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Purification & Characterization of a Deoxyribonuclease from Etiolated Soybean

Cunyong Xu
Western Kentucky University

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Cunyong

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Purification and Characterization of a Deoxyribonuclease from Etiolated Soybean

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Master of Science

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Cunyong Xu
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Purification and Characterization of Deoxyribnonuclease

from Etiolated Soybean

Date Recommended May 17, 1993

Valgene L. Dunham
Director of Thesis

Claire A. Rinehart

Frank R. Jorman

Ward K Houston 6/29/93
College Dean Date

L. E. Alden 7/26/93
Graduate Studies Date

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Purification and Characterization of a Deoxyribonuclease from Etiolated Soybeans

Cunyong Xu May, 1993. 69 Pages

Directed by: Valgene L. Dunham, Frank R. Toman and Claire A. Rinehart

Department of Biology Western Kentucky University

A deoxyribonuclease (DNase) has been isolated from a purified preparation of soybean nuclei. The procedure, involving solubilization of proteins using ammonium sulfate, Sephadex G-75, DEAE-Cellulose and CM-Sephadex chromatography and non-denaturing polyacrylamide gel electrophoresis, resulted in a 554-fold purification with a yield of 6% of the enzyme. The purified enzyme had a molecular weight of 60 KDa as monitored by non-denaturing polyacrylamide gel electrophoresis. In addition, high-molecular-weight (HMW) soybean DNA, essentially free from protein, RNA and other contaminates, has been isolated from soybean to be used as a substrate for the enzyme. Enzyme activity was directly proportional to the concentration of enzyme and substrate as determined by a spectrophotometric assay. In the presence of divalent cations (Mn^{2+}), the enzyme hydrolyzed soybean DNA, calf thymus DNA and pBR322 DNA but was inactive with yeast RNA. The enzyme was partially inhibited by KCl. Endonuclease activity was indicated on agarose gels using covalently closed circular pBR322 DNA and spectrophotometric analysis of assay products following Sephadex G-10 chromatography. The enzyme had a K_m for soybean DNA of 4.5 μg/ml and a V_{max} of 0.02 ΔA_{260}/min.

Introduction

Nucleases are a diversified group of enzymes that are present in various cells and tissues. The mechanism of these enzymes is to hydrolyze phosphodiester bonds in nucleic acids. This degradation of DNA and RNA may play important roles in DNA replication, fidelity and repair, removal of RNA primers, defense against foreign (phage, viral) infection and processing of precursor mRNA. Each of these topics and a classification of nucleases will be discussed in detail below.

Endodeoxyribonuclease Involved in DNA Replication

The progression of the replication fork by separation of two intertwined strands of the double helix automatically leads to the occurrence of positive supercoiling. In prokaryotes this problem appears to be overcome by the action of gyrase which introduces negative supercoils in front of the replication fork (Kornberg, 1980). Gyrase is in fact a special type of topoisomerase, a class of endonuclease which changes the degree of supercoiling of DNA. Plant type I topoisomerases have been isolated from T. aestivum (wheat) embryos (Dyan et al., 1981). This enzyme activity, having the ability to relax both positively and negatively supercoiled DNA, has also been identified in P. sativum (pea; Jenns et al., 1978).

Endodeoxyribonucleases nick double-stranded DNA in AT-rich regions, particularly when the tendency of these regions to exhibit transient

denaturation (i.e,"breathe") is accentuated by supercoiling. Such an enzyme could therefore nick one DNA strand at intervals, allowing free rotation of a stretch of DNA. Re-ligation follows after passage of the replication fork. Single-strand-specific endodeoxyribonucleases have been detected in a number of plants (Bryant, 1980; 1982). Typical is the enzyme from P. sativum which is chromatin-bound and shows some correlation with DNA replication (Weir et al., 1983).

Fidelity of DNA Replication and DNA Repair

The enzyme that synthesizes a new DNA strand using a template strand and a primer strand is called DNA polymerase. The three types of DNA polymerases in E. coli have been shown to have 3' to 5' exonuclease activity. To produce a sequence of bases in the DNA of a daughter cell which is identical to that in the parent, the process of DNA replication should have high fidelity. The 3' to 5' exonuclease of bacterial DNA polymerases can recognize and eliminate mismached base-pair terminus on the primer DNA (Kornberg, 1974).

DNA polymerase I also has a 5' to 3' exonuclease activity (nick translation function) that cleaves base-paired regions of DNA, releasing oligonucleotides from the 5'-end. This nuclease can act on DNA molecules containing mismached bases or a distortion that makes them unsuitable as substrates for polymerase (Kornberg et al., 1969). A typical example of 5' to 3' exonuclease activity is the elimination of thymine dimers from DNA exposed to ultraviolet radiation, leaving a short gap which is filled by DNA polymerase followed by DNA ligase to make the final phosphodiester bond (Haseltine, 1983). In plants, an endonuclease that can excise UV-irradiated

DNA has been detected in cultured cells of Daccus carota (carrot; McLennan et al., 1986).

Removal of RNA Primers

The presence at the 5' end of any nascent DNA strand of an oligoribonucleotide primer must be removed from new Okazaki fragments (especially on the lagging strand of DNA). In prokaryotic cells, this is thought to be accomplished by the 5' to 3' exonuclease activity of DNA polymerases I, which then fills in the resulting gap. Although Euglena contains a 5' to 3' exonuclease activity associated with DNA polymerases (McLennan et al., 1975), the activity has not been found associated with other eukaryotic DNA polymerases leading to the necessity for an additional enzyme to remove these primers. Ribonuclease H is an enzyme that specifically degrades RNA in a DNA/RNA hybrid molecule. The enzyme has been detected in a number of eukaryotic organisms including Sacchararomyces (Wyers et al., 1973), Ustilago (Babks, 1974), and Carrot (Sawai et al., 1978).

Defense Against Foreign (phage, Viral) Infection

It is well established that a biochemical barrier exists to infection of bacterial cells by viral or phage DNA. Deoxyribonuclease acts as such a barrier by attacking invading DNA. The nucleotide sequence of host DNA recognized by restriction endonucleases has been modified by methylation, whereas the foreign invading DNA lacks the characteristic host modification pattern making it susceptible to attack by the restriction enzyme.

Processing of Precursor mRNA Molecules

RNA processing in the nucleus has stimulated interest in nuclease activity in this organelle. Polyribonucleotides of animal cells are synthesized in the nucleus as large molecules that are subsequently cleaved to generate both the RNA molecules in cytoplasm and fragments that are degraded in the nucleus. Some nuclease activity involving RNA processing previously reported may now be recognized to be ribozymes, the activity of which act as catalysts for their own splicing.

Most eukaryotic mRNA has been found to contain a poly (A) sequence at the 3' end. Studies of mRNA degradation in a cell free system that seems to reproduce faithfully in vivo degradation mechanisms show that the stabilizing effect of the poly (A) tail requires poly (A) binding protein (PABP) in a way which suggests that the poly (A) tail is degraded only when it is naked and unassociated with PABP. The poly (A) tails of all cytoplasmic mRNAs in almost all eukaryotes are associated with PABP. Digestion of poly (A)-PABP complexes with nonspecific nucleases results in fragments with PABP bound to a length of 27 residues (Jackson et al., 1990).

Classification of Nuclease

Nuclease is used as a specific name for enzymes that degrade both DNA and RNA; DNase hydrolyses DNA, whereas RNase uses RNA as a substrate. Another property employed in classification of these enzymes is the location of catalytic activity on the substrate. The enzyme that can hydrolyze from within polynucleotides to generate oligonucleotides is called an endonuclease. Exonucleases produce mononucleotides one residue at a

time from the end of polynucleotides. Another important feature used in classification is the mode of phosphodiester bond cleavage at the phosphodiester bond on either side of the phosphate. Cleavage on one side generates 3'-hydroxyl and 5'-phosphate termini. Cleavage on the other side produces 3'-phosphate and 5'-hydroxyl ends. Using the above types of classification, a brief survey of DNases is presented below.

Bacterial DNases

A number of bacterial endodeoxyribonuclease and exodeoxyribonucleases isolated from E. coli have been reported. Some typical properties of the enzymes are presented in Table 1 (Adams, 1986). All endonucleases identified are characterized by having small molecular weights, whereas exonucleases possess large molecular weights and have a preference for single-stranded DNA. The products of nucleases are quite different and include oligonucleotides with 5'-phosphate ends, a nick with 3'-OH and 5'-deoxyribose ends, and mono- and dinucleotides with 5' termini. The most interesting nuclease listed, endonuclease III, shows the combined activities of glycosylase (specific for DNA irradiated with ultraviolet light) and an AP endonuclease that attacks the AP site (site on DNA lacking a base is called an AP site) at which depurination or depyrimidination has occured. The glycosylase produces AP-DNA, and the endonuclease nicks the duplex DNA 3' to the AP site to give a 3'-terminal sugar.

Animal DNases

Two major types of deoxyribonucleases have been purified and characterized from animals. The first type is pancreatic DNase I (Kunitz, 1950) involved in degradating double stranded DNA relatively nonspecifically.

Table 1. Deoxyribonuclease of E. coli.

Name	Mr (KDa)	Substrate	Product
Endonuclease I	12	Duplex DNA	Oligonucleotides with 5'-p end
Endonuclease III	27	AP* site & u/v-irradiated duplex	Nick with 3'deoxyribose 5'-p end
Endonuclease IV	33	AP* site	Nick with 3'-OH 5'-deoxyribose
Endonuclease V	27	ssDNA damaged duplex	Short oligonucleotides
Exonuclease I	72	ssDNA	Mono-& dinucleoside 5'-p
Exonuclease II	109	ssDNA or nicked duplex	Nucleotide 5'-p
Exonuclease V	140	ssDNA	Oligonucleotide
Exonuclease VII	88	ssDNA	Oligonucleotide

Reference: Adams, 1986. AP* site = apurinic site.

The enzyme is produced in the pancreas and used as a digestive enzyme in the small intestine. The enzyme has an optimum pH in the range of 6.8 to 8.2 and is activated by magnesium or manganese ions. The nature of the divalent cation qualitatively affects specificity. Citrate, borate and fluoride inhibit the enzyme by removing the activating divalent ions. DNase I has been used extensively for mapping sites in DNA that were responsive to protein binding and the disposition of DNA in nucleosomes.

The second type of DNase is a lysosomal enzyme found in spleen and thymus and believed to be involved in intracellular breakdown of DNA. The enzyme has a molecular weight of 40 KDa with an optimum pH in the range of 4.5-5.5 and no requirement for magnesium ions. DNase II from porcine spleen contains two subunits with the molecular weights of 10 and 35 KDa (Liao, 1985). The DNase degrades double-stranded DNA to generate oligonucleotides with an average chain length of six units, which have a free 5'-hydroxyl group and a phosphate residue on position 3'. Endodeoxyribonuclease activity occurs in many mammalian tissues including liver, spleen, kidney and pancreas. Although the enzymatic properties of DNase II from different tissues and species are very similar, the chemical properties can vary greatly (Liao et al., 1988).

Characteristics of Plant Nuclease

Wilson (1975) has classified plant nucleases into four categories: RNase I, II, Nuclease I and Exonuclease I. The following criteria were used to classify the enzymes: substrate (sugar specificity), reaction products, mode of action (endo- or exonuclease), base specificity, molecular weight, pH optima and ethylenediaminetetraacetic acid (EDTA) sensitivity (Table 2). RNase I and II

Table 2. Characteristics of plant nucleases.

	RNase I	RNase II	Nuclease I	Exonuclease I
1. Substrate	RNA	RNA	RNA, DNA	RNA, DNA
2. Products	N>P	N>P	pN, 3'-OH end	pN
3. Mode of action	Endo nuclease	Endo nuclease	Endo nuclease	Exonuclease
4. Base specificity	G>A=U>C	G>A=U>C	A>U(T),G,C	None
5. Mol. Wt (KDa)	20-25	17-20	35-54	>100
6. pH optimum	5-6.0	6-7.0	5-6.5	7-9.0
7. EDTA inhibition	--	--	High	High

Reference: Wilson, 1975.

are RNA specific endonucleases that release 2': 3'-cyclic nucleotides with guanosine 2': 3'-cyclic monophosphate as the major early product (Jervis, 1974). RNase I has a pH optimum near 5.0 with a molecular weight of 20-25 KDa. RNase II has a molecular weight of 17-20 KDa with an optimum pH of 6.5. Nuclease I is also an endonuclease which produces 5' nucleotides with a preference for adenosine in both DNA and RNA, having a pH optimum of 6.0 and a molecular weight of 45 KDa. Exonuclease I breaks down polynucleotide chains from the 3' end releasing 5'-mononucleotides and has a pH optimum near 8.0 with a molecular weight of 100 KDa. RNase activity is not affected by EDTA, whereas DNase activity is inhibited by EDTA which removes a metallic ion required for the enzyme activity.

A number of plant nucleases discussed here have been isolated, and some of these enzymes have also been separated from each other (Table 3). For example, mung bean DNase was separated from nuclease I and RNase on a Sephadex G-100 column (Johnson et al., 1968). The enzyme activity exhibited a preference for ApN and TpN linkages in single-stranded DNA. Also, the mung bean DNase was called "region specific" endonuclease because a transient stage was observed in which native lambda DNA was cleaved at the AT-rich central region of genome (Johnson et al., 1970).

Developmental Changes in Activity

Changes in levels of nucleic acid and nuclease have been found during various phases of active plant growth (Kessler et al., 1961; Bryant et al., 1976). Partial suppression of the increased RNase activity by actinomycin-D suggested that de novo enzyme synthesis could be partially responsible for additional activity during senescence (McHale et al., 1968).

Table 3. Species and Name of Plant nucleases

Species	RNase I	RNase II	Nuclease I	Exonuclease I
1. Barley	RNase		DNase	Exophospho-diesterase
2. Carrot				Phospho-diesterase
3. Corn	RNase A	RNase II	RNase B	
4. Muskmelon			Nuclease	
5. Mung bean	RNase M1		Nuclease I	
6. Oats	Relative purine specificity Endo-	RNase	DNase*	Alkaline pho-sphodiesterase
7. Peas	RNase		DNase*	
8. Potato			Nuclease I	
9. Ryegrass	RNase			
10. Soybean	RNase			
11. Spinach	RNase			
12. Sugar beet				phospho-diesterase
13. Sugar cane	RNase			
14. Tobacco	RNase	RNase	Extracellular nuclease	Alkaine pho-phodiesterase
15. Wheat	RNase		Nuclease	

Reference

Wilson, 1975. 5*. Johnson et al., 1968. 7*. Jenns et al., 1978.

In addition, suppression of the activities of chromatin associated DNase and RNase by kinetin in detached leaves as compared to attached leaves has been demonstrated in cotyledons of barley (Srivastava et al., 1968). Senescence in the first seedling leaf of barley is characterized by declines in the level of DNA. Control leaves exhibited DNase activity but leaves floated on a kinetin solution resulted in a retardation of senescence. The results suggested that kinetin drastically suppressed the increases in DNase during senescence.

