PURIFICATION AND CHARACTERIZATION OF

OVINE PANCREATIC DEOXYRIBONUCLEASE

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ABBREVIATIONS

A_{280}	-	Absorbance at 280 nm
Asn	-	Asparagine
^{o}C	-	Degree centigrade
cm	-	Centimeter
CM	-	Carboxymethyl
Con A	-	Concanavalin A
DEAE	-	Diethylaminoethyl
DNA	-	Deoxyribonucleic acid
DNase	-	Deoxyribonuclease
EDTA	-	Ethylenediaminetetraacetate
gm	-	Gram
hr	-	Hour
M	-	Molar
µl	-	Microliter
mg	-	Milligram
ml	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
N	-	Normal
nm	-	Nanometer
PMSF	-	Phenylmethanesulfonyl fluoride
SDS	-	Sodium dodecyl sulfate
V	-	Volts

CHAPTER I

INTRODUCTION

Two types of deoxyribonuclease are known in animals. One of these, designated DNase I, has been found only in secretory glands such as the pancrease and parotid. The enzyme has a pH optimum near 7, requires divalent metal ion and produces nucleotides with 5' phosphate. The other type, designated DNase II, is a lysosomal enzyme occurring in almost all cells. It has an acidic pH optimum and produces nucleotides with 3' phosphate. DNase from bovine pancrease, available in relatively pure form, has received a great deal of study not only as an enzyme but also as a protein. Bovine DNase consists of four forms which separate on phosphocellulose. Multiple forms of DNase are due to a histidine residue replacing a proline in the primary sequence, and the presence or absence of a sialic acid residue in the carbohydrate side chain. The primary structure of one of the four forms is known.

Multiple forms of DNase occur not only in the bovine species, but also in other mammals as well. Malt DNase also has been reported to show multiple forms. The chemical basis of multiplicity for species other than bovine has not yet been established. The biological significance of this multiplicity is not understood. We wanted to study ovine pancreatic DNase, because this enzyme has been reported to exist in multiple forms. The most formidable problem in this context

is to obtain the enzyme in pure form. However, because of protease contamination, the purification strategy is the removal of proteases quickly from the preparation and then further purification. The method described herein seems to be suitable, and has the potential for application to all DNase of type I.

CHAPTER II

LITERATURE REVIEW

Araki in 1903 (1) was first to report that many tissues and secretion extracts, such as from pancreatic juice, had the property of liquifying DNA gels without liberating either inorganic phosphate or purine and pyrimidine bases, but with liberation of oligonucleotides. In 1905, Sachs (2) demonstrated that the liquifying action was caused by an enzyme distinct from trypsin and presented evidence that trypsin rapidly inactivates the nuclease. De la Blanchardière (3) confirmed the finding of Sachs, and made several unsuccessful attempts to separate the nuclease from the inactivating tryptic enzymes.

Purification

Bovine pancreatic DNase was independently purified by Fischer (4), Laskowski (5) and McCarty (6) in the 1940's. In 1950, Kunitz (7) made it available in relatively pure and crystalline forms. Baumgarten et al. (8), in 1958, and Polson (9), in 1956, reported modified purification procedures giving better yields of material of equal purity. The separation of DNase I from contaminating RNase by chromatography on DEAE-cellulose was reported by Zimmerman and Sandeen in 1966 (10). All preparations of purified bovine pancreatic DNase were contaminated with proteases (11) which readily inactivated the enzyme above pH 6. This

concern led Hugli (12), 1973, to use a combination of DEAE-Sephadex and phosphocellulose chromatography, Liao (13), 1974, used affinity elution with Ca^{2+} on DEAE-cellulose, Otsuka and Price (11), 1974, tried lima bean protease inhibitor affinity chromatography, and Wang and Moore (14), 1978, designed a simple, one step procedure using affinity chromatography and salting-out adsorption on lima bean protease inhibitor bound to Sepharose. Wadano (15), 1979, reported removal of proteases and their zymogens from DNase preparation by using Con A-Agarose affinity chromatography. However, DNase can be protected completely from cleavage by trypsin or chymotrypsin with 10 mM $CaCl_2$ (16).

General Properties of the Enzyme

Kunitz (7) was first to report that DNase is a soluble protein of the albumin type having an isoelectric point of pH 4.7. Molecular weight of the protein was determined to be 31,000 gm/mole by Lindberg (17). Price et al. (16) in 1969 found that it is a single chain protein consisting of 270 amino acid residues with two disulfide bonds. They also established DNase to be a glycoprotein which contained glucosamine and mannose in the carbohydrate moiety. Catley et al. (18) in 1969 reported that the carbohydrate side chain was a heptasaccharide consisting of 2 N-acetylglucosamine and 5 mannose residues attached to a single point on the peptide chain to an asparagine residue through an asparatamido linkage. Liao, Salnikow, Moore and Stein (19, 20) completely sequenced bovine pancreatic DNase I, and found that it consists of a single polypeptide chain of 257 amino acid residues with two disulfide bonds. The disulfide bond essential for activity links

residues 98 and 101. The carbohydrate moiety is attached to asparagine-18.

