Weil, L., James, S., and Buchert, A. R. (1953), *Arch. Biochem. Biophys. 46,* 226–278.
Weil, L., and Seibler, T. S. (1954), *Arch. Biochem. Biophys. 54,* 368–377.
Westhead, E. W. (1965), *Biochemistry 4,* 2139–2144.
Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2,* 935–941.
Zamenhof, S. (1957), *Methods Enzymol. 3,* 702.
Zewe, V., and Fromm, H. J. (1962), *J. Biol. Chem. 237,* 1668–1675.
Zimmerman, T. P., Gersten, N. B., Ross, A. F., and Meich, R. P. (1971), *Can. J. Biochem. 49,* 1050–1054.

Mung Bean Nuclease I. Physical, Chemical, and Catalytic Properties†

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ABSTRACT: A simplified purification procedure for mung bean nuclease has been developed yielding a stable enzyme that is homogeneous in regards to shape and size. The nuclease is a glycoprotein consisting of 29% carbohydrate by weight. It has a molecular weight of 39 000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme contains 1 sulfhydryl group and 3 disulfide bonds per molecule. It has a high content (12.6 mol %) of aromatic residues. Approximately 70% of the enzyme molecules contain a peptide bond cleavage at a single region in the protein. The two polypeptides, 25 000 and 15 000 daltons, are covalently linked by a disulfide bond(s). Both the cleaved and intact forms of the enzyme are equally active in the hydrolysis of the phosphate ester linkages in either DNA, RNA, or adenosine 3′-monophosphate. The enzymatic activity of mung bean nuclease can be stabilized at pH 5 in the presence of 0.1 mM zinc acetate, 1.0 mM cysteine, and 0.001% Triton X-100. The enzyme can be inactivated and reactivated by the removal and readdition of Zn^{2+} or sulfhydryl compounds.

Mung bean nuclease is a member of a class of nucleases isolated from plants which possess a pronounced specificity towards nucleic acid substrates lacking ordered structure. Other so-called "single-strand specific" nucleases that are well characterized have been isolated from *Neurospora crassa* (Linn and Lehman, 1965), *Aspergillus oryzae* (S_1) (Ando, 1966; Sutton, 1971; Vogt, 1973; Rushizky et al., 1975), *Penicillium citrinum* (P_1) (Fujimoto et al., 1974a,c), and wheat seedlings (Hanson and Fairley, 1969; Kroeker et al., 1975). These enzymes are sugar unspecific endonucleases (*N. crassa* also possesses a single-strand specific exonuclease) which release 5′-phosphoryl-terminated products.

Mung bean nuclease, originally prepared by Sung and Laskowski (1962), and purified to homogeneity by Ardelt and Laskowski (1971), possesses DNase,[1] RNase, and ω-monophosphatase activities (Mikulski and Laskowski, 1970). The enzyme exhibits a preference for A↓pN and T↓pN linkages in denatured DNA (Sung and Laskowski, 1962). The enzyme was called "region specific" because a transient stage was observed in which native λDNA was cleaved at the A–T rich central region of the genome (Johnson and Laskowski, 1970). Mung bean nuclease has also been shown to release products enriched in A and T from early melting regions of native DNA (Kedzierski et al., 1973).

Due to the increasing importance of single-strand specific nucleases as probes of DNA structure and function, we have decided to simplify our method for preparation of mung bean nuclease and further characterize the physical, chemical, and catalytic properties of the enzyme.

Experimental Procedures

Materials. Calf thymus DNA was either prepared by the method of Kay et al. (1952) or purchased from Sigma (type V). Yeast RNA (type VI), adenosine deaminase (type I), and Triton X-100 were obtained from Sigma. Triton X-100 consists of mainly *p*-(1,1,3,3-tetramethyl)butylphenyl poly(oxyethylene glycol) with an average of 9.6 ethylene oxide units.

Ion-exchange celluloses used were Whatman DE-52 and CM-52. Frozen mung bean sprouts were supplied by R. J. Reynolds Foods, Inc., Jackson, Ohio.

General Methods. Analytical gel electrophoresis was performed on 5% acrylamide gels at pH 9.4 and run at 2 mA/gel (Kowalski and Laskowski, Jr., 1972). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 10% acrylamide gels according to the method of Weber and Osborn (1969). Gels stained with Coomassie blue were scanned at 550 nm using a Gilford Model 2400 spectrophotometer equipped with a linear transport. Gels were stained for carbohydrate using the periodic acid–Schiff reagent as described by Segrest and Jackson (1972). The content of sulfhydryl groups was determined according to the method of Habeeb (1972). Amino acid analysis was performed on a Beckman 121 M analyzer. The protein was oxidized with performic acid according to the procedure of Hirs (1956) and hydrolyzed for 22 h according to the procedure of Moore and Stein (1963).

