11

Endonucleases Specific for Single-Stranded Polynucleotides

I. ROBERT LEHMAN

I. Introduction

Endonucleases with a high degree of specificity for polynucleotides that lack an ordered structure have been isolated from a variety of fungi, including *Neurospora crassa* (*1, 2*), *Aspergillus oryzae* (*3, 4*), *Penicillium cit-*

1. S. Linn and I. R. Lehman, *JBC* **240**, 1287 (1965).
2. S. Linn, and I. R. Lehman, *JBC* **240**, 1294 (1965).
3. T. Ando, *BBA* **114**, 158 (1966).
4. V. M. Vogt, *EJB* **33**, 192 (1973).

THE ENZYMES, Vol. XIV

ISBN 0-12-122714-6

rinum P (*5*, *6*), and *Ustilago maydis* (*7*, *8*). Similar enzymes have been purified from mung bean sprouts (*9*, *10*) and wheat seedlings (*11*). Because of their ability to discriminate between single- and double-stranded polynucleotides, these enzymes have become exceedingly useful as reagents for the analysis of nucleic acid structure. Two of these nucleases, from *N. crassa* and *U. maydis*, appear to be required for DNA repair and recombination *in vivo*. A summary of the properties of the six single-stranded polynucleotide specific endonucleases is given in Table I. The enzymes to be considered in detail are those from *N. crassa*, *Aspergillus oryzae*, and mung bean.

II. *Neurospora crassa* Endonuclease

A. Physical Properties

The *N. crassa* endonuclease was the first of the single-strand-specific endonucleases to be discovered (*1*, *2*). Though purified extensively from both conidia and stationary phase mycelia (approximately 10,000 fold), the enzyme has not yet been obtained in homogeneous form. As judged by sedimentation in sucrose density gradients, it has a sedimentation coefficient, $s_{20,w}$, of 3.7 and a molecular weight (assuming a globular structure) of 55,000 (*12*).

The important feature of the enzyme, and that which has made it a useful reagent, is its high degree of selectivity for single-stranded DNA or single-stranded regions within duplex DNA. Although the 10,000-fold purified enzyme preparation attacks native DNA at 2% the rate of denatured DNA, it is clear that hydrolysis of native DNA is due largely to the activity of a contaminating nuclease, which can be selectively inactivated by incubation at 55°, or by brief exposure to thiols. After such treatment, native DNA is attacked at less than 0.1% the rate found with denatured DNA.

The partially purified enzyme is active under a variety of conditions including temperatures ranging from 25° to 65°, pHs ranging from 6 to 9 in

5. M. Fujimoto, A. Kuninaka, and H. Yoshino, *Agr. Biol. Chem.* **38**, 777 (1974).
6. M. Fujimoto, A. Kuninaka, and H. Yoshino, *Agr. Biol. Chem.* **38**, 785 (1974).
7. W. K. Holloman, and R. Holliday, *JBC* **248**, 8107 (1973).
8. W. K. Holloman, *JBC* **248**, 8114 (1973).
9. D. Kowalski, W. D. Kroeker, and M. Laskowski, Sr. *Biochemistry*, **15**, 4457 (1976).
10. W. D. Kroeker, D. Kowalski, and M. Laskowski, Sr. *Biochemistry*, **15**, 4463 (1976).
11. W. D. Kroeker and J. L. Fairley, *JBC* **250**, 3773 (1975).
12. S. Linn, "Methods in Enzymology," Vol. XII, Part A, p. 247, 1967.