Role of Metal Ions on DNase

It was recognized early that relatively high concentrations of an activating metal were needed for maximum activity of DNase (21, 4, 6). McCarty (6) noted that $MgSO_4$ "in order of 0.003 M" was required in his system; Kunitz (21) found that the optimal concentration of $MgSO_4$ was 0.01-0.02 M, varying in the same direction as the concentration of DNA; Laskowski and Seidel (22, 23) found maximum rates with concentrations of $MgSO_4$ of 0.01-0.02 M. This was confirmed by Wiberg (24) and by Shack and Bynum (25). Several other metal ions were found to activate DNase (26) although the highest enzyme activity was reached with Mg^{++} plus Ca^{++} or Mn^{++} (26). Wiberg (24) showed that the activity of DNase in the presence of both metal ions, at a Mg^{++}:Ca^{++} ratio of 10, is 4- to 5-fold greater than the sum of the activity of DNase with Ca^{++} alone plus Mg^{++} alone. He suggested that DNase may have at least two requirements for metal ions. Ca^{++} satisfies one function better than Mg^{++}, and Mg^{++} satisfies the other better than Ca^{++}.

The rate of DNA hydrolysis by DNase is a complex process which results from the interaction of diverse factors such as pH, ionic strength, DNA concentration, and the nature and concentration of the activating metal ion (25). When Mg^{++} is used as the activating metal ion, DNase hydrolyzes native DNA by producing random single strand nicks (27, 28). If Mg^{++} is replaced by Mn^{++} or Ca^{++}, native DNA is hydrolyzed by simultaneous cleavage of both strands (29). The addition of calcium to a reaction mixture that contains Mg^{++} produces

double strand breaks (29). Price (30) suggested that Ca^{++}, Mg^{++}, and Mn^{++} have several specific binding sites on DNase: one strong and about five weak Ca^{++} binding sites at pH 5.5, and at least four strong binding sites at pH 9.0. At pH 7.5, DNase binds strongly two Ca^{++} and another three Ca^{++} are weakly bound; Mg^{++} and Mn^{++} compete with one of the two strong Ca^{++} binding sites (30). On the other hand, at pH 7.5 the enzyme binds strongly two Mn^{++} ions, as determined by electron paramagnetic resonance; Ca^{++} displaces Mn^{++} from both of the two specific sites (31). Addition of Ca^{++} at pH 7.5 induces changes in the circular dichroism and optical rotatory dispersion spectra of the enzyme (32) and a positive ultraviolet difference spectrum (33). It is also required to prevent the reduction of one of the two disulfide bonds (34) and to protect DNase from proteolytic inactivation (16, 12). Price (35) suggested that one of the essential roles of Ca^{++} on DNase activity is to enable the enzyme to bind substrate. The apparent stokes radius of DNase increases progressively when the pH is raised from 4.7 to 9.5 (36). This hydrodynamic change was interpreted as the unfolding of the protein molecular structure. Addition of Ca^{++} produces reversal of the pH effect (36). Lizárraga et al. (37) suggested the double role of Ca^{++}. First a specific influence in the correct refolding of denatured DNase and second an unspecific role increasing the ionic strength which determines the extent of compaction of the various conformers probably through solvent effects alone. Tullis et al. (38) suggested that DNase exists in three conformational states at $25^{o}C$ and pH 7.5.

Inhibitors of DNase

The presence of a protein inhibitor of DNase was first observed by Dabrowska et al. (39), who found that tissue extracts of the crop gland of a pigeon had an inhibitory effect on the activity of DNase. Inhibitors were later found in tissue extracts of several animals (40, 41). In 1970, Lindberg and Skoog (42) purified the inhibitor from calf thymus and determined that the interaction between the DNase and the inhibitor gave rise to a stable complex consisting of one molecule of each of the protein species. Lazarides and Lindberg (43) demonstrated that the widely occurring DNase I inhibitor was actin. 2-Nitro-5-thiocyanobenzoic acid has been reported to be the specific inhibitor for DNase I (44). Liao et al. (45) have suggested that inhibitor forms covalent bonds with certain serine or threonine residues of the enzyme. Protein modifying agent p-nitrobenzenesulfonyl fluoride, which is specific for tyrosine residues is able to inactivate DNase I (71). It was found that Ca^{++}-DNase was inactivated faster than Ca^{++} free DNase by this reagent.

Various Forms of DNase

Salnikow et al. (46) showed that bovine pancreatic DNase was present in four distinct forms separable by chromatography on cellulose phosphate. The major component, DNase A, contains a single polypeptide chain to which is attached a neutral carbon side chain. The covalent structure of the polypeptide chain of 257 amino acid residues, with the carbohydrate attached at Asn-18 has been elucidated (19, 20). The neutral carbohydrate side chain contains, on the average, 6 mannose and

2 N-acetylglucosamine residues (46). Catley (47) has shown that the 2 N-acetylglucosamine residues are adjacent to the asparagine residue and are followed by a variable number of mannose residues. Salnikow et al. (46, 48) showed that the polypeptide chain of DNase B is indistinguishable from that of DNase A, but that the molecule differs from DNase A in that it contains an acidic carbohydrate side chain consisting of 3 N-acetylglucosamine and 5 mannose residues and 1 residue each of galactose and sialic acid. DNase C, on the other hand, has the same type of neutral carbohydrate side chain as does DNase A, but its polypeptide chain differs from that of DNase A only by the substitution of histidine-118 by a proline residue (46, 48). DNase D was found to bear the same relationship to DNase C that B bears to A, namely that it is a sialylate derivative of C (49).