Enzyme Assays. The standard mung bean nuclease assay employs acid precipitation of heat-denatured calf thymus DNA (Sung and Laskowski, Sr., 1962). One unit of mung bean

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[1] Abbreviations used are: 3′-AMP, adenosine 3′-monophosphate; DNase, deoxyribonuclease; RNase, ribonuclease; 3′-AMPase, adenosine 3′-monophosphatase; DEAE, diethylaminoethyl; CM, carboxymethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

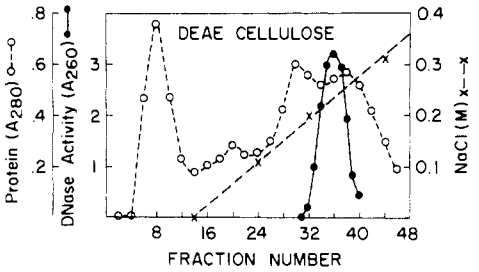

FIGURE 1: DEAE-cellulose chromatography of the dialyzed material from the $(NH_4)_2SO_4$ step. The column (3.2 × 15 cm) was eluted by means of a peristaltic pump at 2 ml/min and 15-ml fractions were collected. The DNase activity (●—●) was measured with the standard DNase assay as described under Experimntal Procedures. The protein (O- - -O) was estimated by measuring the absorbance at 280 nm. The NaCl gradient is represented by the broken line.

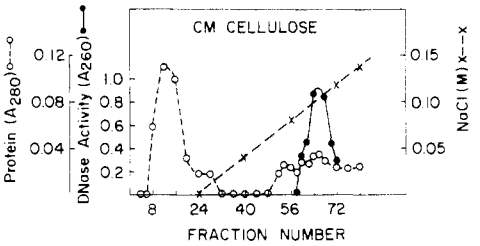

FIGURE 2: Carboxymethylcellulose chromatography of the pooled and dialyzed material from the DEAE-cellulose step. The column (2.1 × 15 cm) was eluted by means of a peristaltic pump at 2 ml/min and 7-ml fractions were collected. Prior to collection, 0.35 ml of 1 M sodium acetate, pH 5.0, was placed into each tube. DNase activity (●—●) was determined as in Figure 1. (O- - -O) absorbance at 280 nm. The NaCl gradient is represented by the broken line.

nuclease is that amount of enzyme which liberates acid-soluble material at a rate of 4.0 A_{260} units[2]/min at 37 °C. Assays are carried out in 1 ml of 0.025 M ammonium acetate (pH 5.0) containing 0.5 mg of DNA. Units used throughout this paper refer to the enzyme activity on denatured DNA, unless otherwise stated.

RNase activity was measured using the standard assay (above), except that 0.5 mg of RNA/ml was used as the substrate. The definition of one unit is the same as that of the standard assay.

The 3′-AMPase activity was measured using the adenosine deaminase coupled assay described by Ipata (1967). The reaction mixture consisted of 0.9 ml of 0.025 M sodium succinate (pH 6.0), 0.05 ml of 1 mM 3′-AMP, and 0.03 ml of adenosine deaminase (diluted 1 to 500 in 0.025 M ammonium acetate, pH 8). One unit is defined as that amount of enzyme which converts 1 μmol of 3′-AMP to adenosine per min per ml of reaction mixture at 25 °C.

Purification. The initial steps in the preparation of mung bean nuclease (i.e., extraction by blending, ammonium sulfate precipitation, and ethanol precipitation) remain essentially unchanged from the previously published method (Ardelt and Laskowski, 1971). However, since an appreciable quantity of nuclease activity was lost upon washing the sprouts with water, the frozen sprouts were ground directly in the blender with 2 l. of hot (55 °C) water per 2 kg of frozen sprouts. By this method, starting with 17 kg of frozen sprouts, approximately 1 g of lyophilized material could be obtained following the ethanol step. This represents a two- to threefold increase in yield over the previous procedure. The mung bean nuclease activity in this lyophilized material is stable in storage at 4 °C and may be accumulated by pooling a number of preparations. Normally, 2 g was used as starting material for the procedure to be described.

All operations were conducted at 4 °C, unless otherwise stated. Centrifugations were done at 15 000 rpm for 20 min.

(*1*) *Heat Step.* To 2 g of material from the ethanol step was added 200 ml of 0.02 M ammonium acetate, pH 6.0. The mixture was titrated to pH 6.0 with ammonium hydroxide and stirred at room temperature until dissolved. Next, 37 ml of 0.5 M sodium acetate, pH 4.5, containing 5 mM zinc acetate and 10 mM cysteine was added. The mixture was then placed in a 500-ml flask and heated in a 70 °C water bath for 15 min, cooled in ice, centrifuged, and filtered through Whatman No. 1 paper.

(*2*) *Ammonium Sulfate Step.* The solution from the previous step was brought to 50% saturation with ammonium sulfate by the addition of solid ammonium sulfate (298 mg/ml) over a 20-min period with stirring. After an additional 20 min of stirring, the precipitate was eliminated by centrifugation and the supernatant was brought to 80% saturation with ammonium sulfate (198 mg/ml) in the same manner. After an additional 20 min of stirring, the precipitate was collected by centrifugation and dissolved in 40 ml of buffer A (0.05 M sodium acetate, pH 6.0, 1 mM cysteine, 0.1 mM zinc acetate) and dialyzed overnight against 4 l. of the same buffer.

