Purification and Properties of Nuclease SP†

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ABSTRACT: Single-strand-specific nucleases are a diverse and important group of enzymes that are able to cleave a variety of DNA structures present in duplex molecules. Nuclease SP, an enzyme from spinach, has been purified to apparent homogeneity, allowing for the unambiguous characterization of a number of its physical properties as well as its DNA strand cleavage specificities. The effects of ionic strength, pH, divalent metal cations, and temperature on nuclease SP activity have been examined in detail. Nuclease SP was found to be quite thermostable and could be stimulated by Co^{2+}. In addition, the cleavage of UV-damaged and undamaged supercoiled plasmid substrates under a variety of conditions suggests that at least two types of structures are recognized and processed by nuclease SP: UV photoproduct-induced distortions and unwound "nuclease hypersensitive sites". These studies indicate that nuclease SP is functionally related to other single-strand-specific nucleases and is a potential enzymatic tool for probing and manipulating various types of DNA structures.

Nucleic acids are hydrolyzed by a wide variety of enzymes that participate in a number of important biological processes including DNA synthesis, repair, and recombination, RNA synthesis and processing, and also bacterial restriction [for a review, see Linn and Roberts (1982)]. Single-strand-specific nucleases comprise a diverse group of enzymes from a wide variety of organisms that hydrolyze single-stranded DNA and RNA and are thought to mediate certain steps in DNA repair and recombination as well as other events in nucleic acid metabolism (Shishido & Ando, 1982). Although there is a great deal of uncertainty regarding the specific biological roles mediated by single-strand-specific nucleases, the physical and enzymatic properties of several of these enzymes have been studied in some detail (Holloman & Holliday, 1973; Kowalski et al., 1976; Chow & Fraser, 1983). Several single-strand-specific nucleases such as S1 nuclease, Bal 31 nuclease, and mung bean nuclease have been widely utilized for a number of nucleic acid structural manipulations (Berk & Sharp, 1977; McCutchan et al., 1984; Brown et al., 1986; Legerski et al., 1978). Although the individual physical properties of single-strand-specific nucleases vary considerably, they share certain common features including a requirement for divalent metal cations for activity and the production of DNA and RNA strand scission products containing 3′-hydroxyl and 5′-phosphoryl groups (Shishido & Ando, 1982).

We have previously reported the identification and partial purification of an endonuclease from spinach leaves that cleaves single-stranded DNA and RNA as well as duplex DNA damaged by UV light or modified with *cis*-diamminedichloroplatinum(II) (Doetsch et al., 1988, 1989). This enzyme, which we have termed nuclease SP, is inactivated following dialysis against EDTA and appears to cleave duplex DNA preferentially at positions of adenine near areas of helical distortion induced by damaging agents. Nuclease SP generates DNA scission products containing 3′-hydroxyl and 5′-phosphoryl groups (Doetsch et al., 1988).

We wished to further characterize this enzyme with respect to its physical and enzymatic properties as well as to determine whether or not the single-strand DNA and RNA nuclease activities as well as the DNA damage endonuclease activity were mediated by the same protein. We report here the purification of nuclease SP to apparent homogeneity and present some of its physical properties. We have also examined the effects of pH, ionic strength, temperature, and transition metals on nuclease SP activity. In addition, the rate at which this enzyme processes UV-damaged supercoiled DNA substrates modified with ultraviolet light compared to undamaged substrates has been determined. These studies allow a more detailed characterization of nuclease SP and provide further insight into the relationship of this enzyme to other single-strand-specific nucleases.

Materials and Methods

Enzymes and Chemicals. Restriction enzymes and DNA polymerase I (Klenow fragment) were purchased from Bethesda Research Laboratories. Deoxyribonucleotide [α-^{32}P]triphosphates ([α-^{32}P]dNTPs)[1] (specific activity 3000 Ci mmol^{-1}) were purchased from Amersham. Plasmid DNA (pUC19) was isolated as described previously (Yanisch-Perron et al., 1985) or purchased (pBluescript KS+, Stratagene) and subsequently isolated from large-scale preparations (Sambrook et al., 1989). All other chemicals were of reagent grade or better.

Purification of Nuclease SP. All enzyme purification steps were performed at 0–4 °C unless stated otherwise. The purification of the nuclease SP was monitored by using DNA nicking assays (described below) and is summarized in Table I.

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[1] Abbreviations: dNTPs, deoxyribonucleotide triphosphates; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; bp, base pair; TE buffer, 10 mM Tris·HCl, pH 7.5, and 1 mM EDTA; IEF, isoelectric focusing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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Table I: Purification of Nuclease SP[a]

fraction	purification step	protein (mg)	specific activity (units/mg)[b]	total units
I	homogenate	294000	4.6×10^5	1.35×10^{11}
II	35% ammonium sulfate supernatant	226800	2.3×10^5	5.22×10^{10}
III	60% acetone pellet	10780	1.0×10^5	1.08×10^9
IV	DEAE-cellulose	6600	5.7×10^5	3.76×10^9
V	Affi-gel Blue	158	8.6×10^5	1.36×10^8
VIa	poly(U)–Sepharose (0.3 M NaCl)	6.3	5.9×10^6	3.72×10^7
VIIa	Sephadex G-75	0.016	5.0×10^8	8.00×10^6
VIb	poly(U)–Sepharose (0.1 M NaCl)	67	1.8×10^6	1.21×10^8
VIIb	concanavalin A–Sepharose	0.5	6.6×10^7	3.32×10^7
VIIIb	Superose 12	0.036	8.6×10^8	3.10×10^7

[a] Nuclease SP was purified by a series of chromatography steps as described under Materials and Methods. [b] Specific activity was determined by using a nicking assay employing UV-irradiated, supercoiled pUC19 DNA (Materials and Methods).

Crude extracts of *Spinacia oleracea* were prepared from 30 kg of washed, deveined spinach leaves (purchased from the DeKalb County Farmer's Market, Decatur, GA). The leaves were homogenized batchwise in a 4-L blender. Each batch contained 250 g of spinach leaves and 300 mL of homogenization buffer (10 mM KH_2PO_4, pH 7.4, 1 mM EDTA, and 10 μM PMSF). The resulting homogenate was passed through four layers of cheesecloth, pooled, and centrifuged at 12000*g* in a GSA Sorvall rotor for 45 min. The resulting supernatant constituted fraction I.