TABLE I

PROPERTIES OF SINGLE-STRANDED POLYNUCLEOTIDE-SPECIFIC ENDONUCLEASES

Enzyme source	Degree of purity (%)	Molecular weight	Ratio DNase/RNase	Final products of hydrolysis	pH optimum	Divalent cations	Inhibitors	Comments
N. crassa (*1*, *2*)[a]	Unknown	55,000	1	>90% Nucleoside 5′-phosphates	7.0–9.0	Zn^{2+} Co^{2+} Mg^{2+}	2-Mercaptoethanol, potassium phosphate, ATP	Preferential attack at G and dG residues
U. maydis (*7*, *8*)	Unknown	42,000	3	>90% Nucleoside 5′-phosphates	8.0	Mg^{2+} Ca^{2+} Co^{2+} Zn^{2+}	2-Mercaptoethanol, potassium phosphate, ATP	Preferential attack at G and dG residues
A. oroyzae (*3*, *4*)	>90	32,000	5	>90% Nucleoside 5′-phosphates	4.0–4.3	Zn^{2+} Co^{2+}	—	—
P. citrinum (*5*, *6*)	>90	Unknown	0.7	>90% Nucleoside 5′-phosphates	5.0	Zn^{2+}	—	Possesses 3′-nucleotidase activity; cold labile
Mung bean (*9*, *10*)	>90	39,000	1.2	>90% Nucleoside 5′-phosphates	5.0	Zn^{2+}	—	Possesses 3′-nucleotidase activity
Wheat seedlings (*11*)	>90	43,000	2	>90% Nucleoside 5′-phosphates	4.8–5.5	Zn^{2+} required for stabilization	Potassium phosphate, NaF	Possesses 3′-nucleotidase activity; preferential attack at A and dA residues

[a] Numbers in parentheses are reference numbers.

the presence of a variety of divalent cations, and at ionic strengths ranging from 0.03 to 0.20. Although the enzyme shows some activity in the absence of added divalent cations (20% of maximal), it is strongly inhibited (95%) by EDTA at 0.1 m*M*. This inhibition cannot be overcome by an excess of Mg^{2+}, but is specifically reversed by Co^{2+}. The enzyme is also inhibited to some extent by potassium phosphate (80% inhibition at 33 m*M*) and ATP (50% inhibition at 0.5 m*M*) (*13*).

The *N. crassa* endonuclease hydrolyzes RNA at approximately the same rate as DNA. The same enzyme is responsible for both activities. Thus, the two cochromatograph on hydroxylapatite, have the same pH and temperature optima, show similar rates of heat inactivation, respond in the same way to EDTA and to divalent cations, and have similar specificities for single-stranded polynucleotide substrates.

B. Reaction Catalyzed

The *N. crassa* endonuclease hydrolyzes single-stranded DNA and RNA to a mixture of mono- and oligonucleotides. As judged by the formation of acid-soluble products, the hydrolysis of single-stranded DNA proceeds in two phases: an initially rapid phase that yields a digest composed of a mixture of 5′-mononucleotides (approximately 30%) in which dGMP predominates, and oligonucleotides of various lengths ranging from dinucleotides to larger than pentanucleotides. At the end of the second phase, mononucleotides comprise approximately 45% of the digest and the remainder is mainly in the form of di- and trinucleotides. More prolonged incubation yields greater than 90% mononucleotides.

The *N. crassa* nuclease can very effectively remove single-stranded regions in double-stranded DNA. This point is illustrated in Fig. 1. In the experiment shown, duplex bacteriophage T7 DNA was treated with *E. coli* exonuclease III to remove from 25 to 5000 nucleotides from the 3′ termini of the duplex. Treatment of these DNAs with the *N. crassa* endonuclease resulted in an amount of material made acid-soluble that was comparable to that removed initially by exonuclease III. The value of the slope (0.86) is consistent with the extent of conversion to acid-soluble nucleotide expected under the conditions of the experiment. The *N. crassa* enzyme is thus able to remove single-stranded stretches in double-stranded DNA to within ten nucleotides of the hydrogen-bonded nucleotides; the value of ten represents the experimental error in the analysis.