(*3*) *DEAE-Cellulose Step.* A column (3.2 × 15 cm) was packed with DE-52 cellulose and equilibrated with buffer A. The dialyzed enzyme from the previous step was applied to the column and washed with 60 ml of buffer. Next, a 500-ml gradient from 0 to 0.35 M NaCl in buffer A was applied to the column, resulting in the protein–activity profile shown in Figure 1. As a consequence of the NaCl gradient, the pH of the effluent increased from 6 to 6.8. This superimposed pH gradient was found to enhance the resolution of the proteins eluting in the gradient portion of the column when compared with a constant pH elution using a sodium acetate gradient. The central fractions representing greater than 80% of the eluted activity were pooled and dialyzed overnight against 6 l. of buffer B (0.01 M sodium acetate, pH 5.0, containing 1.0 mM cysteine, 0.1 mM zinc acetate).

(*4*) *CM-Cellulose Step.* A column (2.1 × 15 cm) was packed with CM-52 cellulose and equilibrated with buffer B. The dialyzed protein solution from the previous step was charged on the column and washed with 50 ml of buffer. Next, a 400-ml gradient from 0 to 0.15 M NaCl in buffer B was applied to the column giving the protein–activity profile shown in Figure 2. A 0.35-ml aliquot of 1 M sodium acetate, pH 5.0, was placed in all tubes prior to collection in order to maintain a pH of 5 in the collected fractions. The pH of the column effluent dropped from 5.0 to 3.9 as a function of the increasing salt gradient. The formation of a pH gradient again markedly enhanced the separating potential of the column during the salt gradient. The central fractions in the activity peak were pooled, accounting for greater than 75% of the eluted activity. The pooled fractions (96 ml) were concentrated to about 5 ml by removing buffer through a dialysis bag with solid Ficoll 400.

(*5*) *Sephadex G-100 Step.* A column (1.6 × 96 cm) was packed with Sephadex G-100 and equilibrated with at least 1 l. of buffer C (0.05 M sodium acetate, pH 6.0, 0.05 M NaCl,

[2] One absorbance unit of a substance is the amount of material which if dissolved in 1.0 ml would have an absorbance of 1.0 at a particular wavelength and 1-cm long light path. The term potency is used instead of specific activity, and is defined as activity units/1 A_{280} unit.

TABLE I: Summary of the Mung Bean Nuclease Purification Procedure.[a]

Step	Total A_{280} Units	Total Act. (units)	Potency (Act. Units/ A_{280} Unit)	Yield (%)	A_{280}/A_{260}	% Act. Remaining after 6-week Storage in Solution at 5 °C
Crude extract	537 000	32 200	0.06	100		
Ammonium sulfate and ethanol	2 820	22 600	8	70		96
70 °C treatment	520	21 000	40	65		99
Ammonium sulfate	186	17 300	93	54	1.36	97
DE-52 Cellulose	27.9	10 500	378	33	1.43	100
CM-52 Cellulose	3.26	6 100	1870	19	1.55	82
G-100 Sephadex	1.66	4 760	2870	15	1.69	91

[a] Values listed in the table are based on averages of five purifications and were found to vary only slightly between preparations. Units of activity on denatured DNA are defined under Experimental Procedures.

0.1 mM zinc acetate). The extensive washing was necessary to remove small amounts of carbohydrate from the swollen Sephadex beads. As can be seen from Figure 3, the mung bean nuclease is eluted between 1 and 2 column void volumes of buffer. Furthermore, a high degree of purity of the eluted mung bean nuclease is evidenced by the constant potency (activity units/A_{280} unit) across the peak. The pooled fractions (12–15 ml) were concentrated to 1–2 ml using Ficoll 400, as described above, and dialyzed vs. 10 mM sodium acetate (pH 5.0), 0.1 mM zinc acetate, 1 mM cysteine. Losses of activity on concentration of mung bean nuclease solutions at pH 5 in an Amicon diaflo apparatus could be prevented by addition of 0.001% Triton X-100. This was not done with the purification procedure reported here in order to avoid unknown concentrations of Triton X-100 which would contribute to the absorbance at 280 nm. Likewise, losses of activity in both the Amicon apparatus and glass containers could be prevented by adjusting the pH to 7–8 in the absence of sulfhydryl compounds.

Table I summarizes the purification procedure based on 34 kg of frozen mung bean sprouts. Starting with the lyophilized material of the ethanol step, the purification may be readily accomplished in 4 days. As can be seen from Table I, the enzyme is stable in solution at 5 °C at each step in the purification. The purified enzyme stored in 50% glycerol (v/v) at −20 °C for 6 months showed no loss of activity.

Results

Physical and Chemical Properties of the Enzyme.