In general, nuclease activity in cotyledons increases as germination proceeds in parallel with the decrease in nucleic acid content. However, in the embryonic axis of pea seeds, the increase in RNase activity with time is accompanied by increasing RNA content (Fumio et al., 1978). Parallelism between the RNA content and RNase activity suggests that RNase may have some positive function in the synthesis of RNA in addition to degrading RNA.

Increased Nuclease Activity Resulted from Infection or Mechanical Damage

Plants infected with pathogens have elevated nuclease levels. Both DNase and RNase activities increased in wheat seedlings infected with compatible strains of puccinia rust (Grunward, 1975). Mechanical damage to potato tubers and leaves (Pitt et al., 1971), tobacco leaf tissue and aging bean endocarp tissue is associated with an increase in RNase activity. Evidence indicates this increase in tobacco is a result of de novo synthesis of the enzyme (Bagi et al., 1967).

Nucleases play many important roles in DNA and RNA metabolism, DNA replication, DNA repair and RNA processing. Recently, some

important nucleases have been summarized to give a further understanding for their typical properties (Singer et al., 1991). As noticed in Table 4, the nucleases are from many different organisms, and most of the important enzymes are endonucleases with the exception of Exo VII. In addition, these nucleases prefer single-stranded DNA except for RNase H. The specificity of the nucleases is greatly variable, including DNA, RNA, and DNA-RNA hybrid specificity. All seven nucleases generate the product 5'p(Np)nN.

Although there have been reports of numerous nuclease properties from different organisms, little information concerning the properties of plant DNase is available. The relatively high activity of DNase in various germinating seeds suggests an important role in seed sprouting. Therefore, the purpose of this research is designed to investigate the presence, purification and characterization of an endodeoxyribonuclease in soybean germinating tissues.

Table 4. Summary of Typical Nucleases

Name	Source	Specificity	Strand Preference	Endo or Exo	product
Bal 31	Altermona espejiana	DNA	s.s.	endo	5'-p(Np)nN
Exo VII	E.coli	DNA	s.s	exo { 5' to 3' / 3' to 5' }	5'-p(Np)nN
Mungbean nuclease	Mungbean sprouts	DNA,RNA	s.s	endo	5'-p(Np)nN
Neurospora nuclease	Neurospora crassa	DNA,RNA	s.s	endo	5'-p(Np)nN
RNase H	Cellular	RNA·DNA	d.s	endo	5'-p(Np)nN
RNase H	Retrovirus	RNA·DNA	d.s	endo	5'-p(Np)nN
S_1 nuclease	Aspergillus oryzae	DNA,RNA	s.s	endo	5'-p(Np)nN

Note: N denotes any nucleotide. (Np)n denotes a polynucleotide chain that is n residues long.
Reference: Singer et al., 1991.

Materials and Methods

Plant Growth Conditions

Soybean seeds were purchased from Stewart Seed Company, Greensburg, Indiana. Seeds were spread on the surface of a very moist, 2 inch layer of vermiculite in a 12" x 24" plastic non-drain flat and lightly covered with a thin layer of vermiculite. Seeds were germinated in the dark at room temperature (25°C) for 5 days with proper watering. Hypocotyl sections (2cm) from the five-day old etiolated soybeans were harvested and used as a source of DNA and DNase.

Chemicals

Unless otherwise specified, all chemical materials were purchased from Sigma Chemical Company, St. Louis, Missouri.

Soybean DNA Isolation and Purification

Purification of high-molecular-weight (HMW) DNA was accomplished by the following procedure outlined in Figure 1.

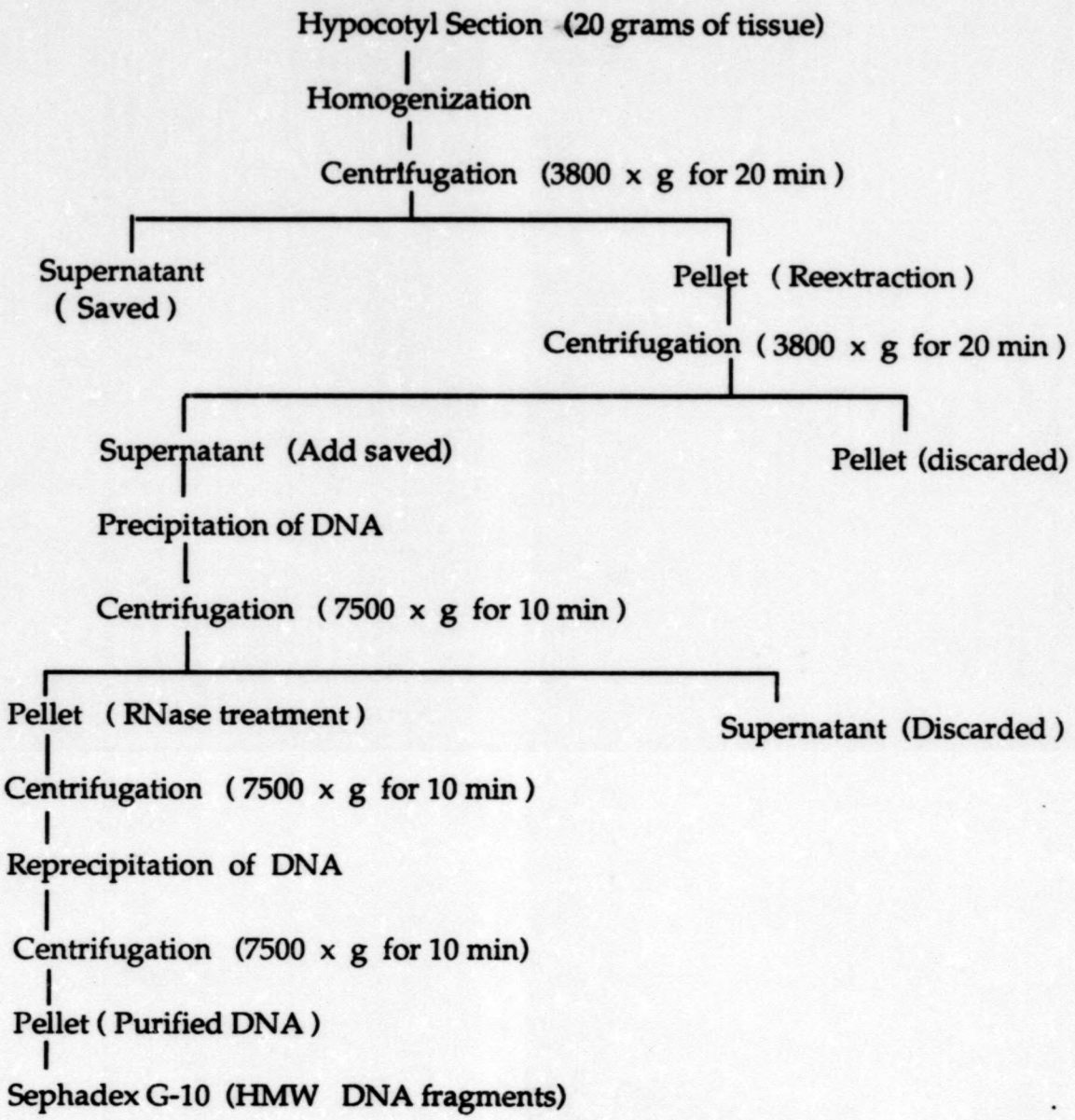
Tissue Homogenization

Hypocotyl sections (2 cm) were harvested and placed in a -20° C freezer overnight. Twenty grams of tissues, suspended in 200 ml of extraction buffer and 40 ml of phenol, were rigorously ground with a prechilled mortar and pestle in a cold room. The extraction buffer consisted of 0.1M Tris-HCl (pH 8.0), 0.05M ethylenediaminetetraacetate (EDTA; to inactivate DNase activity), and 1% sodium dodecyl sulfate (SDS; to denature proteins). The mixture was filtered through 2-layers of prewetted and chilled cheesecloth and then centrifuged at 3,800 x g with a Beckman Centrifuge JA 20 rotor for 20 min at 4°C.

Deproteinization

The most commonly used method for deprotenizing a solution of DNA is extraction with phenol that efficiently denatures proteins. The supernatant was saved from the previous centrifugation, and the pellet was reextracted with 20 ml of extraction buffer and 20 ml of phenol then centrifuged as above. The combined supernatants were then recentrifuged at 3,800 x g for 10 min at 4°C. The top aqueous phase containing nucleic acid was carefully collected, and the lower layer phase containing denatured proteins and phenol liquid was discarded. Forty mls of phenol were mixed into the saved supernatant and recentrifuged at 3,800 x g (repeated twice at this step for deproteinization).

Figure 1. Isolation and Purification of Soybean DNA.


Hypocotyl Section (20 grams of tissue)
Homogenization
Centrifugation (3800 x g for 20 min)
Supernatant (Saved)
Pellet (Reextraction)
Centrifugation (3800 x g for 20 min)
Supernatant (Add saved)
Pellet (discarded)
Precipitation of DNA
Centrifugation (7500 x g for 10 min)
Pellet (RNase treatment)
Supernatant (Discarded)
Centrifugation (7500 x g for 10 min)
Reprecipitation of DNA
Centrifugation (7500 x g for 10 min)
Pellet (Purified DNA)
Sephadex G-10 (HMW DNA fragments)

Nucleic Acid Precipitation

Ethanol precipitation was used for concentrating DNA solutions and for removing residual phenol from the deproteinized aqueous solution. Following the deproteinization, DNA was precipitated by addition of 0.6 volume of isopropanol, gently mixed and placed in a freezer at -20°C for 4hr. The above solution was recentrifuged at 7,500 x g for 10 min at 4°C. The supernatant was discarded and the pellet quickly suspended in 10 ml of 0.1 M Tris-HCl buffer (pH 7.0) containing 0.05 M EDTA (TE buffer). The DNA solution was reprecipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol and then placing in a freezer for 2 hr. The precipitated DNA was recentrifuged at 7,500 x g for 10 min at 4°C.

RNase Treatment

The pellet from the previous centrifugation was suspended in 10 ml of Tris-HCl buffer (pH 7.0) containing 5 m $MnCl_2$. RNase (1 unit to 50 µg/ml RNA) was added to the sample solution and then incubated for 30 min at 37°C (Hattory, 1986). Following RNase treatment, the DNA solution was reprecipitated as described above.

Spectrophotometric analysis

The absorbance of the DNA solution was read in the range of 220 to 300 nm with a Gilford Response Spectrophotometer. DNA yield was calculated based on absorbance at 260 nm (A_{260} of 1.0 = 50 µg of DNA). The OD_{260}/OD_{280} absorbance ratio was employed to determine the purity of DNA.

Electrophoresis

Agarose gel electrophoresis was a simple and effective method for separating large molecular weight fragments of DNA. A gel was prepared with an 1% agarose concentration for the size of soybean DNA fragments to be separated. The samples, containing 3.3 µg of soybean DNA, 0.05% bromphenol blue and 0.1 M TE buffer in a total volume of 30 µl per lane, were loaded into the sample wells, and then electrophoresed for 1 hr and 20 min at a 100 volts until the blue index reached the end of the gel.

Gel Staining and De-Staining

When the electrophoresis was completed, the gel was stained in ethidium bromide (EB) solution (1µg/ml) for 5 to 10 min until the separated bands were clearly illuminated on the UV light box. For de-staining, the gel was rinsed 3 times with 300 ml d.H_2O for 45 min each rinse until the unbound dye was removed.

Photography of DNA in Agarorose Gel

DNA bands were photographed in agarose gel stained with EB by illumination with UV light. Photography included an orange filter (Kodak wratter#23A) and polaroid type 667 film and was accomplished at setting of F = 5.6 for 1 second in the dark. It is necessary to use gloves and protective eyewear at all time while handing ethidium bromide and viewing gels under UV light.

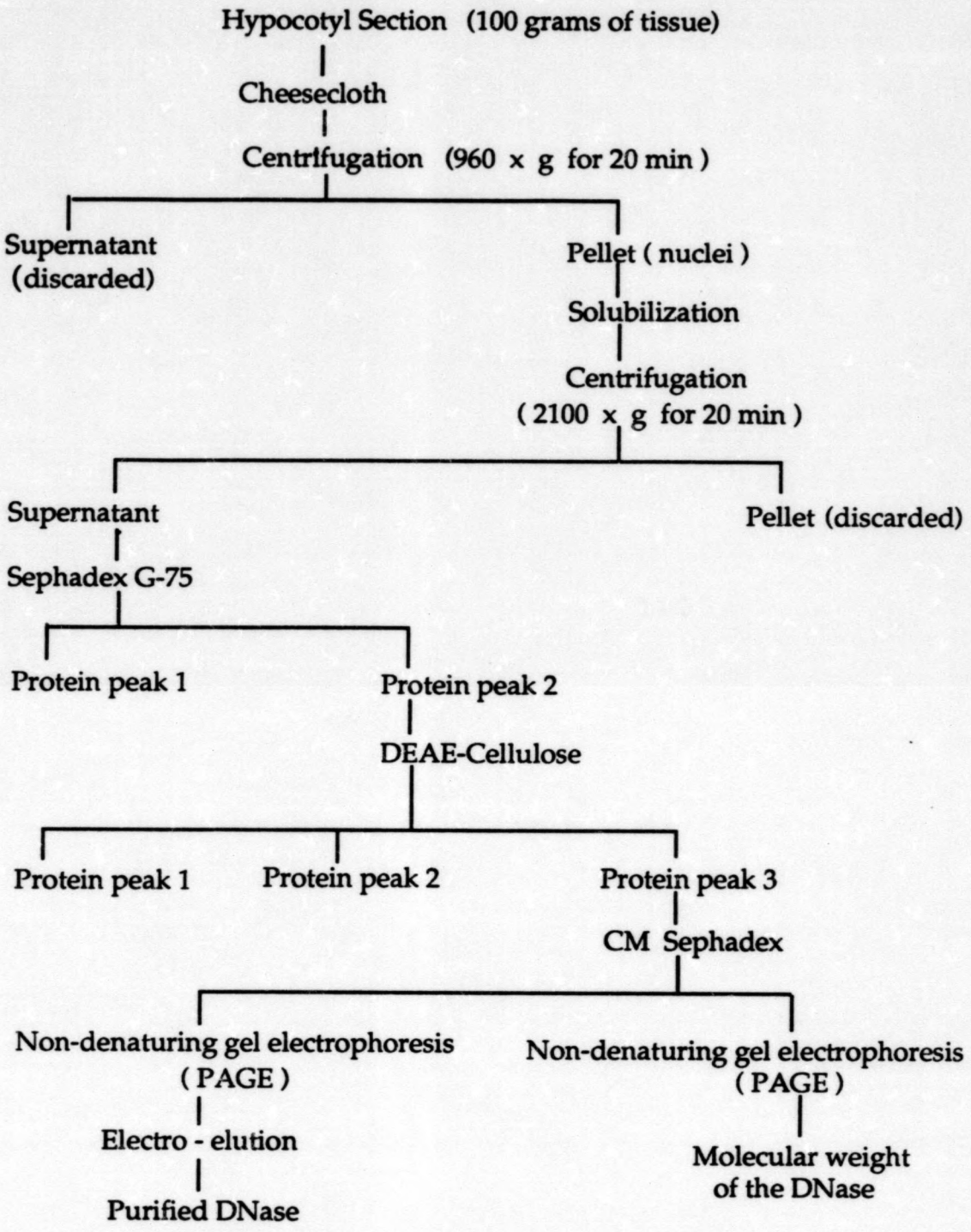
Gel Filtration

To obtain HMW soybean DNA fragments as substrate for DNase, the purified DNA solution was applied to Sephadex G-10 gel filtration. The resin was swollen in 10 mM Tris-HCl wash buffer (pH 7.0) for one day in the cold room. A diluted DNA solution (1: 10) was loaded on the top of the column (1.5 x 15 cm) which had been washed previously with 2 volumes of the wash buffer. Fractions were collected at a flow rate of 20 drops/min with 30 ml of a total elution volume. Void volume of the column was calibrated using blue dextran with a molecular weight of 2,000 KDa.