The pooled homogenate supernatants (fraction I) were brought to 35% saturation with ammonium sulfate, and the resulting precipitate was removed by centrifugation as described above. The remaining supernatant (fraction II) was then brought to 70% ammonium sulfate saturation, and the precipitate, recovered as described above, was resuspended in 2.25 L of 10 mM KH_2PO_4, pH 7.4, 1 mM EDTA, and 10 μM PMSF. The resuspended ammonium sulfate pellet was then dialyzed for 48 h against the same buffer described above. The dialyzed 70% ammonium sulfate pellet (3.75 L) was brought to 30% acetone saturation with continuous stirring, and the precipitate was recovered by centrifugation. The remaining 30% acetone supernatant was then brought to 60% acetone saturation, and the precipitate, recovered as above, was resuspended in 1.2 L of 50 mM KH_2PO_4, pH 7.0, and dialyzed against the same buffer for 48 h. After dialysis, it was observed that not all of the resuspended pellet had dissolved; therefore, the dialyzed material was centrifuged again at 1000*g* for 0.5 h. The resulting supernatant (fraction III, 2.2 L) was utilized in the subsequent purification steps.

Fraction III was applied to a DEAE-cellulose column (4.5 × 50 cm) equilibrated with 50 mM KH_2PO_4, pH 7.0. After the column was loaded, it was washed with 2 L of equilibration buffer followed by stepwise elution (12-mL fractions) of the bound proteins with 1 L each of 0.2 and 0.4 M KH_2PO_4, pH 7.0, respectively. Nuclease SP eluted as a sharp peak of activity with 0.2 M KH_2PO_4. The active fractions were pooled, concentrated, and dialyzed against 25 mM KH_2PO_4, pH 7.0, and constituted fraction IV.

Fraction IV was divided into two equal volumes (81.4 mL) and applied simultaneously to two Affi-Gel Blue (Bio-Rad, 100–200 mesh) columns (3.0 × 18 cm). Each column was washed with 540 mL of equilibration buffer (until the absorbance at 280 nm was zero). The bound proteins on each column were eluted stepwise (12-mL fractions) with 0.2 M (324 mL) and 0.4 M KH_2PO_4 (288 mL), respectively. Nuclease SP activity was observed in the 0.4 M KH_2PO_4 fraction. The samples containing nuclease SP were pooled, concentrated, and dialyzed against 25 mM HEPES, pH 7.0, and constituted fraction V.

Fraction V was applied to a poly(U)–Sepharose 4B (Sigma) column (2.0 × 5.0 cm) equilibrated with 25 mM HEPES, pH 7.0. The column was washed with 0.7 L of equilibration buffer (until the absorbance of the eluent at 280 nm was zero). The bound proteins were eluted stepwise (5-mL fractions) with 0.1 M (95 mL), 0.3 M (35 mL), and 0.5 M (20 mL) NaCl/25 mM HEPES, pH 7.0, respectively. The majority of nuclease SP activity was eluted at 0.1 M NaCl with an additional, residual amount eluting at 0.3 M NaCl. No nuclease SP activity was detected in the 0.5 M NaCl eluant. The 0.1 and 0.3 M NaCl/25 mM HEPES, pH 7.0, eluants, after pooling and concentration, constituted fractions VIb and VIa, respectively. Fractions VIa and VIb exhibited identical DNA nicking and base-specific DNA endonuclease activities.

Fraction VIa was brought to 0.5 M NaCl and was applied to a Sephadex G-75 superfine (Pharmacia) column (1.8 × 94 cm) which had been equilibrated with 0.5 M NaCl/25 mM HEPES, pH 7.0, and calibrated with the following proteins: bovine serum albumin (66 kDa); egg albumin (45 kDa); carbonic anhydrase (29 kDa); and cytochrome *c* (12.4 kDa). The gel filtration column was eluted (2-mL fractions) with 180 mL of the equilibration buffer (Figure 1B). Nuclease SP activity was monitored by using 3′ end-labeled DNA as described previously (Doetsch et al., 1988). The fractions which exhibited the most endonuclease activity (fractions 70–78) were pooled and concentrated (fraction VIIa).

Fraction VIb was brought to 0.5 M NaCl and was applied to a concanavalin A–Sepharose (Sigma) column (1.0 × 9.0 cm) which had been equilibrated with 0.5 M NaCl/25 mM HEPES, pH 7.0. After the application of fraction VIb, the column was washed with equilibration buffer (until the absorbance of the eluant at 280 nm was zero). The bound proteins were then eluted stepwise (2-mL fractions) with 0.1 M (26 mL) and 0.3 M (12 mL) α-D-mannose, respectively. Nuclease SP activity was eluted in the 0.1 M α-D-mannose fraction (fraction VIIb).

Fraction VIIb was applied to a Superose 12 column using a fast protein liquid chromatography system (Pharmacia). The Superose 12 column, equilibrated with 0.5 M NaCl/25 mM HEPES, pH 7.0, was calibrated with the following proteins: bovine serum albumin (66 kDa); egg albumin (45 kDa); carbonic anhydrase (29 kDa); and cytochrome *c* (12.4 kDa). The column was eluted with 25 mL of the same buffer (0.4-mL fractions), and nuclease SP activity was monitored by DNA nicking assays (see below). The peak of nuclease SP activity eluted at a volume corresponding to a protein of 30.8 kDa in size. The active fractions were pooled and concentrated (fraction VIIIb).

DNA Nicking Assay. The standard condition reaction mixture (20-μL final volume) contained 50 mM KH_2PO_4, pH 7.0, 200 ng (approximately 120 fmol) of pUC19 or pBluescript supercoiled DNA, and various quantities of nuclease SP preparations. Supercoiled plasmid DNA samples (10-μL droplets on parafilm over ice) were UV-irradiated (1000 J m^{-2}) with a mineralight shortwave (254 nm) lamp (34-0003-015 W, UV-Products) or left undamaged. The reaction mixtures were incubated for 0.5 h at 37 °C followed by the addition of 3 μL of stop solution (10% SDS, 0.25% Bromophenol blue,

and 25% Ficoll), loaded onto a 1.0% agarose mini-gel (Dan-Kar, Model I), and subjected to electrophoresis at 80 V for 1.5–2 h. The amounts of supercoiled (form I), nicked (form II), and linear duplex (form III) plasmid DNA in a particular sample were determined by densitometric measurements (Bio-Rad Model 620 video densitometer) of photographic negatives obtained from UV light illuminated, ethidium bromide stained agarose gels (Doetsch et al., 1988). The number of DNA nicks per plasmid molecule was determined by the equation $n = -\ln$ (form I fraction) as described previously (Jorgensen et al., 1987). One unit of nuclease SP is defined as the amount of enzyme required to produce 1 fmol of UV-specific nicks in irradiated plasmid DNA in 0.5 h at 37 °C under standard conditions (Doetsch et al., 1988). No correction was made for the differing fluorescence of form I–III DNA (Kowalski, 1979).