Evidence for a Peptide Bond Cleavage in the Enzyme. When subjected to sodium dodecyl sulfate gel electrophoresis without prior reduction of disulfide bonds, the purified protein runs as a single band as shown in Figure 4, gel A. Comparison with unreduced proteins of known molecular weights (see legend of Figure 4) gave a molecular weight for mung bean nuclease of 39 000. A spectrophotometric scan of gel A in Figure 4 at 550 nm showed that 95% of the total Coomassie stain was associated with the 39 000-dalton band. The remaining 5% was distributed between two bands, 25 000 and 15 000 daltons.

When the enzyme is reduced with 2-mercaptoethanol prior to gel electrophoresis with sodium dodecyl sulfate, three bands are seen, as shown in Figure 4, gel B. Comparison with reduced proteins of known molecular weights gave values of 39 000, 25 000, and 15 000 daltons. The simplest explanation for the appearance of these additional bands after reduction is that a large fraction of the molecules are cleaved in a single region of the polypeptide backbone. Without prior reduction, the two species, cleaved and intact, migrate as a single band, since the

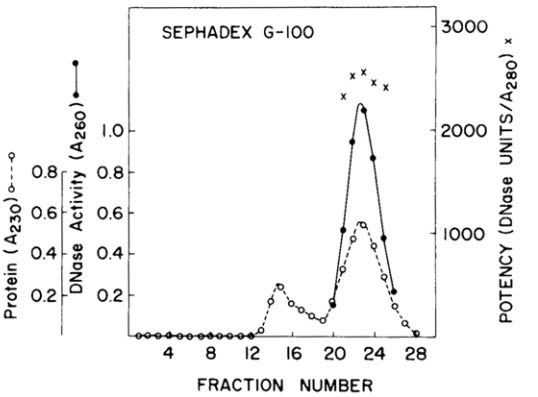

FIGURE 3: Sephadex G-100 gel filtration of the pooled and concentrated material from the CM-cellulose step. Flow rate was maintained at 12 ml/h by means of a Mariotte flask and 5-ml fractions were collected. DNase activity (●—●) was determined as in figure 1; (O- - -O) absorbance at 230 nm; (X) potency (i.e., units/A_{280} unit; $A_{280} = A_{230}/5.0$) of the fractions eluting in the active peak. The void volume of the column is 60 ml (fraction 12).

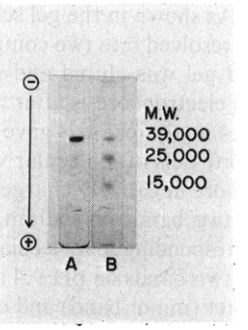

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gels of mung bean nuclease. (A) No prior reduction of disulfide bonds. (B) Prior reduction of disulfide bonds. Solutions of mung bean nuclease in 1% sodium dodecyl sulfate both with and without 1% 2-mercaptoethanol were incubated at 100 °C for 2 min prior to electrophoresis on sodium dodecyl sulfate–polyacrylamide (10%) gels. Twenty-six units (3.5 μg of protein) were applied per gel. Molecular weights, shown at right, were determined using the following standard proteins (reduced and nonreduced): bovine serum albumin (mol wt 68 000), hen egg ovalbumin (mol wt 43 000), bovine pancreatic DNase (mol wt 31 000), Kunitz soybean trypsin inhibitor (mol wt 20 000), and hen egg lysozyme (mol wt 14 300).

25 000- and 15 000-dalton fragments are held together by a disulfide bond(s). Gel scans at 550 nm show that the three bands in the gel of the reduced protein (gel B) account for 92% of the stain in the gel of the unreduced protein (gel A). The bands of the reduced protein are distributed as follows: 31% (39 000), 45% (25 000), and 24% (15 000). The two fragments stain in the ratio of 1.9:1, close to the expected ratio of 1.7:1

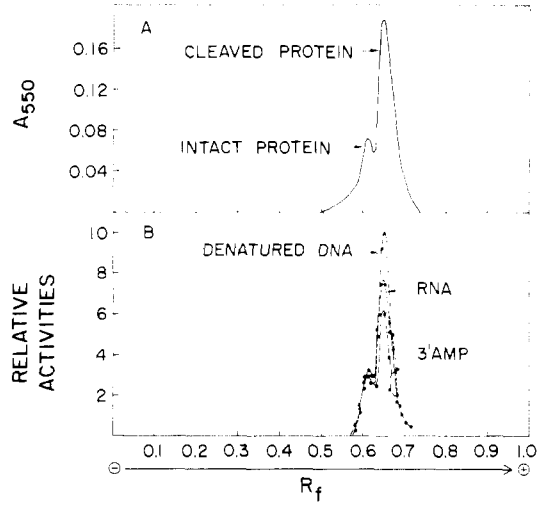

FIGURE 5: Analytical polyacrylamide gel electrophoresis of mung bean nuclease. (A) Scan at 550 nm of a gel stained with Coomassie blue. (B) Activities of mung bean nuclease eluted from a sliced polyacrylamide gel. Electrophoresis and scanning were performed as described under Experimental Procedures. Enzyme activity was eluted from slices (1-mm) of a frozen, 5% polyacrylamide gel in which mung bean nuclease (26 units) was electrophoresed. The slices were placed into tubes containing 0.2 ml of 0.1 M Tris-HCl (pH 8) and stored overnight at 4 °C. The enzyme assays were performed on the substrates listed in the figure as described under Experimental Procedures. The activities toward DNA, RNA, and 3'-AMP listed on the ordinate are multiplied by 0.41, 0.20, and 0.028, respectively, to convert them to enzyme units.