13. M. J. Fraser, R. Tjeerde, and K. Matsumoto, *Can. J. Biochem.* **54**, 971 (1976).

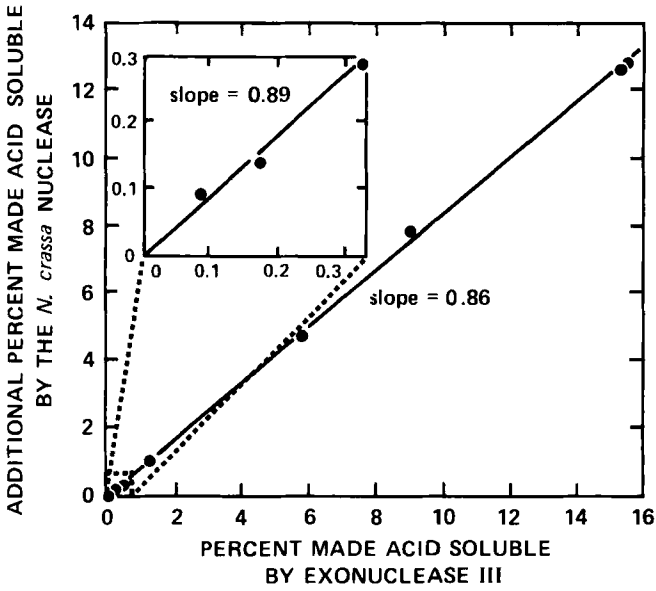

FIG. 1. Effect of pretreatment of ^{32}P-labeled native T7 DNA with exonuclease III on its susceptibility to the *N. crassa* nuclease [from Linn and Lehman (*2*)]. The *abscissa* gives the level of ^{32}P made acid-soluble after exonuclease III treatment; the *ordinate* gives the additional ^{32}P made acid-soluble by subsequent treatment with the *N. crassa* nuclease.

C. BIOLOGICAL ROLE

Recent studies by Fraser and his colleagues have suggested that the *N. crassa* endonuclease represents the end product of a series of proteolytic processing steps beginning with an inactive *pronuclease* (*13*, *14*). Pronuclease can be purified from fresh mycelia. This form of the enzyme, which has a molecular weight of 88,000 based on sucrose density gradient sedimentation, can be activated by treatment with trypsin or by permitting the mycelial extracts to "age." In the latter case activation is presumably a consequence of the action of endogenous proteases. The active enzyme, which has a molecular weight of 61,000, shows both endonuclease activity specific for single-stranded DNA and duplex DNA-specific exonuclease activity. Treatment of this form of the enzyme with trypsin or endogenous proteases results in its conversion to the single-strand-specific endonuclease that is devoid of exonuclease activity and has a molecular weight of

14. S. Kwoong and M. J. Fraser, *Can. J. Biochem.* **56**, 370 (1978).

55,000 (*1, 2*). It therefore appears that the single-strand-specific endonuclease and the double-strand-specific exonuclease activities are associated with a single polypeptide. This polypeptide is released into the culture medium from mycelia during the exponential phase of growth (*15*).

The *endo-exonuclease* may function in recombination and DNA repair *in vivo*. In fact, in several respects it resembles the *recBC* nuclease of *E. coli*, an enzyme that is required for normal recombination in this organism (*16*). Moreover, endo-exonuclease-deficient mutants of *N. crassa* (*uvs*-3 and *nuh*-4) have been isolated that are sensitive to ultraviolet light and to radiomimetic agents, and show an abnormally low frequency of mitotic recombination (*17*). In these two instances the level of inactive endo-exonuclease precursor is higher than the wild type; thus the two mutations may result in the inability of the precursor to undergo conversion to the active form of the enzyme (*18*).

A single-stranded DNA-specific endonuclease with properties identical to the *Neurospora* enzyme has been purified from the smut fungus *Ustilago maydis* (*7*). It is also present at reduced levels in an ultraviolet-sensitive and recombination-defective mutant (*8*).

III. Nuclease S1

Nuclease S1 was first identified by Ando (*3*) in preparations of "Takadiastase" from *Aspergillus oryzae* and purified some 1000-fold. It has subsequently been purified to near homogeneity by Vogt (*4*). The purified protein has a sedimentation coefficient, $S_{20,w}$ of 3.3 and a molecular weight of 32,000. Like the *N. crassa* endonuclease, nuclease S1 appears to be a metalloenzyme. Dialysis of the enzyme against 1 m*M* EDTA results in its inactivation. Activity can be largely restored by the addition of either Co^{2+} or Zn^{2+}.