Enzyme Isolation and Purification

Purified nuclei were prepared from 100 grams of hypocotyl section (2cm) from five-day old etiolated soybeans.The purification procedure is outlined in Figure 2. Hypocotyl sections were harvested and placed in cold homogenization buffer (pH 8.0) consisting of 100 mM Tris-HCl, 0.4 mM phenylmethyl sulfonylfluride (PMSF), 5mM magnesium chloride ($MgCl_2$), and 600 mM sucrose (Dunham et al., 1986). The volume of the buffer used was 2 : 1 (vol /weight of tissue) and included 3 drops of antifoam agent. Homogenization was performed using a Sorval Omni-Mixer at setting 3 for 30 seconds and then at setting 5 for 30 seconds. Tissue homogenates were filtered through two layers of pre-wetted and chilled cheesecloth placed so that the fibers of cloth were perpendicular to each other. The filtrates were then centrifuged at 960 x g for 20 min at 4° C. The nuclei pellet was suspended in 20 ml of solubilization buffer consisting of 10 mM Tris-HCl

Figure 2. Isolation and Purification of Soybean DNase.


Hypocotyl Section (100 grams of tissue)
Cheesecloth
Centrifugation (960 x g for 20 min)
Supernatant (discarded)
Pellet (nuclei)
Solubilization
Centrifugation (2100 x g for 20 min)
Supernatant
Pellet (discarded)
Sephadex G-75
Protein peak 1
Protein peak 2
DEAE-Cellulose
Protein peak 1
Protein peak 2
Protein peak 3
CM Sephadex
Non-denaturing gel electrophoresis (PAGE)
Electro - elution
Purified DNase
Non-denaturing gel electrophoresis (PAGE)
Molecular weight of the DNase

(pH 8.0), 1.5 mM ammonium sulfate, 5 mM β–mercaptoethanol (BME), 0.02 mM $MgCl_2$ and 0.4 mM PMSF. Proteins were solubilized from the nuclei by stirring on ice for 2 hr. The solubilized solution was centrifuged at 2,100 x g for 20 min at 4°C, the pellet was discarded and the supernatant was collected. Two ml of the crude homogenate were saved and assayed for protein concentration and enzyme activity, the rest of the crude homogenate was used for further purification.

Sephadex G-75

Sephadex G-75 resin was swollen for two days in the equilibration buffer consisting of 100 mM Tris-HCl (PH 8.0), 5 mM BME, 0.4 mM PMSF and 25% (v/v) glycerol. Sixteen ml of the crude homogenate fraction was layered on the top of the column bed (2.5 x 15 cm) which had been washed with 300 ml of equilibration buffer. Two protein peaks were collected at a flow rate of 20 drops/min, 60 drops/tube. Each (2 ml) of them (protein $peak_A$ and $_B$) was saved and assayed for protein concentration and enzyme activity at A_{595} nm and A_{260} nm on a spectrophotometer. The Sephadex G-75 protein $peak_B$ contained most of enzyme activity and was used for further purification.

Anion Exchange Chromatography

DEAE-cellulose resin (Whatman DE 52 purchased from Fisher Company. Pittsburgh. PA.) was swollen in equilibration buffer for one day. The column (2.5 x 20 cm) was packed in a cold room at 4°C, and then equilibrated with 400 ml of the above buffer. Fourteen ml of the pooled fraction protein $peak_B$ eluted from Sephadex G-75 were layered on the top of the column. Fractions were collected at a flow rate of 16 drops/min. Bound proteins were eluted by a 0.1 and 0.6 M KCl step gradient wash. Each of the

resulting three peaks was collected and assayed for protein concentration and enzyme activity. The DNase activity, eluted at 0.6 M KCl, was desalted using Sephadex G-25 chromatography. The pooled fraction (12 ml) of DEAE protein peak 3 were applied to a Sephadex G-25 column (1.5 x 18 cm) which had been prepared and washed with 2 times volume of equilibration buffer with pH 8.0. Protein peaks were collected and assayed for protein concentration and enzyme activity.

Cation Exchange Chromatography

A CM Sephadex column (2.5 x 15 cm) was equilibrated at 4°C with 0.01 M sodium acetate buffer (pH 6.0) containing 1.0 mM cysteine. Nine ml of enzyme preparation (desalted DEAE protein peak3 pooled fraction) were applied to the column which was then washed with 60 ml of the buffer. One protein peak (30 ml) was collected and concentrated to 5 ml with an immersible-CX ultrafilter purchased from Millipore Company, Bedford, MA.

Following elution of the protein peak containing high enzyme activity, the column was washed with 160 ml of CM buffer containing 0.15 M sodium acetate. Almost no proteins were eluted with the step gradient wash as monitoring at A_{280} nm. The salt eluate (39 ml) was collected (60 drops/tube), pooled and applied to a Sephadex G-25 chromotography column for desalting. Each resulting fraction was collected (60 drops/tube, 20 tubes) and assayed for enzyme activity.

Non-Denaturing Polyacrylamide Gel Electrophoresis

Discontinuous polyacrylamide gel, consisting of a lower resolving gel and an upper stacking gel as described by Laemmli (1970), was used for further protein purification. All gel compositions were derived from a mixture of three stock solutions: Stock A, stock R, and stock S. Stock A solution contained 29.2% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide in a total volume of 100 ml with d.H_2O to give a 30% acrylamide/bis-acrylamide stock solution. Stock solution R, resolving buffer, was a 1.5 M Tris-HCl solution with pH 8.8. Stock S, stacking buffer, was a 0.5 M Tris-HCL solution with a pH 6.8. Ten percent resolving gels were fabricated as follows: 2.5 ml stock A, 1.875 ml d.H_2O, 3.12 ml of stock R, 0.025 ml of freshly made 10% ammonium persufate (APS), and 0.005 ml N'N'N'N'-tetramethylethylenedanine (TEMED) to give a total gel volume of 7.53 ml. The stacking gels were prepared as follows: 0.325 ml of 30% acrylamide/ 0.8% bis-acrylamide, 0.625 ml of stock S, 3.05 ml d.H_2O, 0.0125 ml of freshly made 10% APS and 0.0025 ml of TEMED for a total volume of 2.5 ml. In gel preparation procedure APS and TEMED are added last since their addition initiates polymerization of the gel. Polymerization of the stacking gel solution was completed after 45 min at room temperature. Samples were prepared as follows: 5 parts of protein solution to 1 part of 6X sample buffer containing 7 ml of stock S, 3 ml of 100% (v/v) glycerol and 1.2 mg bromphenol blue bring to 10 ml with d.H_2O. The sample mixture containing 0.08 µg proteins in 20 µl of a total volume was loaded into each well after removing the teflon comb without tearing the edges of the gel well. The upper and lower chambers of the electrophoretic apparatus were filled with 1X running buffer (pH 8.3) diluted from 5 X electrophoresis buffer consisting

of 0.12 M Tris-HCl and 0.96 M glycine. The gels were electrophoresed for 60 min at 4°C at 20 Amps through stacking and separating gel. It was necessary to use a pump for maintaining water circulation between the chamber of the electrophoretic apparatus. This was accomplished by using a cold water container linked with silicon tubes which were immersed in a container filled with ice in a cold room. Non-denaturing polyacrylamide gels were also employed for molecular weight determination of soybean DNase.

Electro-Elution of Protein Bands

The concentrated enzyme preparations from CM Sephadex chromatography were applied to 10% non-denaturing polyacrylamide gels with a preparatory comb and then electrophoresed for 60 min at 4°C. Three areas of the gel, the protein band and areas both above and below the protein band, were cut out from the non-stained gels. The excised gel slices were individually electro-eluted using a Bio-Rad Electro-Eluter apparatus at 4°C in a cold room. A current strength of 10 mA/tube was used for 6 hr.

It was important to wear gloves at all times while assembling the Electro-Eluter apparatus and while putting the excised gel slices into the glass tubes. A frit was inserted into the bottom of each of the glass tubes needed, making sure that the frit fitted flush at the bottom of the tube. The new membrane cups were soaked in elution buffer consisting of 25 mM Tris-HCl, and 192 mM glycine for 1 hr at 60°C prior to use. The pre-wetted membrane cups were placed in the bottom of each silicone adapter needed . Each adapter with membrane cup was then filled with 400 µl of elution buffer. The buffer was pipeted up and down to remove air bubbles around the dialysis membrane for optimum elution yields. The silicone adapter with the

membrane cup was then secured to the bottom of the glass tube with frit and then the glass tube with attached adapter, frit, and membrane was inserted into a grommet of the electro-eluter module. The top of the tube was then made even with the grommet. All empty grommet holes were filled with stoppers.

Each tube was filled with elution buffer and gel slices inserted. It was important for gel slices not to fill the tube higher than 0.5 cm from the frit suface for proper elution. If the gel slices exceeded the 0.5 cm height, they were macerated and packed into the tube with a glass rod to the lowest possible height. Care was taken not to pack with such a force as to dislodge the frit from the bottom of the tube.

Each module was placed into the buffer chamber containing 600 ml of the elution buffer. The lower buffer level had to be above the top of the silicone adapters otherwise air bubbles would form on the surface of the dialysis membrane and disrupt elution. The upper buffer chamber needed to be filled with 100 ml of elution buffer. A stirring bar was placed in the lower buffer chamber and the entire unit placed on a magnetic stirrer. Vigorous stirring during the run prevented bubbles from sticking to the bottom of the dialysis membranes.

At the end of the elution run, it was necessary to first remove liquid down to the frit level from the tube before removing the adapter. The silicone adapter with membrane cup was carefully removed, making sure not to dislodge the frit from the bottom of the tube. Eluted protein in the adapter and cup was carefully removed with a micropipette. The adapter and cup were then rinsed with 100 µl of elution buffer for each, and then these buffer

rinses were pooled with the protein elution and labeled properly.

Gel Staining

Non-denaturing polyacrylamide gels were stained by silver reagent purchased from Bio-Rad Chemical Company. The dye for staining protein is 100 times more sensitive than coomassie brilliant blue R-250. Gels to be silver stained were fixed in 200 ml of 40% methanol/10% acetic acid for 30 min and then in 200 ml of 10% ethanol/5% acetic acid for 15 min. This step was repeated twice. The gels were then oxidized in 100 ml of oxidizer for 15 min. Following a wash with distilled water, the gels were then stained in 100 ml of one to ten diluted silver reagent for 20 min. Gels were then developed with 100 ml of developer for 5 min (in this case development was repeated twice because the protein concentration was low). The development reaction was stopped by a 5% acetic acid solution for 5 min. In addition, only two lanes as reference were fixed and stained since this procedure would inactivate any enzyme activity associated with the protein bands after gel staining.

DNase Assay Procedure

Kunitz Spectrophotometric Enzyme assay

Kunitz's spectrophotometric analysis (1950) is the most convenient for enzyme assay and kinetic studies of purified DNase. The method is based on the colorimetric determination of the acid-soluble deoxypentose compounds released in the course of enzyme reaction.

The enzyme was incubated in the presence of 0.1 M Tris-HCl buffer (pH 7.0) in a total volume of 1.5 ml containing 5 mM $MnCl_2$ and 50 µg/ml of soybean DNA at 37°C for 45 min and then was terminated by adding 1 ml of 3 M trichloroacetic acid (TCA). These assay tubes were chilled in ice for 15 min and then centrifuged at 7,800 x g for 15 min at 4°C, the supernatant from the previous centrifugation was read at A_{260}nm on a Gilford Response Spectrophotometer with d.H_2O as a blank. A control carried out with each assay contained all assay components but minus enzyme. Average control readings were subtracted from experimental readings. The difference indicated the enzyme's ability to hydrolyze DNA.

DNA-gel electrophoresis for assay of DNase activity

The DNA-gel electrophoresis assay was a sensitive and visual method for detecting DNase activity to hydrolyze DNA. Enzyme was incubated at 37°C for 45 min in 0.1 M Tris-HCl buffer (pH 7.0) in a total volume of 30 µl containing soybean DNA (3.3 µg /well) and 5 mM $MnCl_2$ and then the enzyme catalysed reaction was stopped by adding 3 µl of killing buffer

consisting of 13.5% (w/v) SDS, 0.05% (w/v) bromphenol blue and 10% (v/v) glycerol. Samples (30 µl/well) were electrophoresed in 1% agarose gels for 1 hr and 20 min at 100 volts until the blue index reached the end of the gels. The gel was stained in ethidium bromide (EB) solution with the concentration of 1µg/ml d.H_2O for 5 to 10 min until the DNA bands were clearly illuminated on the UV light box. For de-staining, the gel was rinsed with 3 changes of 300 ml d.H_2O for 45 min each rinse and then photographed on UV light box as described earlier.

Enzyme Activity Calculation

One unit of soybean DNase was defined as the amount of enzyme which liberated acid-soluble nucleotides from acid insoluble polynucleotides at a rate of ΔA_{260} nm 0.005/45 min at 37°C. The following equation was utilized in calculation of activity. Units = (ΔA_{260}/0.005) x (ml total volume) / (ml enzyme sol.assayed) x min.

Protein assay

Protein was estimated according to the method of Bradford (1976), using the microanalysis protein kit of Bio-Rad. Bovine serum albumin was used as a standard.

Endonuclease Activity Indicated on DNA- Gel Assay

Endodeoxyribonuclease activity was assayed on 1% agarose gels using covalently closed circular pBR322 DNA. The soybean DNase was incubated in the presence of 10 mM Tris-$MnCl_2$ buffer (pH 7.0) containing 2.5 µg of pBR322 DNA in a total volume of 30 µl for 45 min at 4°C. The enzyme-catalysed reaction was terminated by adding 3 µl of the killing buffer as mentioned

before. Reaction samples were electrophoresed in 1% (w/v) agarose gels for 1 hr and 20 min at 100 volts. The DNA gel was stained with ethidium bromide (1 μg/ml) for 5 min and then de-stained with 300 ml of destilled water for 45 min with each rinse (repeated three times). DNA bands were visualized on a UV light box and then photographed.

Characterization of Endodeoxyribonuclease Activity

Soybean DNase activity was determined by Kunitz's assay as described previously. The assay product was applied to a Sephadex G-10 gel filtration column to identify endonuclease-generated oligonucleotides. Sephadex G-10 resin was prepared, poured and equilibrated in the same manner as mentioned before. The column (1.5 x 14.5 cm) was calibrated with the following: The void volume of the column was determined by using blue dextran with M.W. 2000 KDa. HMW soybean DNA and adenosine triphosphate (ATP) were used as controls. The blue dextran peak was visualized by eye, the HMW DNA peak, oligonucleotides peak and ATP peak were collected separately and the absorbances read at A_{260} nm with a Gilford Response Spectrophotometer.