(25 000/15 000), assuming equal staining per unit weight.

Further evidence for both the purity of the preparation and for the presence of a peptide bond cleavage was obtained by analytical gel electrophoresis at pH 9.4. If a single peptide bond cleavage was present, the cleaved form of the protein should possess one more negative charge than the intact form at pH 9.4, due to the presence of an additional carboxylate anion in the cleaved form. As shown in the gel scan in Figure 5A, the purified protein is resolved into two components. The protein from an unstained gel was eluted and subjected to sodium dodecyl sulfate gel electrophoresis after reduction. The minor band on the pH 9.4 analytical gels gave a single band on sodium dodecyl sulfate gels of molecular weight 39 000, while the major band (more negatively charged) on the pH 9.4 analytical gels gave two bands on sodium dodecyl sulfate gels after reduction corresponding to molecular weights 25 000 and 15 000. Thus, the two bands on pH 9.4 analytical gels do indeed represent intact (minor band) and cleaved (major band) protein. Also, the cleaved form of the protein is more negatively charged than the intact. Finally, the percentages of the total areas under the peaks in Figure 5A, representing intact and cleaved protein, are 30 and 70%, respectively. These values are in quantitative agreement with the percentage of the total areas determined for the peaks representing the intact (31%) and cleaved (45 + 24 = 69%) protein from the sodium dodecyl sulfate gels after reduction (Figure 4, gel B). Concerning the purity of the protein, it should be mentioned that no other bands of protein were detectable by spectrophotometric scanning elsewhere on the pH 9.4 analytical gel.

Protein eluted from slices of the pH 9.4 analytical gel, shown in Figure 5A, was tested for the three known activities of mung bean nuclease: DNase, RNase, and ω-monophosphatase (Mikulski and Laskowski, 1970). The results are shown in Figure 5B. Both the intact and cleaved forms are active against denatured DNA, RNA, and 3'-AMP. Furthermore, since the three activity profiles closely parallel the protein profile shown in Figure 5A, the specific activities of the intact and cleaved forms of mung bean nuclease are approximately the same.

The Enzyme Is a Glycoprotein. Staining of the sodium dodecyl sulfate gels of the reduced protein with the periodic acid–Schiff reagent reveals three bands corresponding to molecular weights 39 000, 25 000, and 15 000. A control in which periodic acid was omitted showed no stain. Thus, the intact form and both of the fragments of the cleaved form contain carbohydrate. Qualitatively, the amount of carbohydrate is appreciable, since the staining is much more intense than for similar quantities of pancreatic DNase I (2–4% carbohydrate) and ovalbumin (3.2% carbohydrate).

A quantitative measure of the carbohydrate content was obtained using the phenol–sulfuric acid method of Dubois et al. (1956). Using glucose as a standard, mung bean nuclease is 29% carbohydrate by weight.

Molecular Weight. The molecular weight of the native, active enzyme was estimated using two methods. First, the protein was sedimented in a 5–20% sucrose gradient (Martin and Ames, 1961). The purified protein cosedimented with ovalbumin (mol wt 43 000) and gave a single symmetrical peak across which the DNase units/A_{280} unit was constant. Second, the protein was chromatographed on a column (1.6 × 94 cm) of Sephadex G-100 on which the elution volumes of bovine serum albumin (mol wt 68 000), chymotrypsinogen (mol wt 25 700), and Kunitz pancreatic trypsin inhibitor (mol wt 6500) had been previously determined. A semilogarithmic plot of molecular weight vs. elution volume gave a molecular weight of 42 000 for mung bean nuclease.

As presented earlier, the molecular weight of the intact form of mung bean nuclease, under conditions which denature the protein (gel electrophoresis with sodium dodecyl sulfate), is 39 000. Glycoproteins that show impaired sodium dodecyl sulfate binding due to the carbohydrate moieties give decreasing molecular weight values with increasing acrylamide concentrations between 5–15% in gel electrophoresis with sodium dodecyl sulfate (Segrest and Jackson, 1972). The molecular weight values of the intact form of mung bean nuclease, as well as the two fragments (25 000 and 15 000) of the cleaved form, are unchanged using acrylamide concentrations of 7.5, 10, 12.5, and 15%.

Amino Acid Composition. The amino acid composition of mung bean nuclease, expressed as residues per molecule of 38 600 molecular weight, is presented in Table II. A substance that cochromatographs with methionine sulfone was detected in 22-h hydrolysates of both native and performic acid oxidized mung bean nuclease and is reported as such in Table II. Noteworthy is the high content of aromatic residues, amounting to 12.6 mol % of the protein.

Absorption Spectrum and Extinction Coefficient. The ultraviolet absorption spectrum (data not shown) of mung bean nuclease is typical of that of proteins, showing a maximum at 280 nm and a 280:260 nm ratio of 1.7. A pronounced shoulder on the 280-nm peak is seen at 290 nm due to the high tryptophan content.