Preparation of Defined-Sequence DNA Damage Substrates. A 3′ end-labeled [^{32}P]DNA fragment of defined sequence, 125 bp (fragment 1), was generated by *SalI–PvuII* restriction digestion of pUC19 DNA and labeling with [α-^{32}P]dNTPs and DNA polymerase I (Klenow fragment) as described previously (Doetsch et al., 1986). The end-labeled restriction fragment [specific activity (5–15) × 10^6 cpm (μg of DNA)$^{-1}$] was isolated on preparative, nondenaturing 8% polyacrylamide gels as described previously (Doetsch et al., 1985) and, following recovery, was resuspended in TE buffer. The resulting defined-sequence DNA fragment was either UV-irradiated (2000 J m^{-2}) as described above or left undamaged and then used as a substrate for nuclease SP.

Base-Specific DNA Endonuclease Assay. The nucleotide location of nuclease SP mediated DNA cleavage was determined by incubation of the enzyme with UV-damaged, end-labeled defined-sequence DNA substrates (3–9 ng) in reaction mixtures (20-μL final volume) containing 50 mM KH_2PO_4, pH 7.0, for 0.5 h at 37 °C. The reactions were terminated by bringing the samples to 0.3 M NaOAc (250 μL) and extracting 3 times with 500 μL of phenol/chloroform/isoamyl alcohol (20:19:1, v/v). The resulting nuclease SP cleavage products were ethanol-precipitated and analyzed on DNA sequencing gels. The base-specific DNA cleavage reactions (Maxam & Gilbert, 1980) were run alongside each set of nuclease SP mediated DNA cleavage products. The DNA samples were loaded onto 20% polyacrylamide 7 M urea (denaturing) DNA sequencing gels and subjected to electrophoresis and autoradiography as described previously (Doetsch et al., 1985).

Protein Analysis. Protein determinations were performed by using a Bio-Rad protein assay kit (Bradford method) and a bovine serum albumin standard. SDS–PAGE was performed on a Bio-Rad Protean II system using 12% polyacrylamide gels (Laemmli, 1970). The following proteins were used as SDS–PAGE molecular size standards: α-lactalbumin (14.2 kDa); trypsin inhibitor (20.1 kDa); trypsinogen (24.0 kDa); carbonic anhydrase (29.0 kDa); glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa); egg albumin (45.0 kDa); and bovine serum albumin (66.0 kDa). SDS–PAGE gels were silver-stained to allow detection of proteins (Merril et al., 1984). Isoelectric focusing (IEF) experiments were performed on a Phast System (Pharmacia) using precast IEF gels with a pH 3–9 gradient. The following proteins were used as standards: amyloglucosidase (p*I* 3.5); soybean trypsin inhibitor (p*I* 4.55); β-lactoglobin A (p*I* 5.2); bovine carbonic anhydrase (p*I* 5.85); human carbonic anhydrase (p*I* 6.55); horse myoglobin (p*I* 7.35); lentil lectin (p*I* 8.15); lentil lectin (p*I* 8.45); lentil lectin (p*I* 8.65); and trypsinogen (p*I* 9.3). Typically, 50–200 ng of protein, loaded onto the gel, was subjected to electrophoresis for approximately 0.5 h. Proteins were visualized by silver staining.

Single-Stranded DNA and RNA Hydrolysis Assay. Fraction VIIa was used to examine the hydrolysis of single-stranded DNA or RNA by nuclease SP. The reaction mixture and protocol were identical with those used in the nicking assay (described above) except that ϕX174(+) single-stranded DNA (0.3 μg) or calf liver 18S and 28S rRNA (0.5–5.0 μg) was used in place of supercoiled plasmid DNA.

Effect of EDTA Dialysis, Metal Ions, pH, Salt Concentration, Temperature, and Nucleotides on Enzymatic Activity. The effect of EDTA dialysis, metal ions, pH, salt concentration, and nucleotides on nuclease SP activity was investigated by utilizing either UV-damaged or undamaged pUC19 DNA that was incubated with nuclease SP under various conditions. Nuclease SP activity was determined by DNA nicking assays. An aliquot of fraction VIa was dialyzed against 2 mM EDTA, 25 mM HEPES, pH 7.0, and 0.5 M NaCl for 36 h and then incubated at 4 °C for 48 h in the presence of each of the following metal salts (10 mM final concentration): $ZnCl_2$; $NiSO_4$; $CoCl_2$; and $MnCl_2$. In the DNA nicking assays, 100 ng of the metal-incubated enzyme was used to assess activity.

The effect of pH (range 3.5–9.0) on nuclease SP activity was monitored by using the following buffers (pH range): KOAc (3.5–5.0); MES (5.5–6.5); HEPES (7.0–8.0); and Tris·HCl (8.5–9.0). In the DNA nicking assays, 50 mM NaCl (final concentration) was added to the reaction mixture with nuclease SP (0.5 ng, fraction VIIa). The samples were then incubated for 0.5 h at 37 °C and subjected to electrophoresis on agarose minigels. DNA nicking was quantified by densitometric scans of the photographic negatives as described above.

Temperature effects on nuclease SP activity were determined for both UV-irradiated and unirradiated substrates by DNA nicking assays under standard conditions with the reactions carried out at 0, 4, 14, 21, 37, 50, 65, and 75 °C. The effect of various deoxyribonucleotide 5′-monophosphates on nuclease SP nicking activity was determined by utilizing DNA nicking assays. Fraction VIIIb (0.1 ng) was incubated with UV-damaged pUC19 DNA (50 mM KH_2PO_4) in the presence of various concentrations of 5′-dAMP, -dGMP, -dCMP, and -TMP. The effect of NaCl, $NaNO_3$, and KH_2PO_4 concentration on nuclease SP activity was assessed by DNA nicking assays. Fraction VIIa (0.15 ng) was incubated with UV-damaged pUC19 DNA in the presence of various concentrations (0–200 mM) of NaCl or KH_2PO_4 or $NaNO_3$ in 25 mM HEPES, pH 7.0.