RESULTS

Isolation and purification of soybean DNA

A high yield of large-molecular-weight (HMW) soybean DNA, essentially free from protein, RNA and other contaminates, has been isolated and purified from etiolated soybean seeds.

The results of a spectrophotometric scan of the purified soybean DNA at various wavelengths are shown in Figure 3. The average of DNA readings at A_{260} nm and A_{280} nm was 1.7 and 0.9 respectively. The purity of the DNA was based on the ratio of the absorbance at A_{260} nm/A_{280} nm (Kamalay et al., 1990), they typically showed a purity of 1.9 (A_{260} nm of 1.7/A_{280} nm of 0.9 = 1.9). The yield of the purified DNA preparations was determined spectrophotometrically (A_{260} nm of 1.0 = 50µg/ml of DNA). The typical yield was 659 µg/ml, when using 20 grams of hypocotyls. These values were based on the average of five purifications and were found to vary only slightly between preparations.

The size of soybean DNA fragments was estimated using 1% agarose gel electrophoresis. DNA bands were detected by ethidium bromide fluorescence. The migration pattern of the soybean DNA indicated that the top bands were HMW DNA as shown in Figure 4. In addition, staining by EB was directly proportional to the amount of DNA as indicated in Figure 5.

In order to obtain HMW DNA fractions and eliminate small fragments, the DNA solution was subjected to Sephadex chromatography.

Figure 3. Typical spectrophotometric scan of purified soybean DNA. The DNA sample diluted 1: 10 was scaned in the range of 220 to 300 nm. The average of DNA readings at A_{260} nm and A_{280} nm was 1.7 and 0.9 respectively, using 20 g of hypocotyls.

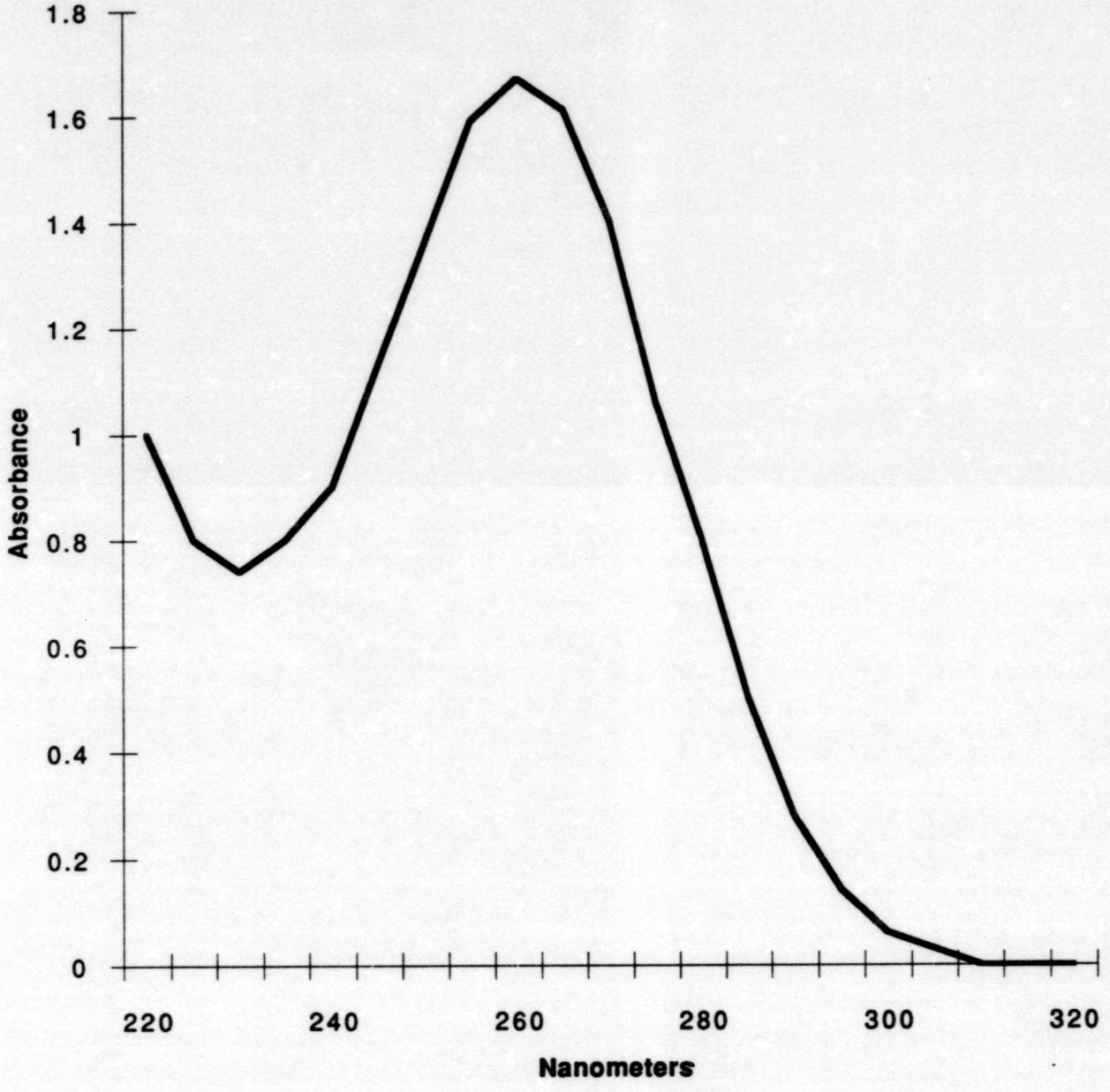

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
220
240
260
280
300
320
Nanometers

Figure 4. Determination of the relative size of soybean DNA fragments. Each lane contained 3.2 μg DNA in 30 μl of TE buffer (pH 7.0).

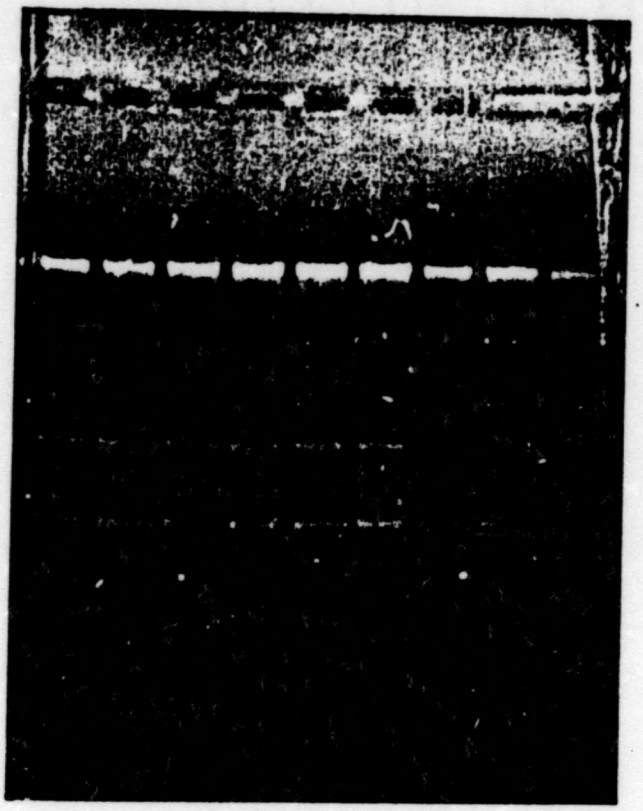
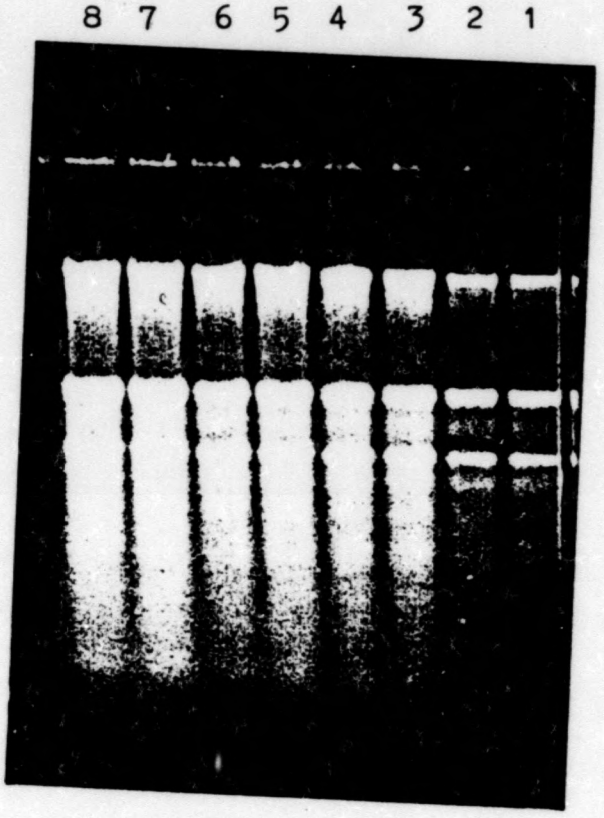


Figure 5. Effects of DNA concentration on gel staining. Duplicated lanes contained 3.2, 6.4, 9.6 and 12.8 μg DNA in 30 μl of TE buffer per lane from lane 1 to 8.


8 7 6 5 4 3 2 1

Following a 1: 10 dilution, the DNA was loaded on a Sephadex G-10 column (1.5 x 15 cm). Fractions were monitored at A_{260} nm and two peaks were collected. The first peak (Figure 6) HMW DNA fraction was eluted in the void volume (calibrated by using blue dextran with molecular weight of 2000 KDa). The two fractions with the highest absorbance at A_{260} nm were saved to be used as substrate for enzyme assays. The second small peak indicated the presence of deoxyribonucleotides and was discarded.

Isolation and Purification of Soybean DNase

Purified nuclei were prepared from 100 g of hypocotyl tissue from etiolated soybean seeds, followed by homogenization, centrifugation and solubilization of proteins from DNA. The supernatant from centrifugation (2100 x g) of solubilized nuclei fraction was designated as a crude homogenate. Two small aliquots of the crude homogenate fraction were saved and assayed for protein concentration and enzyme activity. The average protein concentration was 800 µg/ml. The other aliquot was assayed for enzyme activity at A_{260} nm using the Kunitz method and resulted in a typical specific activity of 0.28 units/µg. These data were based on four purifications and were found to vary only slightly between purifications.

The remainder of the crude homogenate fraction was then applied to a Sephadex G-75 column (2.5 x 2.5 cm) for further purification. Collected fractions were monitored at A_{280} nm and the resulting two protein peaks (peak$_A$ and $_B$) were collected and assayed for protein concentration and enzyme activity (Figure 7). The typical protein concentration of G-75 peak$_A$ was 700 µg /ml with a specific activity of 0.2 units/µg of protein. A typical

Figure 6. Sephadex G-10 chromatography of purified soybean DNA. The fractions were monitored at A_{260} nm and two peaks collected. The first peak eluted in the void volume which was calibrated using blue dextran(2000 KDa)

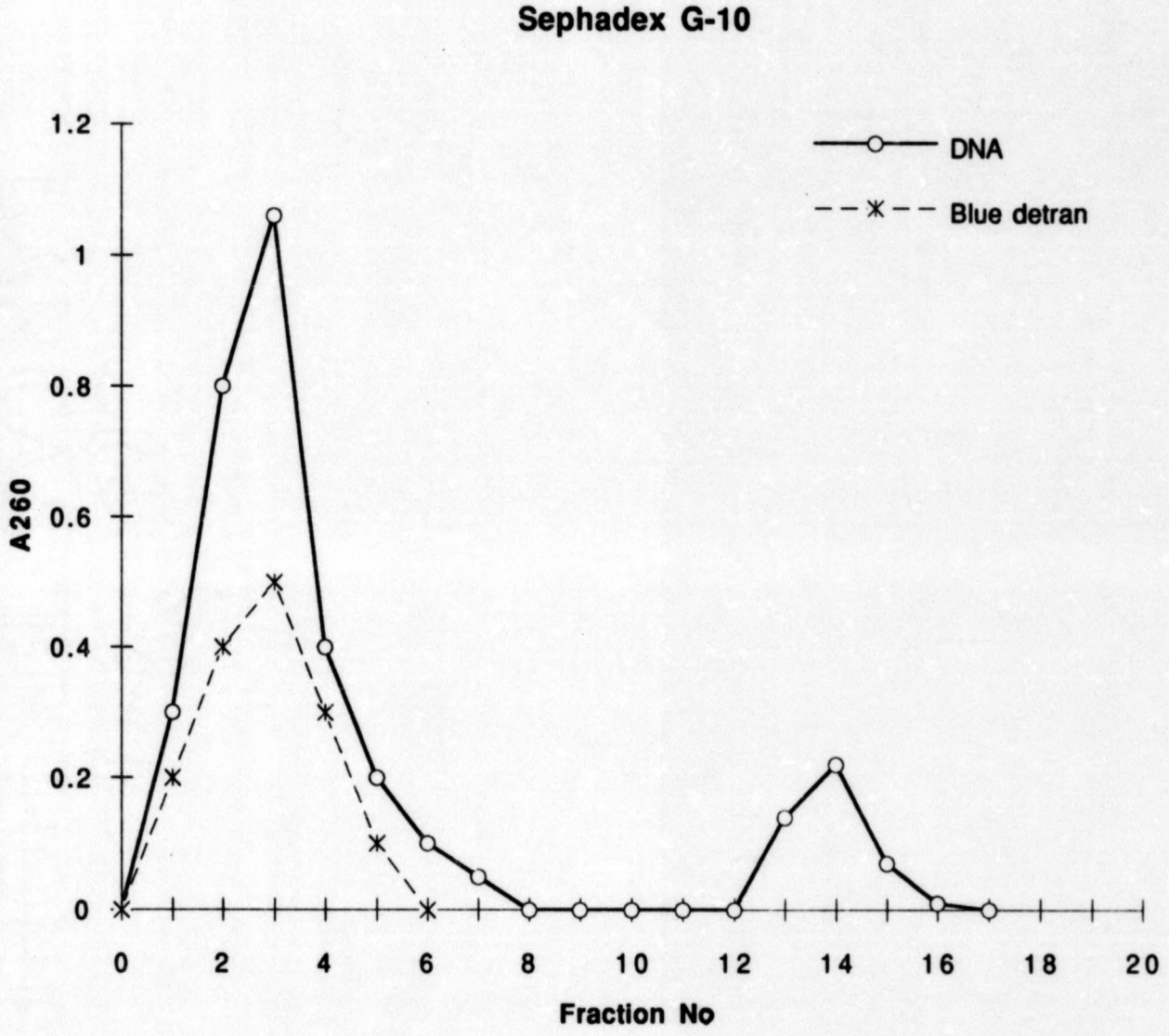
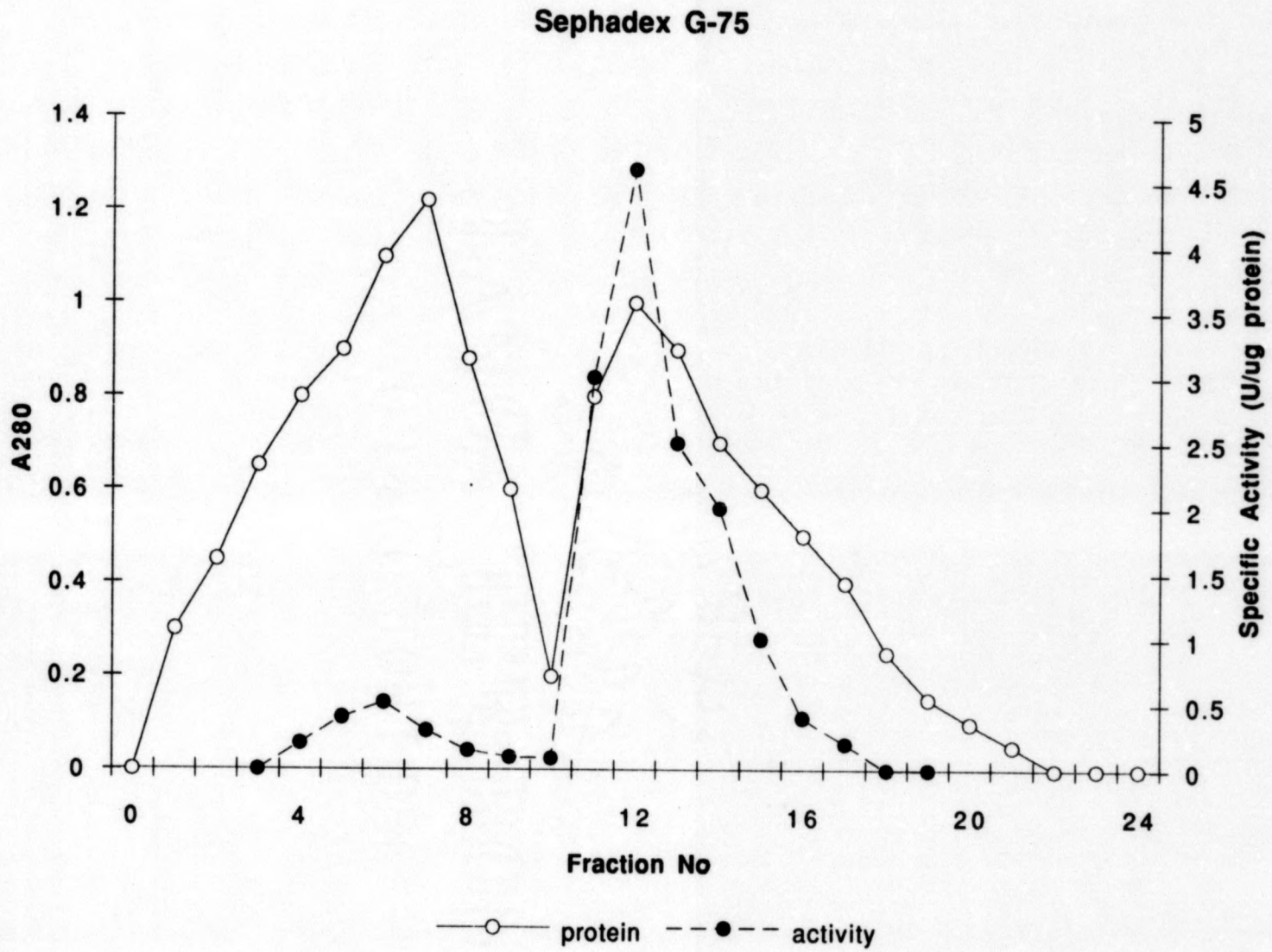