The extinction coefficient was calculated using the protein concentration determined by amino acid analysis. At 280 nm, $E_{1cm}^{0.1\%} = 2.6$ (not corrected for carbohydrate).

Sulfhydryl and Disulfide Content. With native mung bean nuclease, 0.14 mol of sulfhydryl groups/mol of protein reacted rapidly with 5,5'-dithiobis(2-nitrobenzoic acid). Addition of 1% sodium dodecyl sulfate exposes 0.73 mol of sulfhydryl groups/mol of protein over a period of 1 h, after which no further reaction with the reagent occurs. This slow reaction of the buried sulfhydryl group is presumably due to slow de-

TABLE II: Amino Acid Composition of Mung Bean Nuclease.

Amino Acid	No. of Residues[a]
Cysteic[b]	7.3
Aspartic	45
Methionine sulfone[c]	10
Threonine[d]	21
Serine[d]	27
Glutamic	21
Proline	8.2
Glycine	16
AAlanine	30
Valine[e]	28
Methionine	0.9
Isoleucine[e]	18
Leucine	24
Tyrosine[d]	9.6
Phenylalanine	18
Histidine	9.1
Lysine	14
Arginine	13
Tryptophan[f]	14
Total	334

[a] Based on a molecular weight of 38 600 for the protein portion of the glycoprotein. [b] Determined after performic acid oxidation. [c] Found in hydrolysates of the native protein (see text). [d] Corrected for destruction during hydrolysis using the factors of Hirs et al. (1954). [e] Corrected for incomplete hydrolysis using the factors of Reeck (1970). [f] Determined spectrophotometrically by the method of Edelhoch (1967).

naturation of mung bean nuclease in 1% sodium dodecyl sulfate at 23 °C. Supporting evidence that the protein unfolds relatively slowly under these conditions was obtained using sodium dodecyl sulfate gel electrophoresis without prior reduction of the protein. A smear of protein stain of high apparent molecular weight is seen along with a sharp band at 39 000 daltons. Heating at 100 °C for 2 min eliminates the smear and increases the intensity of the 39 000-dalton band.

Thus, it appears that the native protein contains 1 cysteine residue/molecule which is unreactive (or reacts slowly) with 5,5′-dithiobis(2-nitrobenzoic acid). By difference from the cysteic acid content (7.3 residues/molecule, Table II), the protein contains 3 disulfide bonds/38 600 g.

Catalytic Properties of the Enzyme.

Requirements for Stability. With the previous preparation of mung bean nuclease (Ardelt and Laskowski, 1971), dilutions of concentrated enzyme solutions were stable indefinitely at pH 7–8, but lost activity rapidly at pH 5. This is also true of the present preparation (at 2 units/ml and 23 °C), as shown in Table III. Addition of Triton X-100, a nonionic detergent, retards, but does not prevent, the apparent loss of enzyme activity at pH 5.0 (Table III). Addition of Zn^{2+} and cysteine in the presence of Triton X-100 rendered the activity as stable at pH 5.0, as it is at pH 7.5 without any additions.

Triton X-100 is not required for stability of mung bean nuclease in glass containers at enzyme concentrations around 50–100 units/ml (6.7–13 μg/ml). At lower enzyme concentrations, the detergent could stabilize the activity by preventing surface denaturation of the protein, as is the case for a number of acid phosphatases (Tsuboi and Hudson, 1955; Tsuboi et al., 1957), or by preventing surface adsorption. Surface adsorption appears to be responsible for the loss of activity, since the apparent stability of the enzyme is dependent upon the composition of the container as well as the ratio of the surface of the container to the volume of the enzyme solution. At 2 units/ml (0.27 μg/ml), enzyme activity decreases rapidly in Teflon containers, while it remains constant in dialysis tubing.

TABLE III: Stability of Mung Bean Nuclease at Low Concentration.[a]

	Compound			% Act. Remaining		
pH	$Zn(C_2H_3O_2)_2$	Cysteine	Triton	1.0 (h)	4.0 (h)	24 (h)
7.5	−	−	−	98	102	92
5.0	−	−	−	24	8	0
5.0	−	−	+	56	21	5
5.0	+	+	+	98	98	90
5.0	+	−	+	100	94	86
5.0	−	+	+	92	82	50
5.0	+	+	−	56	23	0

[a] A solution of mung bean nuclease at 2 units/ml in 0.01 M Tris-acetate (pH 7.5) was made 0.05 M in sodium acetate (pH 5.0) immediately after addition of various combinations of the following compounds (in final concentrations): 0.1 mM $Zn(C_2H_3O_2)_2$, 1.0 mM cysteine, 0.001% Triton X-100. The activity on denatured DNA was determined as a function of incubation time and is shown in relation to the zero time activity of the pH 7.5 incubation.