In contrast to the *N. crassa* enzyme, which has a broad pH optimum in the range of 7 to 9, nuclease S1 shows optimal activity at pH 4.0–4.3. It is essentially inactive at pHs higher than 6.0. The enzyme is relatively insensitive to ionic strength; its activity at 0.4 *M* NaCl is 55% that at 0.1 *M*, the ionic strength at which it is optimally active. An exception is sodium phosphate, which at pH 4.6 inhibits the enzyme at concentrations as low

15. M. J. Fraser, *Nucleic Acids Res.* 6, 231 (1979).
16. P. J. Goldmark and S. Linn, *JBC* 247, 1849 (1972).
17. E. Käfer and M. Fraser, *Mol. Gen. Genet.* 169, 117 (1979).
18. T. Y.-K. Chow and M. J. Fraser, *Can. J. Biochem.* 57, 889 (1979).

as 10 m*M*. Although the rate of hydrolysis of denatured DNA is not greatly diminished at high ionic strengths, the extent of hydrolysis is reduced, presumably because of the tendency of DNA to renature under these conditions.

Like the *N. crassa* endonuclease, nuclease S1 hydrolyzes both RNA and DNA; however, it is approximately fivefold more active on DNA than RNA. The products after extensive hydrolysis are nucleoside 5′-monophosphates. The purified enzyme is highly specific for single-stranded DNA. Thus, under conditions where 96% of denatured DNA from bacteriophage lambda is rendered completely acid-soluble, less than one phosphodiester bond scission is introduced per ten molecules of native lambda DNA. On the other hand, both S1 and *N. crassa* endonucleases are able to recognize and cleave partially denatured regions in duplex DNA generated by the superhelicity of covalently closed duplex DNAs (*19, 20*) or by mismatches produced by mutational alterations in a DNA duplex (*21*). These enzymes can also cleave short single-stranded regions in duplex DNA (*2, 22, 23*) as well as the nonhydrogen-bonded loops in tRNA (*24*).

IV. Mung Bean Endonuclease

A. Physical Properties (*3*)

The mung bean nuclease has been obtained in near homogeneous form. It has a molecular weight of 39,000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Sucrose gradient sedimentation and Sephadex G-100 filtration yield values of 43,000 and 42,000, respectively. The enzyme contains one sulfhydryl group and three disulfide bonds. Approximately 70% of the enzyme molecules contain a peptide bond cleavage at a single site in the protein. Thus, reduction of the enzyme with 2-mercaptoethanol prior to gel electrophoresis in the presence of sodium dodecyl sulfate generates, in addition to the intact 39,000 dalton polypeptide two polypeptides of MW 25,000 and 15,000,

19. P. Beard, J. F. Morrow, and P. Berg, *J. Virol.* **12**, 1303 (1973).
20. A. C. Kato, K. Bartok, M. J. Fraser, and D. T. Denhart, *BBA* **308**, 68 (1973).
21. T. E. Shenk, C. Rhodes, P. W. J. Rigby, and P. Berg, *PNAS* **72**, 989 (1975).
22. J. E. Germond, V. M. Vogt, and B. Hirt, *EJB* **43**, 591 (1974).
23. K. Shishido and T. Ando, *BBA* **390**, 125 (1975).
24. H. Tenehouse and M. J. Fraser, *Can. J. Biochem.* **51**, 569 (1973).

which are presumably linked covalently by a disulfide bond. No difference in enzymatic activity can be detected between the intact and *nicked* forms of the enzyme. The mung bean endonuclease appears to be a glycoprotein containing 29% carbohydrate by weight.