Sephadex G-10
DNA
Blue detran
A260
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
20
Fraction No

Figure 7. Sephadex G-75 chromatography of crude homogenate.
Sixteen ml of crude homogenate from a solubilized nuclei fraction were applied to a 2.5 x 1.5 cm column. The fractions were monitored for protein at A_{280} nm and then two protein peaks$_A$ and $_B$ were collected. DNase activity was assayed at A_{260} nm (Kunitz, 1950).


Sephadex G-75
A280
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Specific Activity (U/ug protein)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Fraction No
protein
activity

protein concentration of peak$_B$ was 32 µg /ml with a specific activity of 4.6 units/µg of protein. These experimental results indicated that 96% of enzymatic activity was eluted in the G-75 peak$_B$.

Similar results were obtained using DNA-gel assays. Fractions of protein peak$_A$ and $_B$ were assayed respectively and then the assay products were applied to 1% agarose gel electrophoresis. Enzyme activity was assayed for peak$_A$ shown in Figure 8. Changes of DNA bands in lane three indicated that G-75 protein peak$_A$ contained DNase activity at a DNase to DNA ratio of 1: 3 (hydrolytic ratio of the amount of DNase to DNA). Enzyme activity of G-75 protein peak$_B$ was assayed using the assay conditions as above (Figure 9). G-75 protein peak$_B$ degradated DNA in lane 5 at a DNase to DNA ratio of 1: 25. These data taken together demonstrated that the proteins containing the majority of enzymatic activity were eluted in the second protein peak of the Sephadex G-75 column and the molecular weight of the enzyme was below 75 KDa.

The next step of purification was anion exchange chromatography. Fourteen ml of G-75 protein peak$_B$ were loaded on a DEAE-cellulose column (2.5 x 20 cm). Each of the resulting three peaks were collected and assayed for enzyme activity. As shown in Figure 10, when the assay products of DEAE protein peak$_1$ and $_2$ were indicated in gel A, there were no obvious differences between DNA bands of control lanes and enzyme-catalysed reaction lanes. Whereas DEAE protein peak$_3$, (Figure 12) DNase activity in lane 4 at a DNase to DNA ratio of 1: 14. The ratio was reduced when compared to previous data of G-75 protein peak$_B$ which had a ratio of 1: 25. This would indicate that enzyme activity of proteins was partially inhibited by high salt concentration because the tightly bound DEAE protein peak$_3$ was

Figure 8. DNA-gel assay of soybean DNase activity. Soybean DNase activity of protein peak$_A$ from Sephadex G-75 chromatography was indicated on 1% agarose gel electrophoresis.

	Lane No.							
	1	2	3	4	5	6	7	8
DNase (μg)	0.4	0.4	1.1	1.9	2.6	3.3	4.1	--
Soybean DNA (μg)	--	3.3	3.3	3.3	3.3	3.3	3.3	3.3

Lane 1 minus DNA as control, Lane 8 minus DNase as control. Lanes 2 to 7 contained increasing amounts of protein.

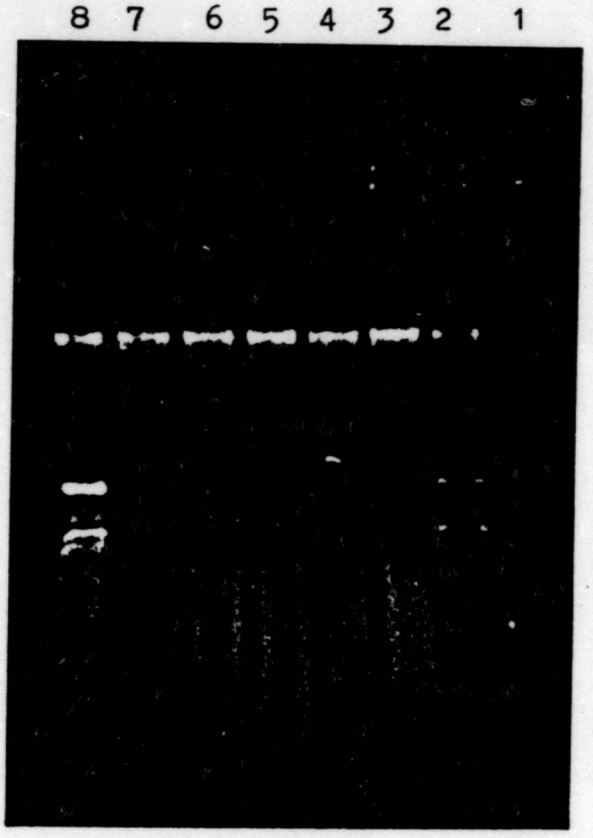

8 7 6 5 4 3 2 1

Figure 9. DNA-gel assay of soybean DNase activity. Soybean DNase activity of protein peak$_B$ from Sephadex G-75 chromatography was indicated on 1% agarose gel electrophoresis.

	Lane No.							
	1	2	3	4	5	6	7	8
DNase (μg)	0.05	0.05	0.09	0.11	0.14	0.18	0.25	--
Soybean DNA (μg)	--	3.3	3.3	3.3	3.3	3.3	3.3	3.3

Lane 1 minus DNA as control, Lane 8 minus DNase as control. Lanes 2 to 7 contained increasing amounts of protein.

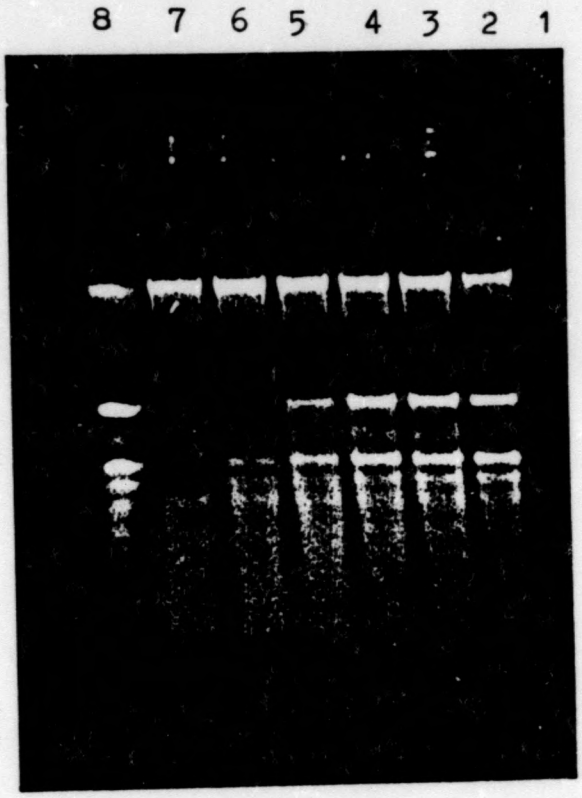

8 7 6 5 4 3 2 1

Figure 10. DNA- gel assay of DNase eluted from DEAE-cellulose protein peak1 and 2. DNase activity of Protein peak1 and 2 from DEAE-Cellulose chromatography was indicated on 1% agarase gel electrophoresis.

	Lane No.							
	1	2	3	4	5	6	7	8
DNase (μg)	0.18	0.23	0.3	--	0.18	0.23	0.3	--
Soybean DNA (μg)	--	3.3	3.3	3.3	--	3.3	3.3	3.3

Lane 1 and 5 minus DNA as controls, Lanes 4 and 8 minus DNase as controls. Lanes 1, 2 and 3 containing protein peak$_1$. Lane 5, 6 and 7 containing protein peak$_2$.

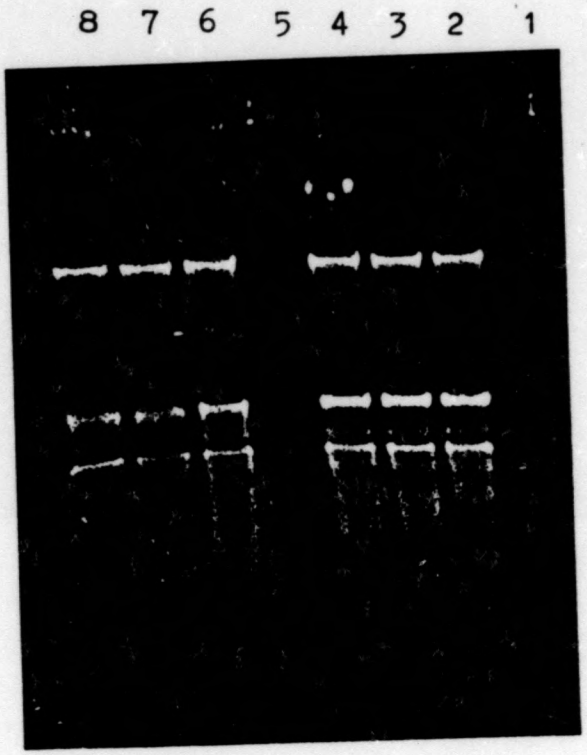

8 7 6 5 4 3 2 1

Figure 11. DNA- gel assay of DNase eluted from DEAE-cellulose. Protein $peak_3$ assay products were applied to 1% agarase gel electrophoresis.

	Lane No.						
	1	2	3	4	5	6	7
DNase (μg)	0.14	0.14	0.14	0.18	0.23	--	--
Soybean DNA (μg)	--	--	3.3	3.3	3.3	3.3	3.3

Lane 1 and 2 minus DNA as controls, Lane 6 and 7 minus DNase as controls. Lane 3 to 5 contained increasing amounts of DNase.

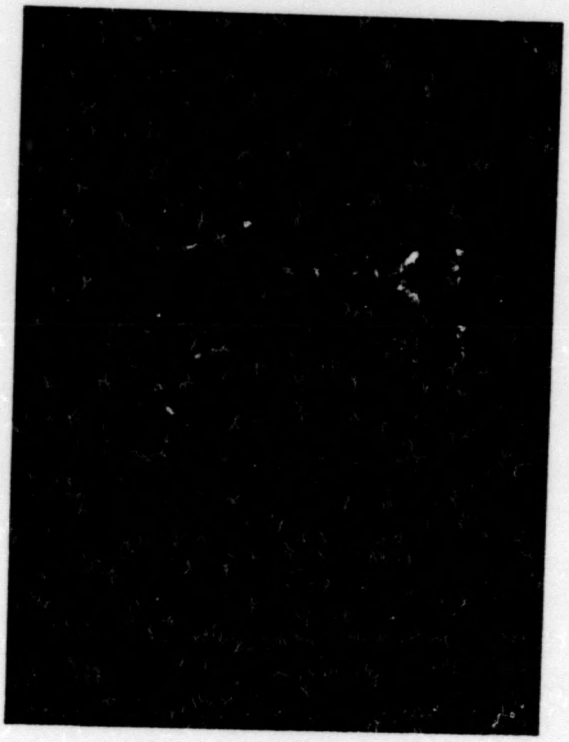

7 6 5 4 3 2 1

Figure 12. Effect of increasing enzyme concentration on DNase activity in the presence of KCl. The DEAE protein peak3 (eluted with 0.6M KCl) assay products were applied to 1% agarase gel electrophoresis.

	Lane No.						
	1	2	3	4	5	6	7
DNase (μg)	0.12	0.12	0.12	0.23	0.35	--	--
Soybean DNA (μg)	--	--	3.3	3.3	3.3	3.3	3.3

Lane 1 and 2 minus DNA as controls, Lane 6 and 7 minus DNase as controls. Lane 3 to 5 contained increasing amounts of protein.