Effect of Divalent Metal Cations on Base Specificity of Cleavage. UV-irradiated and undamaged 3′ end-labeled DNA substrates were reacted with the same metal-incubated nuclease SP preparations utilized in the DNA nicking experiments described above. Incubations were carried out as described above for the base-specific DNA endonuclease assays except that a 10-fold molar excess of EDTA over metal ion concentration was included in the incubation buffer to remove unbound metal and thereby prevent potential metal-induced DNA structural changes. Analysis of the DNA strand scission products was as described for the base-specific endonuclease assays.

Initial Rates of Substrate Cleavage Experiments. Reactions were carried out with 30 ng of nuclease SP (fraction VIIIb) as described above (DNA nicking assay) except that UV-irradiated (1667 J m^{-2}) and undamaged supercoiled pBluescript (Stratagene) DNA (0.7 μg) were used and the incubation

times were varied from 0.5 to 7.5 min. The amounts of forms I (supercoiled), II (nicked circular), and III (linear duplex) DNA were determined as described above for the DNA nicking assays.

Results

Purification and Stability. The purification of nuclease SP is summarized in Table I. The ammonium sulfate and acetone precipitation steps resulted in a preparation (fraction III) that possessed a specific activity approximately 5-fold lower than the crude homogenate (fraction I). However, subsequent purification of nuclease SP (fractions IV–VIIIb) resulted in at least an 8600-fold increase in specific activity. Fraction VIIIb produced a single band on a silver-stained SDS–PAGE gel (Figure 1A), and we conclude that this material corresponded to a homogenous or near-homogeneous preparation of nuclease SP.

The Sephadex G-75 and concanavalin A–Sepharose purified fractions did not significantly lose activity over short periods of time (several days). However, after storage for approximately 2 months at 0 °C in 25 mM HEPES, pH 7.0, and 0.5 M NaCl, a substantial loss in activity was observed. The activity of such fractions could be restored completely in 72 h following the addition of $ZnCl_2$ (10 mM final concentration). Furthermore, storage of nuclease SP in 10 mM $ZnCl_2$ and 50% glycerol at −20 °C resulted in no detectable decrease in activity for at least 5 months.

Physical Properties. Two peaks of nuclease SP activity were eluted from poly(U)–Sepharose at 0.3 M (fraction VIa) and 0.1 M (fraction VIb) NaCl. Gel filtration (Sephadex G-75) of fraction VIa indicated that nuclease SP has an apparent molecular size of approximately 43 kDa (Figure 1B). However, gel filtration (Superose 12 FPLC) of fraction VIIb produced a peak of nuclease SP activity (fraction VIIIb) corresponding to a molecular size of 30.8 kDa. In SDS–PAGE experiments (fraction VIIIb), nuclease SP migrated to a position corresponding to a protein of approximately 32 kDa in size (Figure 1A). These data suggest that fractions VIa and VIb contain physical variants of nuclease SP possessing identical enzymatic properties.

Isoelectric focusing experiments indicated that nuclease SP has a p*I* of 7.7 ± 0.3 (data not shown). Nuclease SP bound to concanavalin A–Sepharose (Materials and Methods), and such a result is an indication that this enzyme is likely to be a glycoprotein (Brattain et al., 1977).

Specificity of Cleavage. We have previously established that partially purified preparations of nuclease SP incise duplex DNA in the vicinity of helical distortions caused by UV photoproducts or *cis*-diamminedichloroplatinum II adducts (Doetsch et al., 1988, 1989). Such partially purified preparations of nuclease SP were also capable of hydrolyzing single-stranded phage DNA as well as 18S and 28S rRNA (Doetsch et al., 1988). Since the above activities could be mediated by several different proteins, it was important to determine whether or not a highly purified nuclease SP preparation possessed the same properties. Under standard conditions, fraction VIIa completely hydrolyzed single-stranded ϕX174(+) DNA and degraded 18S and 28S rRNA, strongly suggesting that the single-strand DNA and RNA nuclease activities are properties of nuclease SP (data not shown).

The base specificity of cleavage of fraction VIIIb was determined by utilizing UV-damaged and undamaged end-labeled DNA substrates of defined sequence (Figure 2). Nuclease SP incised UV-damaged DNA primarily at sites of adenine and thymine, in agreement with the base cleavage pattern observed with partially purified preparations of nuclease SP (Doetsch et al., 1988). Endonucleolytic cleavage of UV-damaged DNA could be detected with a minimum of 50 pg of protein (Figure 2). With larger amounts of enzyme (0.5 and 5.0 ng), a small but detectable amount of cleavage of undamaged DNA was observed that comprised a subset of the cleavage sites observed for the UV-damaged substrate.