Inactivation and Reactivation of the Enzyme. After removal of Zn^{2+} and cysteine by 24-h dialysis of the active enzyme against 0.05 M sodium acetate (pH 5.0) containing 0.001% Triton X-100 (200-fold excess buffer, three buffer changes, 4 °C), the enzyme loses 70–80% of its original activity against denatured DNA. The dialyzed enzyme can be reactivated to near its original activity by readdition of Zn^{2+} (0.1 mM) and cysteine (1 mM) and 20–30-min incubation at 23 °C.

The rate of reactivation is dependent on the nature and concentration of the sulfhydryl compound. At the 1 mM level, glutathione, cysteine, and dithiothreitol are capable of maximal reactivation (80–100% of the original activity) of the activity on denatured DNA in less than 0.6 h, while with 2-mercaptoethanol, 2 h are required. Given enough time, the extent of the reactivation is approximately the same for all 4 sulfhydryl compounds tested at the 0.1–1.0 mM level. At sulfhydryl group concentrations of 0.01 mM and less, cysteine, dithiothreitol, and 2-mercaptoethanol restore less than 50% of the original activity in 4 h. Glutathione, however, is very effective at low concentrations, since near maximal reactivation is achieved at the 1 μM level in 4 h. The rate of reactivation of the enzyme appears to be more dependent on the structure of the sulfhydryl compound than its reducing power.

Zinc acetate (without sulfhydryl compound) is also capable of reactivating the pH 5 dialyzed enzyme. The rate is very slow, however, since only one-half of the maximal reactivation is acheved in 24 h with 0.1 or 1.0 mM Zn^{2+}. Of other metal ions (Co^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}, and Cu^{2+}) tested under the same conditions, none were capable of reactivating the enzyme.

EDTA Inactivation and Attempted Metal Ion Replacement. Dialysis of the enzyme vs. 0.05 M sodium acetate (pH 5.0) containing 1 mM EDTA followed by further dialysis to remove the EDTA results in complete loss of the activity against denatured DNA. Incubation with 1 mM Zn^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, or Ca^{2+} (all as chlorides) or 1 mM Fe^{2+} or Cu^{2+} (both as sulfates) failed to produce reactivation after 18 h at 23 °C in the presence of 1 mM cysteine and 0.001% Triton X-100. Apparently, a metal ion is removed by the EDTA dialysis, resulting in the irreversible inactivation. This essential metal ion is presumably not removed by pH 5 dialysis without EDTA,

since, under these conditions, both sulfhydryl compounds and Zn^{2+} reactivate the enzyme.

Methods of Inactivation. Besides the addition of EDTA, other useful methods of inactivation exist. First, adjustment of the pH to 8.0 results in less than 0.01% of the activity as compared to that measured at pH 5.0 in 0.05 M sodium acetate. Second, addition of 0.01% sodium dodecyl sulfate at pH 5.0 instantaneously and completely inactivates the enzyme.

Discussion

A simplified purification procedure for mung bean nuclease has resulted in a stable enzyme, homogeneous in regard to size and shape. Under conditions described under Results, the purified enzyme is stable in solution at its pH optimum (pH values around 5) at all stages during and after purification. The criteria for homogeneity are: (1) constant specific activity across symmetrical peaks on both Sephadex G-100 and 5–20% sucrose gradient, (2) a single band of molecular weight 39 000 on sodium dodecyl sulfate gels (without prior reduction of disulfide bonds), and (3) two bands, of approximately equal specific activity, on pH 9.4 analytical gels.

Further characterization of the purified enzyme shows that it is a glycoprotein and that ca. 70% of the molecules contain a cleavage in a single region of the polypeptide backbone. Interestingly, the same 30:70 ratio was found in preparations obtained by the former method (Ardelt and Laskowski, 1971). This ratio is not affected by incubating the purified enzyme with a crude extract of sprouted mung beans. The constancy of the intact:cleaved ratio may be the result of microheterogeneity of the protein or of the carbohydrate. Given the polyploidy of plant cells, the presence of two homologous proteins that differ in primary structure in the protease-susceptible region and are present in a 30:70 ratio is not unlikely. Alternatively, carbohydrate heterogeneity, such that 30% of the molecules possess carbohydrate moieties which prohibit cleavage of a particular region of the protein, could be invoked.

An extracellular nuclease from tobacco cell cultures, which has properties similar to the mng bean enzyme, has also been shown to give two bands of activity and protein on pH 8.9 analytical gels (Oleson et al., 1974). It has not been shown, however, whether these two forms are the result of an internal peptide bond cleavage or of other sources of charge heterogeneity. The peptide bond cleavage in the mung bean nuclease appears to have had little or no effect on the activity of the enzyme, since the cleaved and intact forms have approximately the same specific activities. It is not uncommon for native proteins with unstructured regions to be susceptible to proteolysis in those regions. In some cases, hydrolysis of these unstructured regions has little effect on the activity of the protein (e.g., subtilisin-treated RNase (Richards and Vithayathil, 1959), pepsin-treated immunoglobulin G (Porter, 1959)), while in other cases the activity is drastically reduced (e.g., trypsin-treated micrococcal nuclease (Anfinsen, 1972)).