7 6 5 4 3 2 1



eluted with a 0.6 M KCl step gradient. The pooled DEAE protein peak$_3$ fraction was then applied to a Sephadex G-25 column for desalting. The desalted DEAE protein peak3 was reassayed and the assay product was indicated on the 1% agarose gels containing DNA (3.3 µg/lane, Figure 13). The desalted DNase began to digest DNA in lane five and the hydrolytic ratio of the amount of DNase to DNA greatly increased to 1: 94. Spectrophotometric assays indicated that the specific activity of the protein peak3 was 13.6 units/µg of protein with a purification of 49-fold (Figure 14). Little enzyme activity was found in either DEAE protein peak$_1$ and $_2$. These results taken together indicated that DEAE-cellulose chromatography improves enzyme purity and that KCl inhibited enzyme activity.

Desalted DEAE protein peak3 pooled fraction (9 ml) was then applied to a CM Sephadex G-50 column (2.5 x 15 cm). Only one protein peak containing enzymatic activity was eluted with CM buffer (pH 6.0). The CM protein peak fractions (30 ml) containing enzyme activity were pooled and then concentrated to 5 ml by using a concentrator as described earlier. Spectrophotometric assays indicated the specific activity of CM enzyme fraction to be 41.6 units/µg of protein with a purificationof 148-fold (Figure 15). In addition, CM enzyme assay product was subjected to 1% agarose gels containing soybean DNA (6.9 µg of substrate per lane). The separation band in lane six was accomplished with a ratio of enzyme to DNA of 1:184 (Figure 16). The pooled, concentrated protein fraction was applied to non-denaturing polyacrylamide gels. One protein band was visible on the gel as indicated in Figure 17. Three areas of the gel, band area and areas above and below the protein band were cut out from the non-stained gel. The excised gel slices were individually electro-eluted. The electro-eluted fraction from the protein band slice

Figure 13. Effect of increasing enzyme concentration on DNase activity in the abcence of KCl. Desalted DEAE protein peak3 assay products were applied to 1% agarase gel electrophoresis.

	Lane No.							
	1	2	3	4	5	6	7	8
DNase (μg)	0.005	0.005	0.015	0.025	0.035	0.05	--	--
Soybean DNA (μg)	--	3.3	3.3	3.3	3.3	3.3	3.3	3.3

Lane 1 minus DNA as control, Lane 7 and 8 minus DNase as controls. Lane 2 to 6 contained increasing amounts of protein.

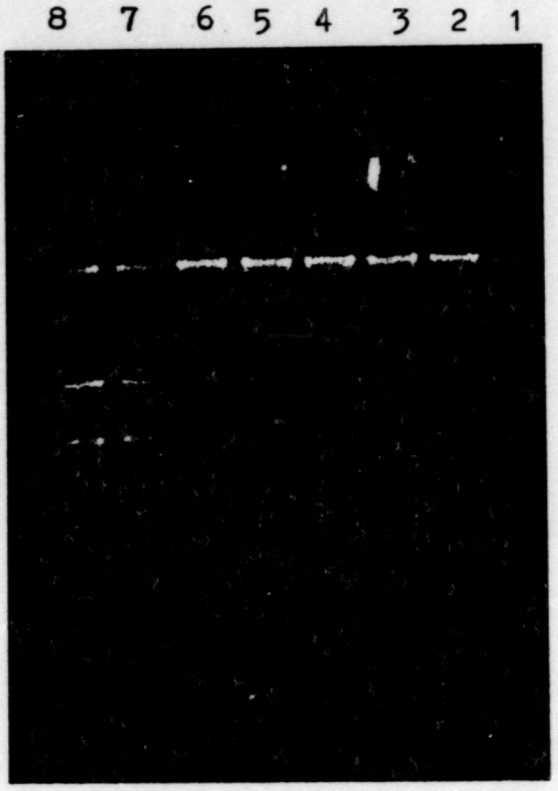

8 7 6 5 4 3 2 1

Figure 14. Anion exchange chromatography of soybean DNase activity. Forteen ml of pooled protein peak$_B$ eluted from Sephadex G-75 were applied to the column. Proteins were eluted with a KCl step gradient (0.1 and 0.6 M KCl). Fractions were monitored for protien at A_{280} nm and assayed for DNase activity at A_{260} nm spectrophotometrically (Kunitz, 1950).

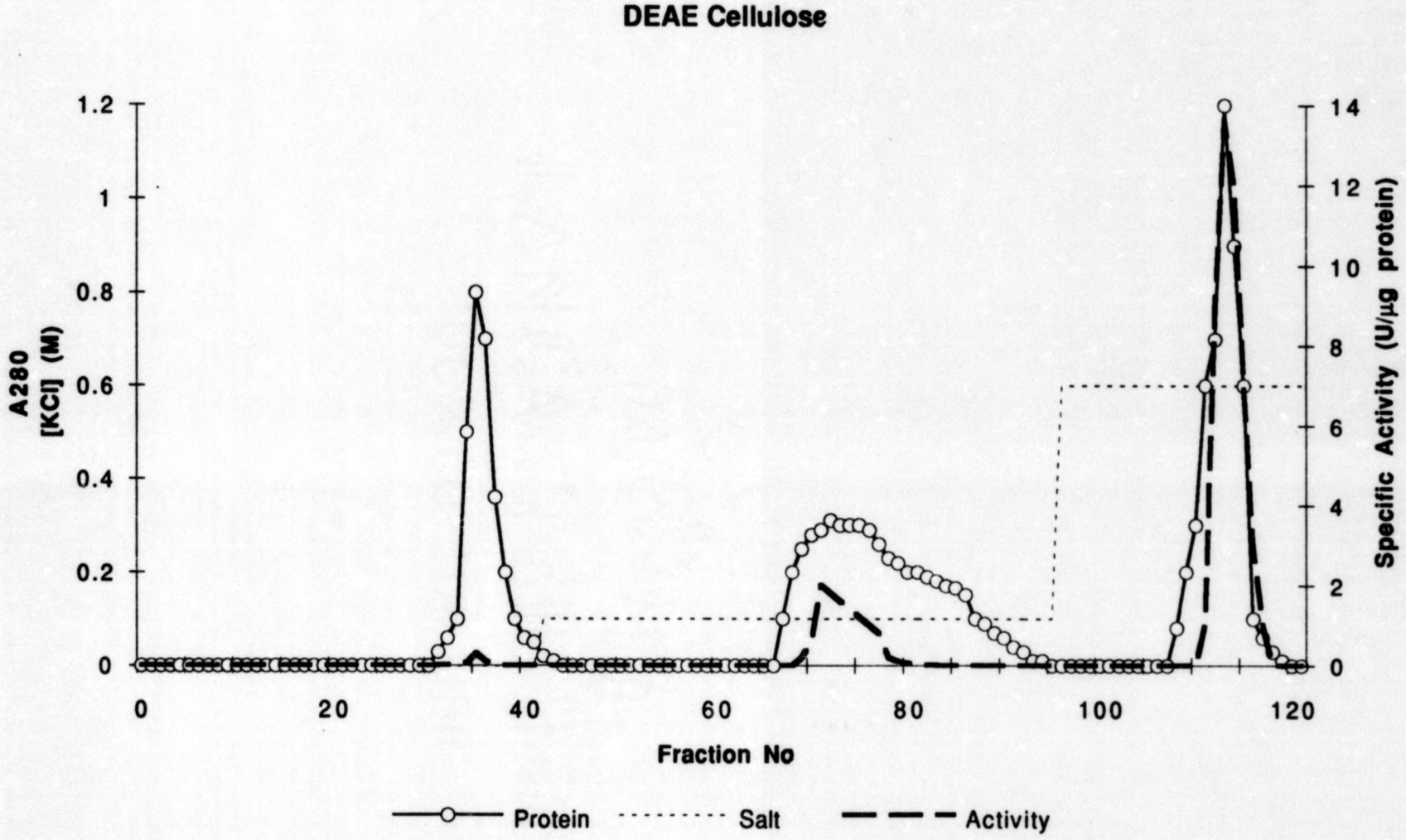

DEAE Cellulose
A280
[KCl] (M)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
Fraction No
14
12
10
8
6
4
2
0
Specific Activity (U/µg protein)
Protein
Salt
Activity

Figure 15. Cation exchange chromatography of soybean DNase activity. Pooled desalted DEAE protein peak$_3$ (9 ml) was applied to a CM sephadex G-50 column (2.5 x 15 cm).

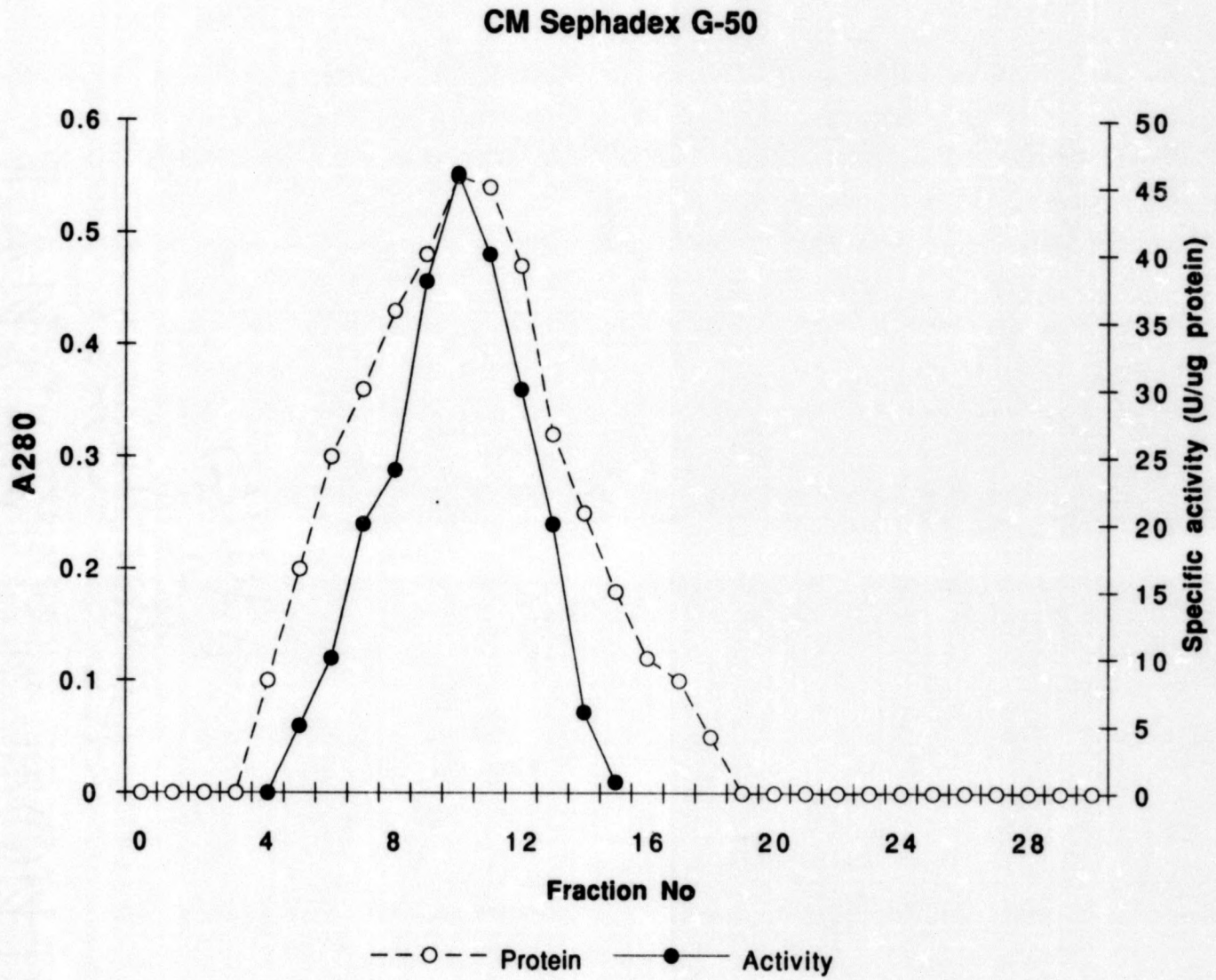

CM Sephadex G-50
A280
0.6
0.5
0.4
0.3
0.2
0.1
0
Specific activity (U/ug protein)
50
45
40
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
28
Fraction No
Protein
Activity

Figure 16. DNA-gel assay of DNase eluted from CM Sephadex chromatography. CM sephadex enzyme assay products were applied to 1% agarose gel electrophoresis.

	Lane No.							
	1	2	3	4	5	6	7	8
DNase (μg)	0.04	0.04	0.12	0.19	0.26	0.38	–	–
Soybean DNA (μg)	–	6.9	6.9	6.9	6.9	6.9	6.9	6.9

Line 1 minus DNA as a control. Line 7 and 8 minus DNase as controls. Lane 3 to 7 contained inceasing amounts of protein.

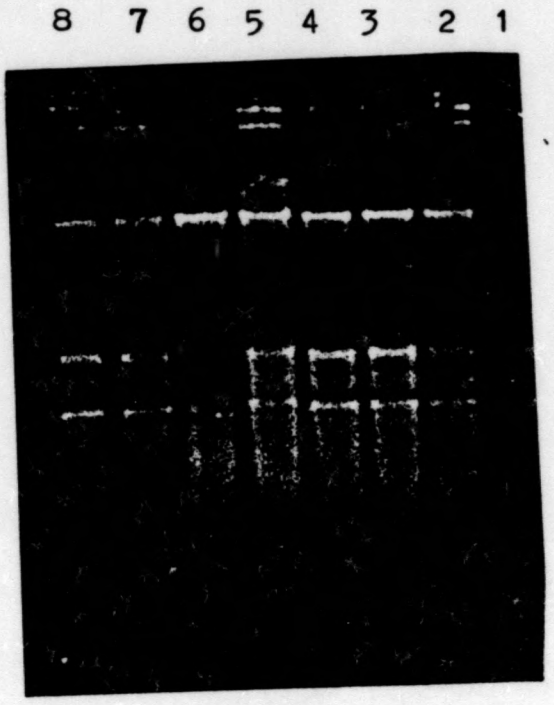
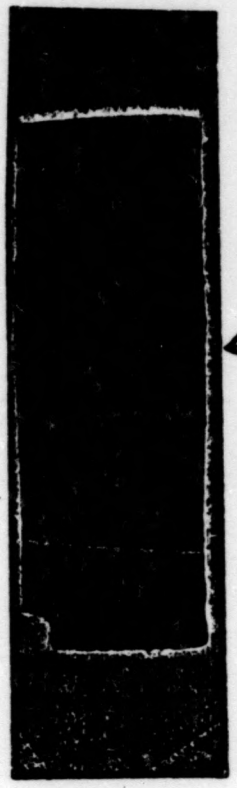

8 7 6 5 4 3 2 1

Figure 17. Non-denaturing Polyacrylamide gel electrophoresis (PAGE) of Soybean DNase. 0.08 μg of DNase per lane.


Protein band

exhibited enzyme activity, whereas the electro-eluted solutions from gel areas of above and below the band had no enzyme activity (Table 5). The most significant increase in purification was obtained in this step which resulted in a 554-fold purification with a yield of 6% of the enzyme (Table 6).

The purified protein, which appeared as a single band on non-denaturing protein gels, had a molecular weight of 60 KDa (Figure 18).

Kinetic Assays of Purified Soybean Deoxyribonuclease

In order to further characterize the soybean DNase activity of the electro-eluted protein, the effects of varying times, enzyme concentrations and the various substrates were assayed.