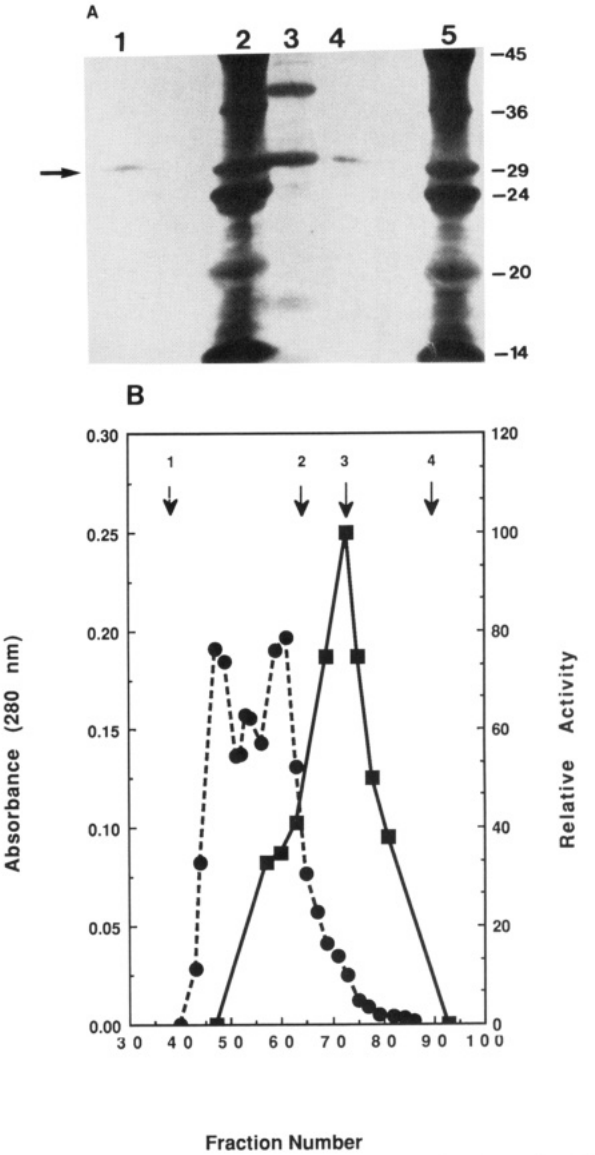

FIGURE 1: Size analysis of nuclease SP. (A) SDS–polyacrylamide gel electrophoresis of nuclease SP. Samples were subjected to electrophoresis on a 12% polyacrylamide gel and silver-stained as described in the text. Lane 1, nuclease SP (0.8 μg, fraction VIIIb); lane 2, marker proteins (14.2, 20.1, 24.0, 29.0, 36.0, and 45.0 kDa) as described in the text; lane 3, nuclease SP (3 μg, fraction VIIb); lane 4, nuclease SP (0.8 μg, fraction VIIIb); lane 5, marker proteins. Arrow indicates position of nuclease SP. (B) Gel filtration chromatography of nuclease SP. Fraction VIa was applied to a Sephadex G-75 (superfine) column and chromatographed as described in the text. UV absorbances (●) of the column fractions were monitored at 280 nm. Arrows indicate size standards used to calibrate the column: (1) blue dextran; (2) bovine serum albumin (66 kDa); (3) egg albumin (45 kDa); and (4) carbonic anhydrase (29 kDa). Nuclease SP activity was determined from densitometric analysis of DNA cleavage products generated by the indicated fraction resolved on a DNA sequencing gel. Activity values (■) were determined relative to fraction 73 (value = 100).

Effect of Transition Metal Ions on Enzymatic Activity. Initial studies (Doetsch et al., 1989) have indicated that dialysis of nuclease SP against 2 mM EDTA resulted in nearly complete loss of enzymatic activity; however, activity could be

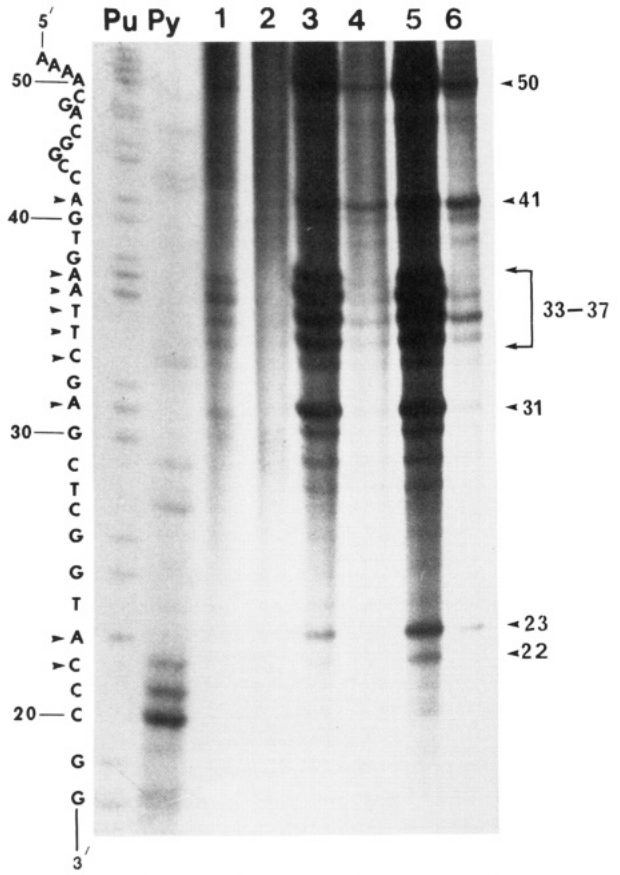

FIGURE 2: Nuclease SP cleavage of UV-damaged DNA. A 3′ end-labeled DNA substrate (fragment 1) was UV-irradiated or left undamaged as described in the text. UV-damaged (lanes 1, 3, and 5) and undamaged DNAs (lanes 2, 4, and 6) were incubated with various amounts of nuclease SP (fraction VIIIb): 0.05 ng (lanes 1 and 2); 0.5 ng (lanes 3 and 4); 5.0 ng (lanes 5 and 6). The reaction products were subjected to electrophoresis on a DNA sequencing gel followed by autoradiography. Approximately 135 kcpm of radioactivity was loaded into each lane. The purine (Pu) and pyrimidine (Py) DNA sequencing reactions were run alongside the enzyme reaction lanes. Arrows denote sites of nuclease SP mediated cleavage (cleavage sites 33 through 37 have been grouped together for simplicity). Uncleaved, full-length DNA fragments located at the top of the gel (accounting for the majority of radioactivity in the sample) are not shown. Base numbering starts from the 3′ end-labeled terminus.