The molecular weight of 39 000 determined for mung bean nuclease by gel electrophoresis in sodium dodecyl sulfate is similar to values obtained by the same method on single-strand specific nucleases from wheat seedlings (43 000) (Kroeker et al., 1975) and *Aspergillus oryzae* (32 000) (Vogt, 1973). The value of 39 000 for mung bean nuclease was assumed to represent the protein portion of the glycoprotein. This value may be somewhat high due to the relatively large amount of carbohydrate (29%).

The stability properties of mung bean nuclease, as well as the facile inactivation and reactivation of the enzyme, are reminiscent of properties of other single-strand specific nucleases. The S_1 nuclease from *Aspergillus oryzae* (Vogt, 1973), the P_1 nuclease from *Penicillium citrinum* (Fujimoto et al., 1974a), and the wheat seedling nuclease (Hanson and Fairley, 1969) are all, like the mung bean enzyme, stable to heat treatment (60–70 °C) at pH 5 in the crude stages of purification in the presence of Zn^{2+} (a sulfhydryl compound must be added in the cases of mung bean and wheat seedling nucleases). All of the above mentioned single-strand specific nucleases in the highly purified state appear also to require the presence of Zn^{2+} in the incubation mixture for long term stability at pH 4–5. Finally, enzyme inactivated at pH 5 in the absence of Zn^{2+} can be readily reactivated by the addition of Zn^{2+} and sulfhydryl compound in the cases of mung bean and wheat seedling nucleases, or Zn^{2+} and a mixture of amino acids in the case of P_1 nuclease (Fujimoto et al., 1974b).

The following paper in this issue (Kroeker et al., 1976) describes the action of mung bean nuclease on essentially native DNA and illustrates further the similarities between the catalytic properties of mung bean nuclease and other single-strand specific nucleases.

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References

Ando, T. (1966), *Biochim. Biophys. Acta 114,* 158.
Anfinsen, C. B. (1972), *Biochem. J. 128,* 737.
Ardelt, W., and Laskowski, M., Sr. (1971), *Biochem. Biophys. Res. Commun. 44,* 1205.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28,* 350.
Edelhoch, H. (1967), *Biochemistry 6,* 1948.
Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974a), *Agric. Biol. Chem. 38,* 777.
Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974b), *Agric. Biol. Chem. 38,* 785.
Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974c), *Agric. Biol. Chem. 38,* 1555.
Habeeb, A. F. S. A. (1972), *Methods Enzymol. 25B,* 457.
Hanson, D. M., and Fairley, J. L. (1969), *J. Biol. Chem. 244,* 2440.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219,* 611.
Hirs, C. H. W., Stein, W. H., and Moore, S. (1954), *J. Biol. Chem. 211,* 941.
Ipata, P. L. (1967), *Anal. Biochem. 20,* 30.
Johnson, P. H., and Laskowski, M., Sr. (1970), *J. Biol. Chem. 245,* 891.
Kay, E. R. M., Simmons, M. S., and Dounce, A. L. (1952), *J. Am. Chem. Soc. 74,* 1724.
Kedzierski, W., Laskowski, M., Sr., and Mandel, M. (1973), *J. Biol. Chem. 248,* 1277.
Kowalski, D., and Laskowski, M., Jr. (1972), *Biochemistry 11,* 3451.
Kroeker, W. D., Hanson, D. M., and Fairley, J. L. (1975), *J. Biol. Chem. 250,* 3767.
Kroeker, W. D., Kowalski, D., and Laskowski, M., Sr. (1976), *Biochemistry 15* (following paper in this issue).
Linn, S., and Lehman, I. R. (1965), *J. Biol. Chem. 240,* 1287.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.
Mikulski, A. J., and Laskowski, M., Sr. (1970), *J. Biol. Chem. 245,* 5026.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Oleson, A. E., Janski, A. M., and Clark, E. T. (1974), *Biochim. Biophys. Acta 336,* 89.
Porter, R. R. (1959), *Biochem. J. 73,* 119.
Reeck, G. (1970), in Handbook of Biochemistry, 2nd Ed., Sober, H. A., Ed., Cleveland, Ohio, Chemical Rubber Co. Press, C-281.
Richards, F. M., and Vithayathil, P. J. (1959), *J. Biol. Chem. 234,* 1459.
Rushizky, G. W., Shaternikow, V. A., Mozejko, J. H., and Sober, H. A. (1975), *Biochemistry 14,* 4221.
Segrest, J. P., and Jackson, R. L. (1972), *Methods Enzymol. 28B,* 54.
Sung, S., and Laskowski, M., Sr. (1962), *J. Biol. Chem. 237,* 506.
Sutton, W. D. (1971), *Biochim. Biophys. Acta 240,* 522.
Tsuboi, K. K., and Hudson, P. B. (1955), *Arch. Biochem. Biophys. 55,* 191.
Tsuboi, K. K., Wiener, G., and Hudson, P. B. (1957), *J. Biol. Chem. 224,* 621.
Vogt, V. M. (1973), *Eur. J. Biochem. 33,* 192.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

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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