The purified DNase was assayed at varying times as shown in Figure 19. The hyperbolic saturation curve indicated that the initial reaction velocity was linear through 20 min.

The purified DNase was assayed at various enzyme concentrations. The results (Figure 20) indicated that there was a linear relationship between enzyme concentration and activity.

The effect of varying substrate concentrations at a constant enzyme concentration (0.05 µg/ml of DNase) is indicated in Figure 21. Saturation of enzyme with substrate occured at 10 µg/ml of soybean DNA. Using a Lineweaver-Burk plot, the enzyme had a K_m for soybean DNA of 4.5 µg/ml and a V_{max} of 0.02 ΔA_{260}/min (Figure 22).

Table 5. DNase activity electro-eluted from a non-denaturing polyacrylamide gel.

Gel Slices	Protein Concentration µg/ml	Specific Activity units/µg
Protein band	0.9	155.2
Area above band	0.0	0.0
Area below band	0.0	0.0

Table 6. Summary of purification of soybean deoxyribonuclease.

Purification step	Total volume ml	Protein µg /ml	Total protein µg	Total activity units	Specific activity units/µg	Purification Fold	Yield %
Crude Homogenate	18	780	14040	3948	0.3	1	100
Sephadex G-75	15	31	469	2169	4.6	16	55
DEAE-cellulose desalted peak$_3$	12	6	72	956	13.6	49	24
CM-sephadex C-50	5	4	19	807	41.6	148	20
Electro-eluted protein	2	1	2	224	155.2	554	6

Total DNase activity was determined in the presence of 0.1 M Tris-HCl buffer (pH 7.0) containing 5 mM $MnCl_2$ and soybean DNA 50 µg/ml incubated in 37°C for 45 min. 1 unit = 0.005 ΔA_{260}/45 min.

Figure 18. Non-denaturing PAGE for molecular weight determinaton of purified soybean DNase. Each lane of enzyme had 0.08μg of proteins. Accompaning molecular weight markers are: Bovine (66 KDa), Chicken Egg (45 KDa), Dehydrogenase (36 KDa), Carbonic Anhydrase (29 KDa), and a-Lactalbumin (14 KDa).

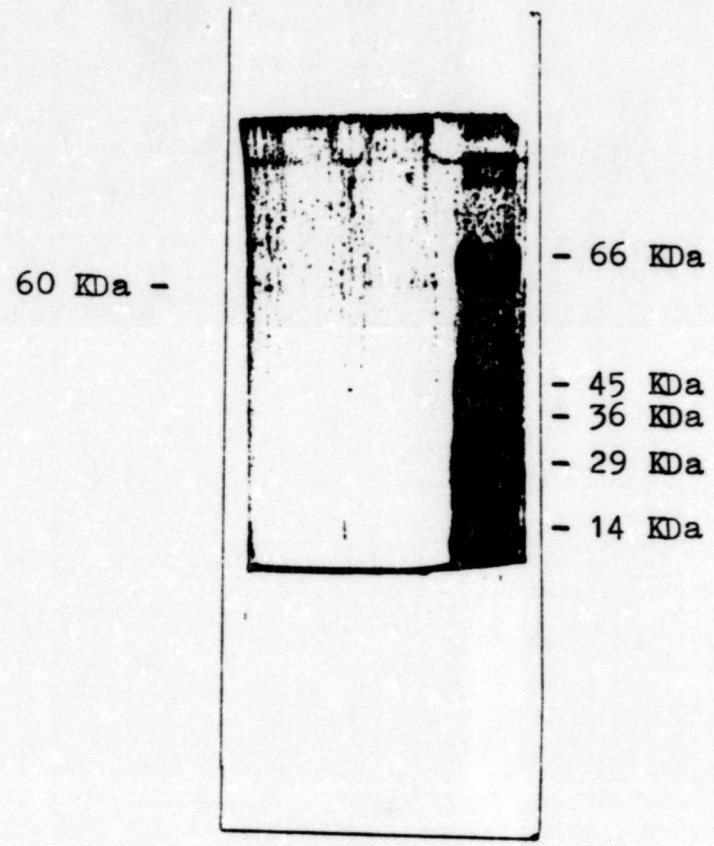

60 KDa -
- 66 KDa
- 45 KDa
- 36 KDa
- 29 KDa
- 14 KDa

Figure 19. Kinetic study of purified soybean DNase. Soybean DNase (1μg/ml) was incubated in the presence of 0.1 M Tris-$MnCl_2$ buffer (pH 7.0) containing soybean DNA (50 μg/ml) at 37°C.

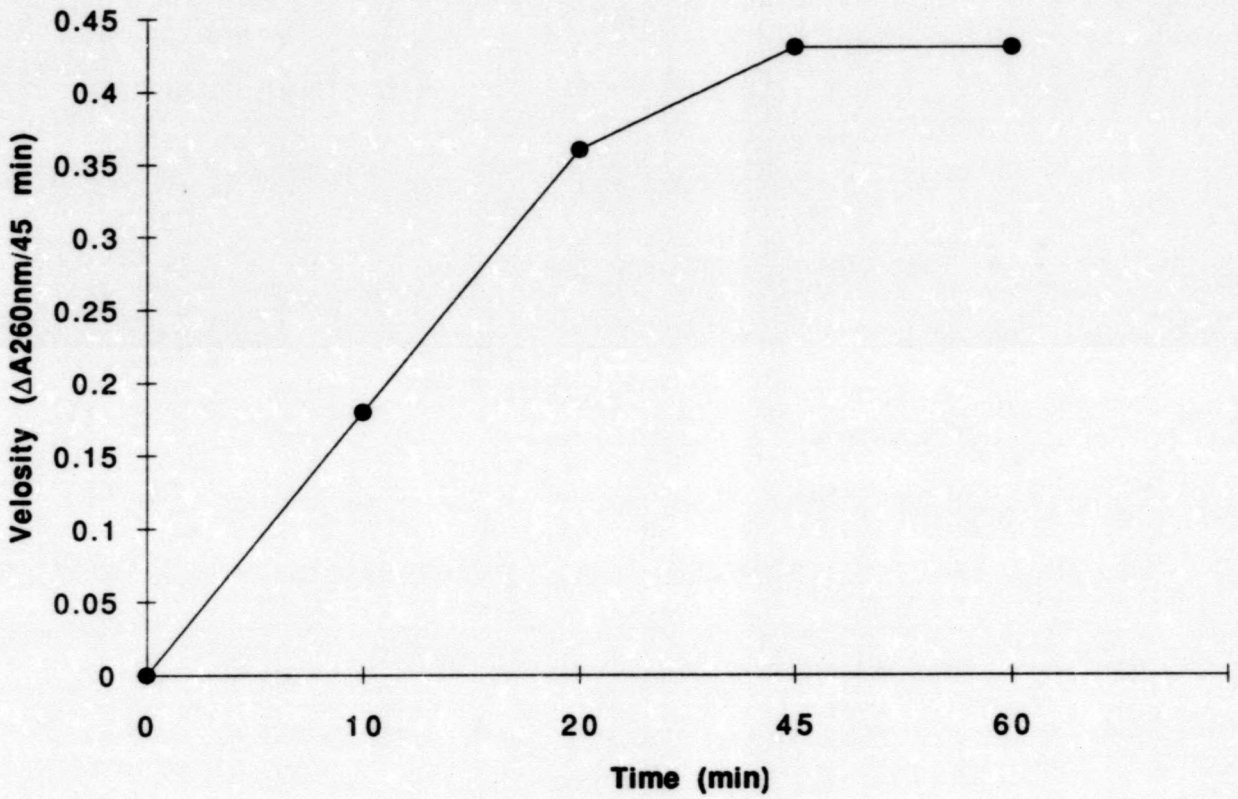

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Velosity (ΔA260nm/45 min)
0
10
20
45
60
Time (min)

Figure 20. The effect of protein concentration on DNase activity. Enzyme was assayed as described in Materials and Methods.

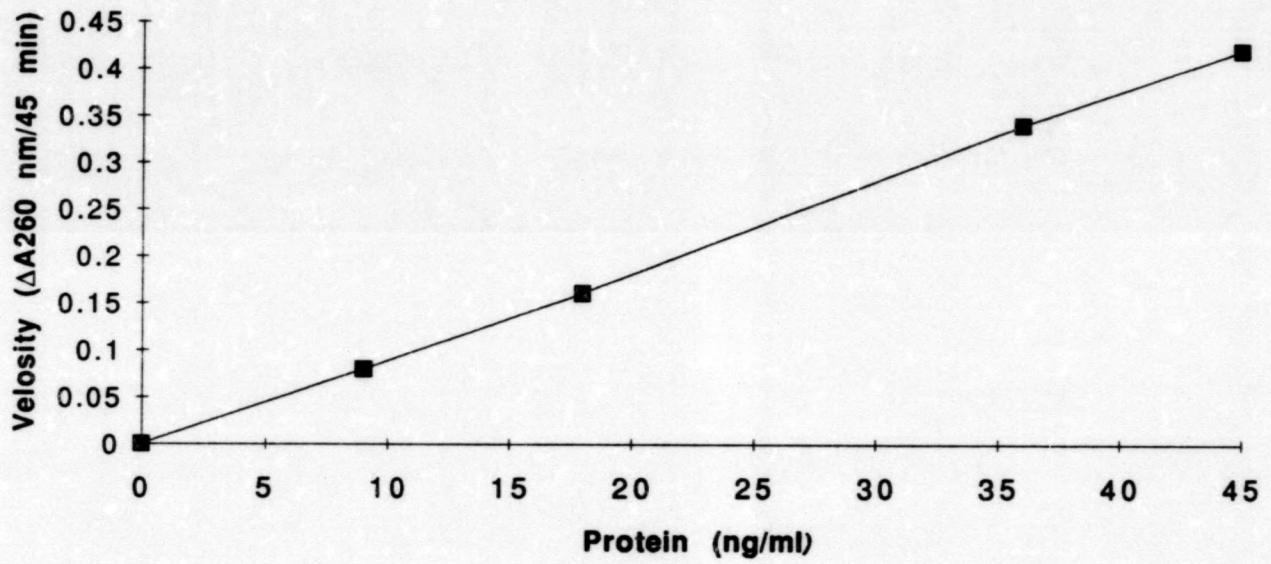

Velosity (ΔA260 nm/45 min)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
40
45
Protein (ng/ml)

Figure 21. The effect of substrate concentration on DNase activity. Each assay tube contained 0.1 µg/ml of soybean DNase.

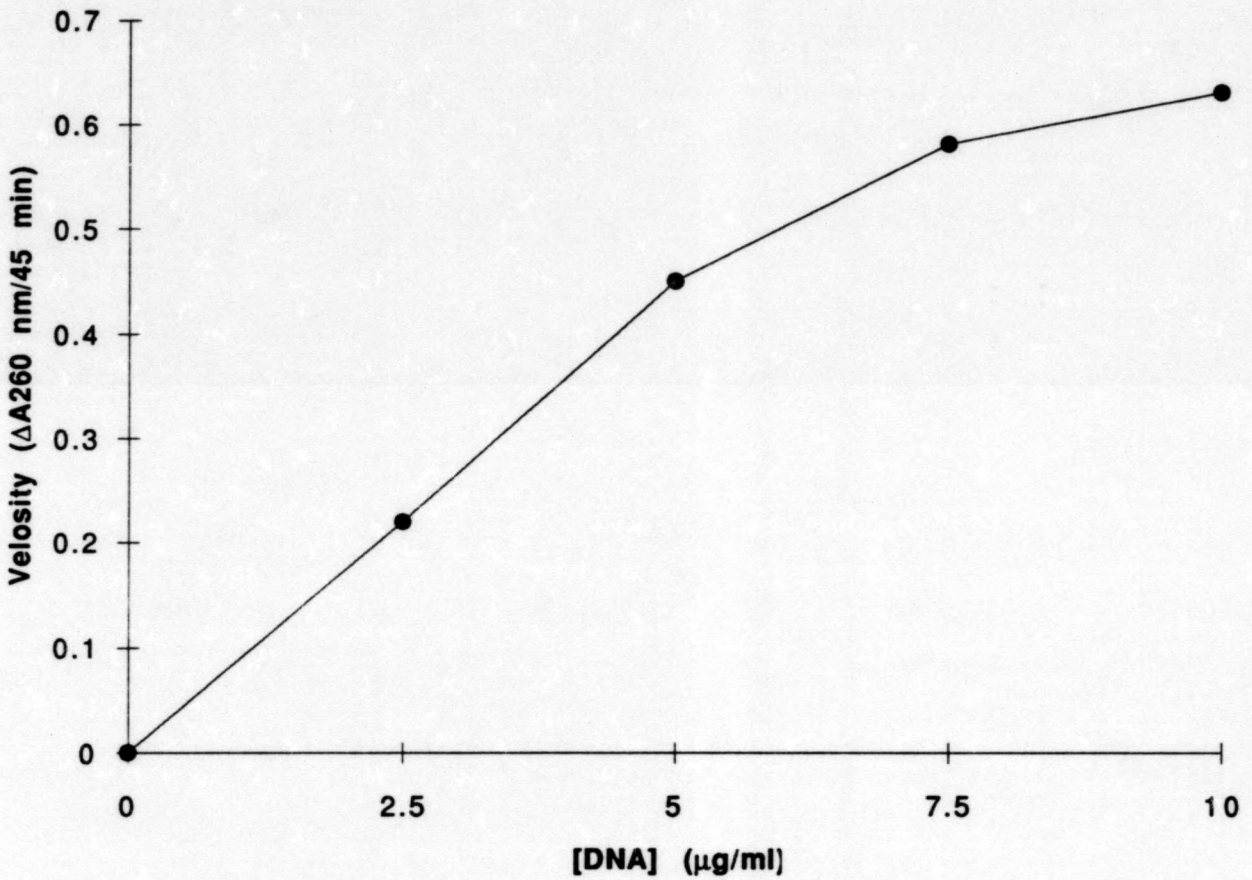
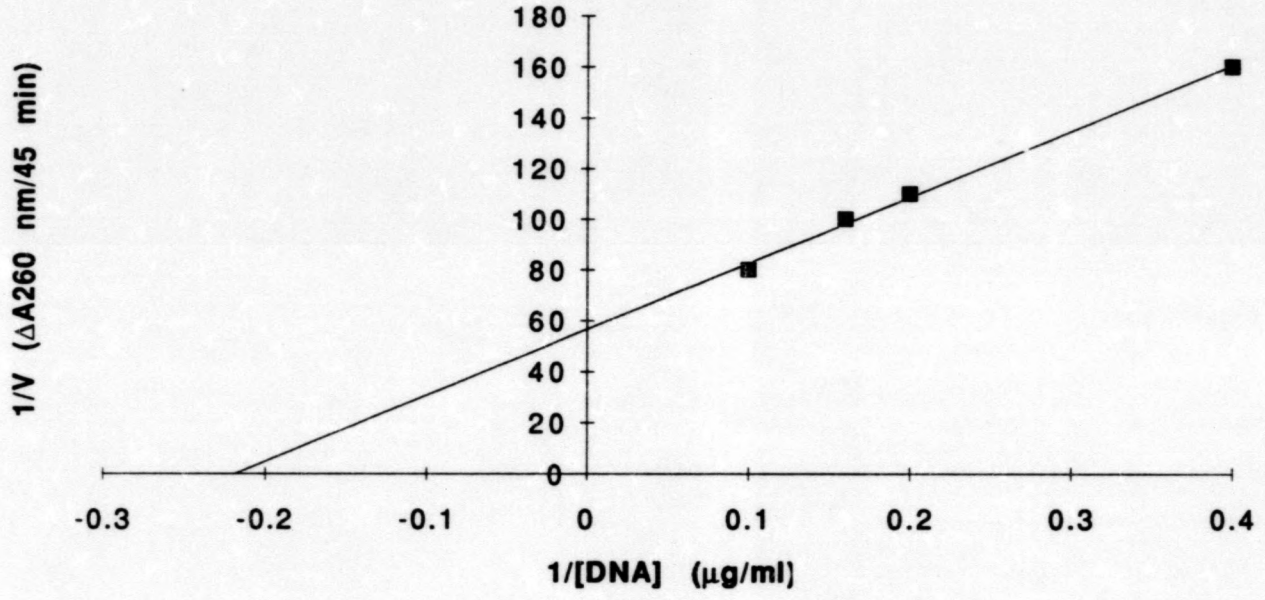

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Velosity (ΔA260 nm/45 min)
0
2.5
5
7.5
10
[DNA] (μg/ml)

Figure 22. Lineweaver-Burk plot of purified DNase using soybean DNA as substrate. Each assay tube contained 0.1 μg/ml of soybean DNase.