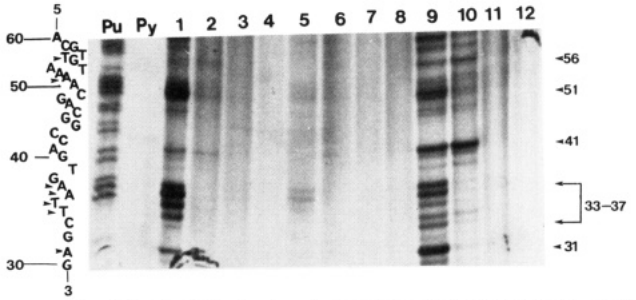

FIGURE 3: Effect of divalent metal cations on base specificity of cleavage. 3′ end-labeled DNA fragment 1 was UV-irradiated (lanes 1, 3, 5, 7, 9, and 11) or left undamaged (lanes 2, 4, 6, 8, 10, and 12) and incubated with 100 ng of nuclease SP (fraction VIa) that was left undialyzed (lanes 1 and 2) or dialyzed with no further additions (lanes 3 and 4) or dialyzed followed by addition of $ZnCl_2$ (lanes 5 and 6), $NiSO_4$ (lanes 7 and 8), $CoCl_2$ (lanes 9 and 10), or $MnCl_2$ (lanes 11 and 12) as described under Materials and Methods. The reaction products were analyzed on a DNA sequencing gel as described in the Figure 2 legend. Approximately 50 kcpm of radioactivity was loaded into each lane, except for lane 5 which was underloaded. Arrows denote sites of nuclease SP cleavage of duplex DNA at the indicated base number from the 3′-labeled end. Uncleaved, full-length DNA fragments located at the top of the gel are not shown.

restored by the addition of $ZnSO_4$ (10 mM final concentration). When undialyzed preparations are stored in the presence of 2 mM EDTA, a loss in enzyme activity is observed over a period of weeks (Doetsch et al., 1989). To further examine the effect of metal ions on enzyme activity, nuclease SP was dialyzed extensively against 2 mM EDTA for 36 h. Samples of the dialyzed nuclease SP preparation were then incubated with various transition metal salts (10 mM) at 4 °C for 48 h, followed by determination of enzymatic activity by DNA nicking assays. Treatment of nuclease SP (fraction VIb) with these transition metals resulted in a varied response with respect to restoration of enzyme activity compared to the undialyzed preparation. Complete restoration (100% relative to undialyzed fraction VIb) of activity (determined by DNA nicking assays) was observed with $ZnCl_2$, whereas $CoCl_2$ stimulated nuclease SP activity to levels 3-fold higher than that of the undialyzed or Zn-containing preparations (not shown). $MnCl_2$ partially restored activity (15%), and little (less than 3%) reactivation occurred with $NiSO_4$.

To determine whether or not reactivation of nuclease SP with the above metals was accompanied by changes in the base specificity of cleavage relative to the native, undialyzed enzyme, a DNA cleavage experiment with end-labeled, defined-sequence substrates was performed with each of the 48-h metal–protein incubation fractions. The enzyme digests were analyzed on a DNA sequencing gel to determine the nucleotide locations of nuclease SP mediated DNA cleavage. For these experiments, a 10-fold excess of EDTA relative to the metal ion concentration was included in the incubations to prevent potential metal-induced DNA structural distortions (Saenger, 1984). Addition of excess EDTA to nuclease SP preparations had a negligible effect on enzyme activity when such preparations were used immediately. Under certain ionic strength and pH conditions, nuclease SP preferentially cleaves UV-damaged DNA at positions of adenine and thymine compared to undamaged DNA (Doetsch et al., 1988). The EDTA-dialyzed enzyme preparations, as expected, did not cleave either UV-damaged or undamaged DNA (Figure 3). Little or no cleavage of either UV-damaged or undamaged DNA was observed with the 48-h Ni- or Mn-incubated enzyme, and these results paralleled those obtained with the DNA nicking assays. Although the overall extent of cleavage was reduced (due to differences in the amounts of radioactivity loaded into lanes 1 and 5), the Zn-incubated preparation cleaved UV-damaged DNA at the same nucleotide locations as the undialyzed preparation. This observation was confirmed upon longer exposures of the gel. In contrast, Co-incubated nuclease SP cleaved both UV-damaged and undamaged DNA at numerous sites, indicating that this metal significantly changes the base specificity of cleavage compared to the undialyzed enzyme (Figure 3). Such Co-incubated preparations cleaved UV-damaged DNA at the same adenine and thymine positions observed with the undialyzed and Zn-incubated enzyme (e.g., A31, T34, T35, A36, and A37). In addition, substantial cleavage was observed at several base positions (e.g., A41 and T56) with either the damaged or the undamaged DNA substrates, and this result may be an indication that Co substantially relaxes the cleavage specificity of nuclease SP.

pH, Salt, Temperature, and Nucleotide Effects. The effect of pH on nuclease SP activity (fraction VIIa, 0.5 ng) was examined over a pH range of 3.5–9.0 utilizing DNA nicking assays. At pH 7.0, optimum cleavage of both UV-damaged and undamaged DNA was observed. Although nuclease SP cleaves UV-damaged DNA to a greater extent than undamaged DNA at pH 7.0, the greatest difference (approximately

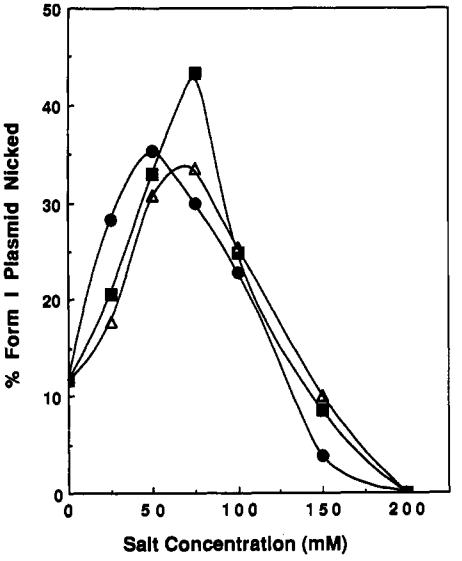

FIGURE 4: Effect of salt concentration on nuclease SP activity. DNA nicking assays were performed on UV-irradiated substrates with nuclease SP (fraction VIIa, 0.1 ng) in the presence of 25 mM HEPES, pH 7.0, and various concentrations of NaCl (△), KH_2PO_4 (■), or $NaNO_3$ (●) under otherwise standard conditions. Enzyme activity is expressed as the percentage of the supercoiled plasmid DNA nicked.

