.

** Calculated using the equations of Radloff and Vinograd (8) for random scission and based on the fraction of intact circles (0 hits) remaining at the indicated incubation times.

using the fraction of intact circles remaining to determine the average number of hits per molecule (Table II).

In an attempt to determine whether the digestion of linear ØX174 RF DNA fragments results from a double-strand clipping activity of the enzyme, as suggested by the kinetic experiments, or by an accumulation of single-strand nicks, ØX174 RF DNA was digested with S_1 nuclease for 20 min under standard assay conditions then examined by analytical centrifugation. This digestion time is sufficient to convert >90% of the circular molecules to linears (Table I). At neutral pH, the DNA in the digest sedimented as a single, symmetric 16S peak corresponding to a molecular weight of 3.4×10^6 daltons. Under denaturing conditions (pH 13), a 17.5S peak, molecular weight 3.5×10^5 daltons, contained about 60% of the DNA. The remainder formed a trailing edge on the peak. Since the average molecular

weight of the denatured DNA strands is considerably less than half the double-strand value, they must have been nicks in the duplex. The data indicate an average of about 2 nicks per strand; however, the peak has a large trailing edge so the actual value may be somewhat higher. Alkaline sedimentation of untreated ØX174 RF DNA showed a symmetric peak with a molecular weight of 2×10^6. This demonstrates degradation of double-stranded DNA can occur by an accumulation of single-strand nicks in the duplex.

CONCLUSIONS

The evidence presented indicates that S_1 nuclease purified by DEAE-cellulose and Sephadex G-100 chromatography has the ability to degrade double-stranded DNA in addition to its activity with single-stranded polynucleotides. The possibility that the observed double-strand activity is the property of a separate enzyme which copurifies with S_1 has not been rigorously excluded. Degradation of native DNA seems to be endonucleolytic. This is contrary to the suggestion of Sutton (1); however, the sensitivity of his assay is uncertain. Hydrolysis of single-stranded material has been clearly shown to be endonucleolytic (1). Sedimentation analyses of ØX174 RF digests indicate that the observed degradation of double-stranded material can occur by an accumulation of single-strand nicks. The kinetic data in Table II suggest but do not prove that the enzyme may also be capable of making double-strand scissions.

It has recently been suggested that S_1 nuclease attacks double-stranded DNA in regions where single-strands occur due to local melting or at free ends adjacent to single-strand nicks in the duplex (9). Our results are consistent with this proposal.

The foregoing observations suggest that caution be exercised when S_1 nuclease is used to measure duplex formation in nucleic acid hybridization experiments. Double-strand clips may render short regions of duplex acid-soluble. Also, use of S_1 nuclease to measure the size of duplex regions may result in underestimates of their true length. The enzyme is clearly very selective for single-stranded DNA; however, it does degrade duplex material at a finite rate. This problem

will be especially critical in studies of short repetitive sequences or length distributions in the presence of large amounts of single-stranded material.

Acknowledgments. This work was supported by U.S. Public Health Service grant no. GM-13762 to J.B. and GM-51175 to J.D.J.

REFERENCES

1. Sutton, W. D. (1971). Biochim. Biophys. Acta 240, 522.
2. Britten, R. J., Graham, D. E. and Neufeld, B. Methods in Enzymology, in press, 1973.
3. Vogt, V. M. (1973). Eur. J. Biochem. 33, 192.
4. St. John, T. S. and Johnson, J. D., unpublished observations.
5. Davis, R. W., Simon, M. and Davidson, N. (1971). Methods in Enzymology, 21, 413.
6. King, J. and Laemmli, U. K. (1971). J. Mol. Biol. 62, 467.
7. Lehman, I. R. and Richardson, C. C. (1964). J. Biol. Chem. 239, 233.
8. Radloff, R. and Vinograd, J. (1971). Biochim. Biophys. Acta 247, 207.
9. Germond, J. E., Vogt, V. M. and Hirt, B. (1973). Experientia 29, 771.