The mung bean enzyme is rapidly inactivated at pH 5, the pH at which it is optimally active. It can, however, be stabilized by the addition of 0.1 m*M* Zn^{2+} and 1 m*M* cysteine or other thiols (glutathione or dithiothreitol). Dialysis of the enzyme at pH 5 results in complete loss in activity. However, activity can be restored by the addition of Zn^{2+} and cysteine. In contrast, dialysis of the mung bean nuclease at pH 5 in the presence of 1 m*M* EDTA results in a loss of enzymatic activity that cannot be restored by the addition of Zn^{2+} and cysteine; other divalent cations (Co^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}) are equally ineffective. Presumably a metal ion is removed by the EDTA dialysis that results in irreversible inactivation of the enzyme.

B. Reactions Catalyzed (*4*)

The mung bean endonuclease catalyzes the hydrolysis of single-stranded polyribo- and polydeoxynucleotides at approximately equivalent rates, to produce 5′-phosphoryl-terminated mono- and oligonucleotides. It possesses an intrinsic 3′-nucleotidase which also acts on both mono- and oligonucleotides. Comparable endonuclease and 3′-nucleotidase activities are associated with a nuclease isolated from wheat seedlings (*11*).

Although showing a strong (up to 1000-fold) preference for polynucleotides lacking ordered structure, the mung bean endonuclease is less specific in this regard than the *N. crassa* or S1 nucleases. Thus, the mung bean endonuclease catalyzes as many as 50 double-strand cleavages in native T7 DNA at levels of enzyme that are required to convert denatured T7 DNA to >90% acid-soluble material. Under conditions that tend to destabilize the DNA duplex (i.e., lower ionic strength and increased temperature, 30° versus 22°) native T7 DNA can be degraded completely. An analysis of the products generated under these conditions during the early phases of hydrolysis of native T7 DNA suggests that following an initially small number of endonucleolytic cleavages (possibly at very A-T rich regions), hydrolysis occurs preferentially from the ends of the duplex, generating a mixture of mono-, di-, and trinucleotides. Such a mode of hydrolysis is consistent with the degree of specificity of the mung bean nuclease for single-stranded polynucleotides. Thus, short single-stranded stretches that are formed transiently at the ends of the duplex, particularly under conditions of low ionic strength and elevated temperature, may be cleaved by the enzyme. Reiteration of this process should then lead to complete degradation of the DNA.

V. Research Applications of Single-Strand-Specific Endonucleases

Because of their high degree of specificity for denatured regions in duplex DNA, all three of the enzymes described have served as useful probes for the identification and, if necessary, elimination of such regions. However, because of its very high degree of specificity for single-stranded as opposed to duplex DNA, its ease of preparation from readily available sources, and its relatively high specific activity the S1 endonuclease has become the enzyme of choice in the structural analysis of DNA and DNA–RNA hybrids. For example, the S1 enzyme has been widely used in DNA and DNA–RNA annealing experiments as a general reagent for the selective removal of nonhybridized, and hence single-stranded polynucleotide. The enzyme has also been utilized in mapping the location of small deletions in viral chromosomes. To cite a specific example, heteroduplex molecules formed from the complementary strands of a deletion mutant and wild-type SV40 DNA contain a single-stranded loop susceptible to the action of S1 nuclease at a position corresponding to the deletion. Incubation of the heteroduplex with S1 nuclease results in hydrolysis of the molecule at the loop, leaving fragments whose length corresponds to the position of the deletion within the SV40 molecule. In fact, by means of this technique S1 nuclease has been used to locate a deletion as short as 32 base pairs in the SV40 genome (*21*).

S1 nuclease has been used widely for the analysis of spliced mRNAs generated as a consequence of intervening sequences in eukaryotic genes (*25*). The procedure consists of hybridization of unlabeled mRNAs to ^{32}P-labeled single-stranded DNA of high specific radioactivity. If the mRNAs are spliced, RNA–DNA hybrids flanked by single-stranded DNA, will result, together with loops of nonhybridized single-stranded DNA, at splice points in the mRNA. When these structures are treated with S1 endonuclease, the single-stranded DNA is hydrolyzed, resulting in a fully duplex structure with discontinuities in the DNA at the splice points. These duplexes can be resolved and their size determined by electrophoresis through agarose gels.

25. A. J. Berk and P. A. Sharp, *PNAS* 75, 1274 (1978).