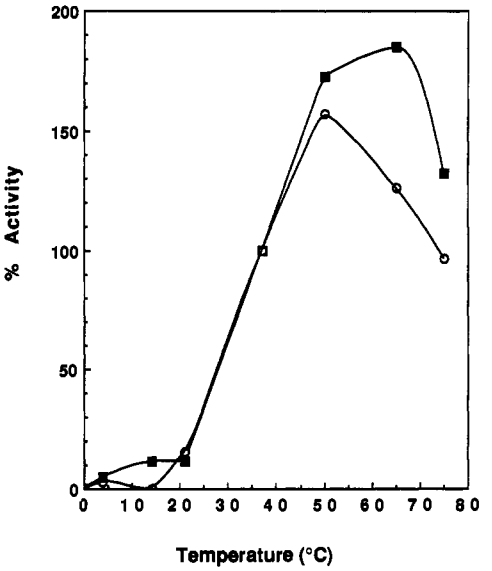

FIGURE 5: Temperature dependence of nuclease SP activity. DNA nicking assays were performed under standard conditions (Materials and Methods) at various temperatures with nuclease SP (fraction VIIa, 0.2 ng) and UV-irradiated (■) or unirradiated (○) substrates. Activity values were determined relative to 37 °C (value = 100%). No adjustment was made for the temperature-dependent change in pK_a for phosphate buffer.

4-fold) in cleavage between these two substrates (UV specificity) occurred in the range pH 7.5–8.0 (not shown).

Nuclease SP mediated cleavage of UV-irradiated supercoiled plasmid DNA occurred optimally within a relatively narrow salt concentration range (50–75 mM) for NaCl, KH_2PO_4, and $NaNO_3$ (Figure 4). At salt concentrations above 100 mM, DNA nicking actvity was inhibited substantially and was completely blocked at 200 mM. At low salt concentrations (0 and 25 mM), nuclease SP activity was 25–80% of that observed at the optimum concentrations (Figure 4). The effects of salt concentration on nuclease SP cleavage of supercoiled plasmid DNA differ somewhat when compared to cleavage of linear duplex DNA substrates. Although nuclease SP incision of UV-damaged and undamaged linear duplex DNA is also greatly inhibited at salt concentrations above 100 mM, the greatest extent of cleavage occurs within the lower (0–25 mM) salt concentration range (Doetsch et al., 1989). Such a difference in the activity at low salt concentrations might be attributed to the differences in the effects of low ionic strength on DNA topology between a supercoiled, covalently closed circular plasmid and a linear duplex DNA molecule (Lilley, 1980; Courey & Wang, 1983). Since nuclease SP is thought to recognize and cleave single-stranded regions contained within duplex DNA, at low salt concentrations, linear duplex substrates would be expected to be more single-stranded compared to supercoiled, circular substrates.

In the presence of 10 mM $ZnCl_2$, nuclease SP exhibited a temperature optimum for nicking UV-damaged or undamaged plasmid DNA in the range of 50–65 °C (Figure 5). At higher temperatures, substantial activity was still observed, indicating a relatively high degree of thermal stability for this enzyme.

Previous studies have indicated that nuclease SP cleaves DNA preferentially at sites of adenine and thymine, and it was of interest to determine whether or not deoxyribonucleotides such as dAMP inhibited the activity of this enzyme as is the case with other single-strand-specific nucleases such as S1 nuclease (Wiegand et al., 1975) The effect of exogenous deoxynucleotide monophosphates on nuclease SP activity was examined. Both dCMP and TMP had no observable effect on nuclease SP activity at concentrations up to 5 mM (data not shown). In contrast, an approximately 50% decrease in the activity of nuclease SP was observed upon addition of dGMP to a concentration of 0.5 mM, and no activity was observed at concentrations greater than 2.5 mM (data not shown). In addition, dAMP (5 mM) completely inhibited nuclease SP activity.

Initial Rates of Substrate Cleavage. We wished to obtain an indication of the difference in the rates of nuclease SP processing of UV-damaged versus undamaged supercoiled plasmid substrates. The amounts of uncleaved, supercoiled plasmid substrate remaining at various times following initiation of reactions with nuclease SP (fraction VIIIb) were determined by DNA nicking assays (Materials and Methods). Plots of the decrease in amounts (femtomoles) of supercoiled (form I) DNA vs time were linear (up to 7.5 min) and allowed an estimation of the initial rates of processing UV-damaged and undamaged supercoiled molecules. The slopes of the lines representing the disappearance of form I molecules were −17.1 fmol min^{-1} (intercept = 280 fmol) and −2.8 fmol min^{-1} (intercept = 260 fmol) for UV-damaged and undamaged substrates, respectively. These data indicate that nuclease SP initially processes supercoiled plasmid substrates containing UV photoproducts at a rate of 570 fmol min^{-1} (μg of protein)$^{-1}$ compared to 92 fmol min^{-1} (μg of protein)$^{-1}$ for substrates containing no UV photoproducts. Such a difference in the rates of cleavage suggests that UV-damaged substrates are more efficiently recognized and processed by nuclease SP due to the presence of UV photoproduct induced helical distortions.

Discussion

The most purified preparations of nuclease SP used in these studies have been enriched at least 8600-fold and confirm our previous suggestions from experiments with partially purified material that this enzyme hydrolyzes single-stranded DNA, RNA, and duplex DNA under a variety of conditions (Doetsch et al., 1988). In addition, several important features of the properties of this enzyme have been determined. This information indicates that nuclease SP is most likely a metalloprotein with single-strand-specific nuclease activities similar

to S1 and mung bean nucleases.

The effects of divalent metal cations on nuclease SP are quite similar compared to a number of nucleases isolated from a diverse range of species. The interaction of Zn(II) and Co(II) [as well as Mn(II) and Ni(II)] with native DNA is very similar, although the effects of these metals on the thermal denaturation/renaturation cycles of DNA are very different (Eichhorn & Shin, 1968; Eichhorn, 1981; Marzilli, 1981; Jia & Marzilli, 1991). The equilibrium constants for the binding of these metals to EDTA [log K = 16.31 for Co(II) and 16.5 for Zn (II)] exceed those for DNA binding [log K = 5.3 for Co(II) and 4–6 for Zn(II)] by a considerable margin (Izatt et al., 1971; Martell & Smith, 1974; Zimmer et al., 1974; Waalkes & Poirer, 1984). The metal ions are labile, and, therefore, the equilibrium should be established immediately. Hence, the greater activity and different cleavage selectivity (Figure 3) of Co–nuclease SP compared to Zn–nuclease SP are due to the metal–enzyme complexes rather than metal–DNA complexes. It should be pointed out that cobalt substitutions for native metals in other enzymes can substantially alter their substrate specificities (Chlebowski & Coleman, 1976).