180
160
140
120
100
80
60
40
20
0
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
1/V (ΔA260 nm/45 min)
1/[DNA] (μg/ml)

Characterization of DNase Catalytic Specificity

Interaction of the enzyme with substrate was characterized by endodeoxyribonuclease assays using covalently closed circular pBR322 DNA (assay condition as described in Materials and Methods). The second DNA band from the top in lanes 4, 5 and 6 were progressively degradated with the increasing amount of DNase, whereas the third band of DNA in lanes 4, 5 and 6 were correspondingly increased when compared to controls (Figure 23). The results clearly indicated that the covalently closed circular pBR322 DNA was degraded into oligonucleotides indicating the presence of an endonuclease.

In addition, soybean endonuclease activity was evidenced by spectrophotometric analysis of enzymatic assay products following separation by Sephadex G-10 chromatography (Figure 24). HMW DNA fragments were eluted in the void volume which was calibrated using blue dextran (molecular weight of 2,000 KDa), a second peak of DNA (small molecular weight) was eluted in the total volume. The assay product was eluted between the HMW DNA fragments and ATP. The products of the assay were oligonucleotides generated from HMW soybean DNA fragments by the action of an endodeoxyribonuclease.

Substrate Specificity Studies

Substrate specificity studies on the purified DNase indicated a preference for soybean DNA and calf thymus DNA with no activity on yeast RNA. In addition, the enzyme had a slight preference for denatured soybean DNA and calf thymus DNA when compared to native DNAs (Table 7). The

Figure 23. Soybean endodeoxyribonuclease activity using covalently closed circular pBR322 DNA as substrate. Assay condition as described in Materials and Methods.

Lane No.	1	2	3	4	5	6	7	8
DNase (μg)	0.015	0.015	0.025	0.075	0.1	0.125	--	--
pBR322 DNA (μg)	--	2.5	2.5	2.5	2.5	2.5	2.5	2.5

Lane 1 minus DNA as control, Lane 7 and 8 minus DNase as controls. Lanes 2 to 7 contained increasing amounts of protein.

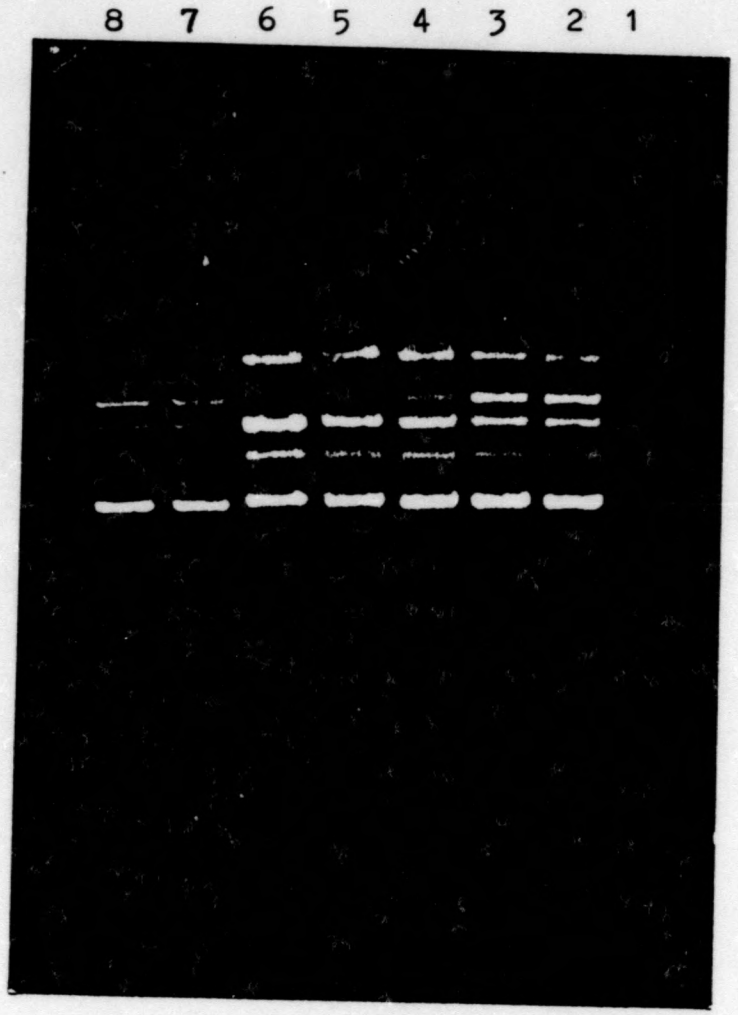

8 7 6 5 4 3 2 1

Figure 24. Characterization of endodeoxyribonuclease activity. Blue dextran, HMW soybean DNA, oligonucleotides (acid soluble assay product), and ATP were used to calibrate the Sephadex G-10 column.

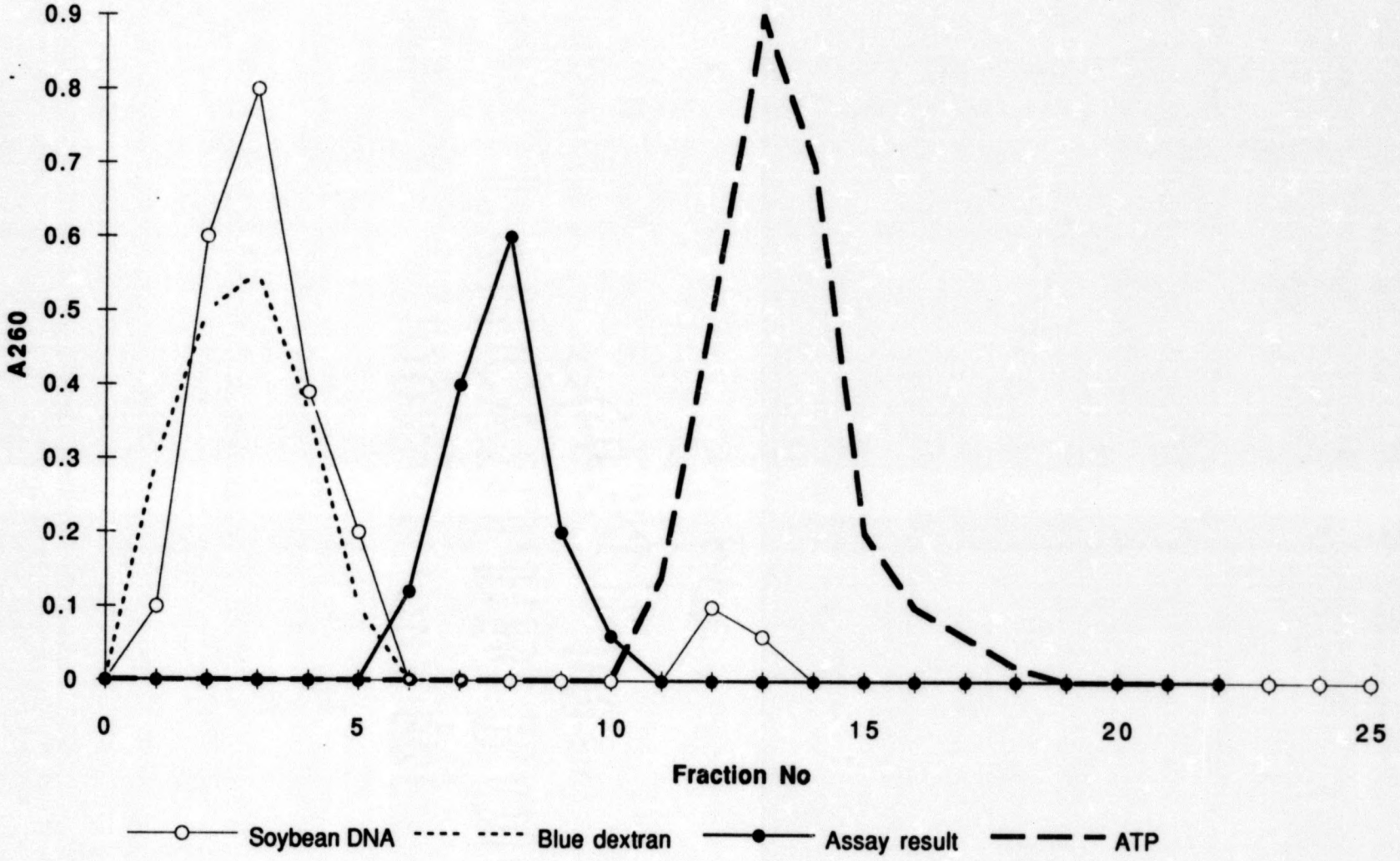

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A260
0
5
10
15
20
25
Fraction No
Soybean DNA
Blue dextran
Assay result
ATP

Table 7. Substrate Specificity of Soybean DNase.

Substrate	Concentration µg /ml	Relative activity %
Native soybean DNA	25	100
Denatured soybean DNA	25	115
Native calf thymus DNA	25	67
Denatured calf thymus DNA	25	87
Yeast RNA	25	0.0

Soybean DNase activity was determined in the presence of Tris-HCl buffer (pH 7.0) containing 5 mM $MnCl_2$ and substrate as indicated at 37° C for 45 min.

assay result showed that the heat denatured DNase lost the ability to hydrolyze DNA (Table 8).

Table 8. Effect of heat denaturation soybean DNase activity

Substrate	Concentration µg/ml	enzyme activity units/µg
Soybean DNA	25	0.0

Heat denatured soybean DNase activity was assayed in the presence of Tris-HCl Buffer (pH 7.0) containing 5 mM $MnCl_2$ and substrate as indicated at 37°C for 45 min.

DISCUSSION

A deoxyribonuclease has been isolated from a purified preparation of soybean nuclei. The purification procedure, involving solubilization of proteins from DNA, Sephadex G-75, DEAE-cellulose, and CM-Sephadex chromatography and non-denaturing polyacrylamide gel electrophoresis, resulted in a 554-fold purification with a yield of 6% of the enzyme.

Nuclear proteins such as DNA-binding protein, topoisomerase, nuclease and other contaminating proteins often aggregate during purification. In an attempt to remove these contaminating proteins, this research employed a series of column chromatography techniques that would effectively extract most of the DNase from the purified nuclei fraction. In addition, the enzyme assay should be reliable at all stages of the purification. This research applied a spectrophotometric method (Kunitz, 1950) and a DNA-gel electrophoresis assay for the identification of soybean DNase activity at each purification step.

Results from initial purification steps indicated that two protein peaks (peak$_A$ and $_B$) were eluted from the Sephadex G-75 column with 96% of the soybean DNase activity in peak$_B$ which had a molecular weight below 75 KDa. The DNA-gel assay demonstrated that DNase was present and DNA-binding proteins had been removed during purification, because DNase no DNA-binding activity as evidenced by retardation of DNA migration during

electrophoresis. DEAE protein peak3 enzyme activity eluted with 0.6 M KCl was partially inhibited by high salt concentration. Following desalting with a Sephadex G-25 column, the initial enzyme activity could be restored, which agreeing with the recent data (Magdalena et al., 1991).

The purified protein, which appeared as a single band on non-denaturing polyacrylamide gel electrophoresis, had a molecular weight of 60 KDa. This molecular weight was similar to the values of DNase obtained from mung bean (65KDa; Balakrishnan et al., 1977), muskmelon (54 KDa; Muschek, 1970), shrimp (46KDa; Chou et al., 1990), pea nuclei (47KDa; Chen et al., 1987), and Myxococcus coralloides D (49 KDa; Magdalena etal., 1991).

Substrate specificity results indicated that this purified DNase hydrolyzed both single stranded and double stranded soybean DNA and calf thymus DNA with no activity on yeast RNA. In addition, the enzyme had a slight preference for denatured soybean DNA and calf thymus DNA when compared to these native DNAs. Soybean DNase has similar properties to germinating barly DNase and pancreatic DNase such as the divalent metal ion requirement (Mn^{++}), temperature optimum at 37°C and the pH optimum near 7.0. These properties differ from RNase with respect to a pH optimum near 5.0 with a molecular weight below 25 KDa for RNase (Wilson, 1975).

The results of kinetic studies of soybean DNase indicated a higher K_m than previously published. For example, the K_m for pea DNase was 0.6 mM (Chen et al., 1987) and the K_m for mung bean nuclease was 0.58 mM (Balakrishnan et al., 1977) when compared to the soybean enzyme (4.5 mM).

It was necessary to differentiate endodeoxyribonuclease from topoisomerase. Both endodeoxyribonuclease and topoisomerase share some common endonuclease activities but their reaction products were completely different. Data from pea chromatin topoisomerase I indicated that, with increasing reaction time, the supercoiled pBR322 DNA was progressively converted to the relaxed form via a specific series of topoisomers which appeared on the agarose gel as a ladder (Weir, 1983). In this present research, endodeoxyribonuclease activity was indicated on agarose gels using covalently closed circular pBR322 DNA. The results clearly demonstrated that the closed circular DNA was nicked and then progressively hydrolyzed to oligonucleotides by the action of the endonuclease that could attack at points within the the pBR322 DNA. Additional support for soybean endodeoxyribonuclease activity was indicated on spectrophotometric analysis of Kunitz assay product followed by Sephadex G-10 gel filtration as shown in Figure 24. High-molecular-weight soybean DNA fragments were eluted in the void volume and ATP eluted in the total volume. The acid soluble assay products were eluted between the HMW DNA fragments and ATP. The assay products were oligonucleotides generated from the high-molecular-weight soybean DNA fragments by the action of endonuclease activity.

These data taken together indicated the presence of an endodeoxyribonuclease in the nucei of rapidly dividing meristematic cells. Although the function of the enzyme is not known, it might be involved in DNA replication, fidelity and repair.

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