It is noteworthy that a yeast DNA repair 3′-diesterase which cleaves the 3′-fragments of deoxyribose resulting from oxidative damage exhibits a stimulation by cobalt similar to that of nuclease SP (Johnson & Demple, 1988). When inactive nuclease SP was restored by the addition of $CoCl_2$, the resulting activity was about 3 times greater than when $ZnCl_2$ was added. However, both $MnCl_2$ and $NiCl_2$ restored the activity of the yeast diesterase to levels comparable to that for $ZnCl_2$, whereas the former two metals had little effect on nuclease SP. Johnson and Demple (1988) also provided evidence that the metal ion may be playing both a structural and a catalytic role with the yeast diesterase. We have no evidence to distinguish these possibilities. S1 nuclease also shows a response to Zn^{2+} and Co^{2+} similar to nuclease SP following inactivation by EDTA dialysis (Vogt, 1973). It is conceivable that nuclease SP could be either a Zn^{2+} metalloprotein (such as mung bean nuclease) or a Co^{2+} metalloprotein (such as *Neurospora crassa* nuclease) since both types of single-strand-specific nucleases have been found in nature (Shishido & Ando, 1982). However, there are examples of enzymes which are stimulated by Co^{2+} despite the fact that it is not the naturally occurring metal for that enzyme (Bjork, 1963; Geidroc & Coleman, 1986; Chlebowski & Coleman, 1976).

Nuclease SP demonstrated a pronounced pH optimum of 7.0 for activity on both UV-damaged and undamaged supercoiled DNA substrates. In contrast, the pH optima for other single-strand-specific nucleases are generally in the range of 4.5–5.0 for linear duplex molecules (Shishido & Ando, 1982). At higher pH values (7.5–8.0), nuclease SP showed substantially higher activity against UV-damaged substrates compared to undamaged substrates. This difference is an indication that the enzyme recognizes at least two types of structures within supercoiled plasmid DNA molecules. The first type of structure recognized is present in both UV-damaged and undamaged substrates and is likely to be unwound AT-rich regions that do not exist in linear duplex molecules (Kowalski et al., 1988). These regions have been shown to exist preferentially at 37 °C under neutral pH conditions and are stably unwound regions as opposed to transiently existing "breathing" regions (Kowalski et al., 1988). Such "nuclease hypersensitive sites" are observed with other single-strand-specific nucleases and are cleaved up to 28 000 times faster than nuclease-sensitive sites present in relaxed DNA (Kowalski & Sanford, 1982). The second type of structure is a region of helical distortion caused by UV-induced photoproducts. We have previously shown that in linear, duplex DNA, (6–4) pyrimidine–pyrimidone UV photoproducts are the likely lesions recognized by nuclease SP (Doetsch et al., 1988). However, in UV-damaged, supercoiled substrates, it is conceivable that both major types of UV photoproducts [i.e., cyclobutane pyrimidine dimers and (6–4) photoproducts] may induce regions of distortion that are recognized by this enzyme.

The optimal range of salt concentration for nuclease SP cleavage of UV-irradiated, supercoiled substrates was approximately 50–75 mM for NaCl, KH_2PO_4, and $NaNO_3$ (Figure 4). In contrast, the action of nuclease SP on UV-irradiated and undamaged, linear duplex DNA is in the range of 0–25 mM (Doetsch et al., 1989). These differences in salt concentration optima can be attributed to the differential effects of ionic strength on the two topological forms of DNA substrates used. At low salt concentrations, the formation of denatured regions, such as hairpin structures, in linear duplex molecules is favored and thus accounts for increased cleavage by nuclease SP on such substrates (Svaren et al., 1987). In contrast, the site and extent of cleavage of supercoiled plasmid DNA substrates by single-strand-specific nucleases, such as mung bean nuclease, change substantially as a function of salt concentration, with some sites being preferentially cleaved at low ionic strengths while other sites are perferentially cleaved at higher ionic strengths (Sheflin & Kowalski, 1984). The cleavage pattern apparently depends on the DNA sequence of the particular plasmid. Evidently, the supercoiled plasmid substrates used in these experiments, pUC19, optimally produce unwound recognition sites for nuclease SP at moderate salt concentrations (50–75 mM).

Nuclease SP is thermostable (Figure 5), a property that further relates this enzyme to other single-strand-specific nucleases such as the S1, P1, *N. crassa*, BAL 31, and mung bean nucleases (Shishido & Ando, 1982). These enzymes all exhibit temperature optima in the range of 50–70 °C. In addition, a number of other physical properties of nuclease SP including its affinity for concanavalin A–Sepharose, size, and inhibition by nucleotides reinforce the notion that it is a member of the single-strand-specific nuclease family of enzymes.

Nuclease SP nicks UV photoproduct-containing supercoiled substrates 6 times faster than undamaged substrates. The relatively high UV dose used in these experiments introduces approximately five (6–4) pyrimidine–pyrimidone photoproducts per plasmid molecule, and such molecules would be expected to contain multiple sites for nuclease SP recognition and cleavage. Nuclease SP nicking of UV-damaged, supercoiled plasmids can be envisioned as a process involving recognition and cleavage near UV photoproduct induced helical distortions competing with the recognition and cleavage of stably unwound nuclease hypersensitive sites (present in both UV-damaged and undamaged substrates) discussed above. On the basis of the initial rates of cleavage observed, helical distortions induced by UV photoproducts [presumably (6–4) pyrimidine–pyrimidone photoproducts] are much better substrates for nuclease SP than the undamaged "nuclease hypersensitive sites". Due to the mixed nature of substrates present on UV-damaged, supercoiled plasmid substrates, a more sophisticated kinetic analysis is not possible.

The purification to homogeneity of nuclease SP makes it possible to obtain amino acid sequence information and provides an important tool for initiating studies for cloning the gene that encodes this enzyme. Similar studies with other

enzymes, such as mung bean nuclease, should indicate whether or not the functional similarity of these single-strand-specific nucleases is also reflected at the level of protein structure and may provide insight into the nature of their biological functions. Single-strand-specific nucleases are valuable tools for nucleic acid structural manipulations in a variety of molecular biological methodologies including transcript mapping, cloning of blunt-end cDNA fragments, and gene excision procedures (Berk & Sharp, 1977; Legerski et al., 1978; McCuthcan et al., 1984; Brown et al., 1986; Upcroft & Healy, 1987). The properties of nuclease SP suggest that it may be a useful addition to the repertoire of enzymes employed for such applications.

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