Ovine Pancreatic DNase

Wadano et al. (15) purified ovine pancreatic DNase and found that it, like bovine pancreatic DNase, exhibits multiplicity due to change in primary structure and sugar structure of the carbohydrate moiety. However, unlike bovine DNase, ovine DNase does not have sialic acid in any of its multiple forms. Both the ovine and bovine enzymes have indistinguishable molecular weights and identical end groups, and probably the same number of amino acids. The amino acid composition indicated that the two enzymes may have six residues of amino acids subjected to substitution (15).

Concanavalin A

Lectins are an assortment of proteins that share the ability to bind stereospecifically and reversibly to carbohydrates, in particular

to the sugar moieties of glycoproteins and glycolipids (50). Lectins include a variety of proteins and glycoproteins with diverse structures, different origins and probably distinct biological functions (51). Concanavalin A (Con A) from jack bean is one of the most abundant legume lectins. The protein was first crystallized by Summer (52) in 1919 and its amino acid sequence and three-dimensional structure were described in the early 1970s (53, 54). Its biological activities have been analyzed in detail, including both its ability to stimulate mitosis in lymphocytes and its dose-dependent ability to induce or inhibit the mobility of cell surface receptors (55).

At physiological pH, Con A is a tetramer of identical polypeptide chains, each containing 237 amino acids, one Ca^{++} and one Mn^{++}. The polypeptide chain of each monomer is folded into two extensive β-pleated sheets, one at the back and one through the center of the molecule. The carbohydrate-binding site is a shallow depression at the top of the molecule near the metal atoms (56, 57). There is also a deep cavity at the back of each monomer that appears to be capable of binding hydrophobic compounds. Below pH 5 or on chemical derivatization of amino groups, the Con A tetramer dissociates to dimers that retain many of the biological properties of the tetramer (58). The dimer differs from the tetramer in that it lacks the ability to inhibit mitosis and receptor mobility at high concentrations. The interaction between Ca^{++} and Mn^{++} ions in Con A has been the subject of considerable investigation. The experiments by Kalb and Levitzki (59) indicated that the transition metal ion must bind first and that its binding results in the formation of a Ca^{++} binding site. Brewer and co-workers (60) proposed that the role of Ca^{++} is merely to enhance the rate of

formation of the Mn^{++}-Con A complex which then does not require Ca^{++} for activity. This is in conflict with the results of other studies (59, 61) which indicated that Ca^{++} is required for full saccharide binding activity.

Numerous workers have shown that the binding of Ca^{++} to Con A induces a change in Mn^{++} liganding as well as protein conformation. Alter et al. found that Mn^{++} binding is strongly cooperative when Con A is preincubated with Ca^{++} and noncooperative when no Ca^{++} is present (62). The Ca^{++}-induced cooperativity increases as the pH approaches 7.0. Ca^{++} binding is significant at pH 7.0 whereas weak at pH 5.2.

Con A binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the C-3, C-4 and C-5 hydroxyl groups for reaction with Con A (63). Affinity chromatography on Con A-Sepharose has been used as an essential step in the purification and characterization of vast numbers of glycoproteins and polysaccharides (64). The binding of the substance with Con A requires the presence of both Mn^{++} and Ca^{++} (64). Elution of the bound substances has been achieved using a gradient of α-D-methylmannoside or α-D-methylglucoside (0-0.5 M) in starting buffer (64). Sequential elution with glucose, mannose, methyl α-D-glucoside, methyl α-D-mannoside, and eluent at pH 9 was used to separate lipoproteins on Con A-Sepharose (65).

CHAPTER III

MATERIALS AND METHODS

Frozen ovine pancrease was supplied by Pel-freez (Rogor, Arkansas). Bovine pancreatic DNase (DP grade) was obtained from Worthington (Washington). Calf thymus DNA (Type V), Con A-Agarose, phenylmethylsulfonyl fluoride (PMSF), N,N,N',N'-tetramethylethylenediamine (TEMED), soybean trypsin inhibitor-Agarose,, Sephadex G-100 (40-120 μ) and acrylamide was purchased from Sigma Chemical Company (St. Louis, MO). N,N'-methylenebisacrylamide (Bis) was from Eastman Kodak Company (Rochester, NY). CM-Cellulose (CM-52) was obtained from Whatman Ltd. (Maidstone, Kent, England). Ampholines, DEAE Bio-Gel A were from Bio-Rad (Richmond, CA). Blue Sephadex was prepared by Frank Wicks according to Ryan and Vestling (70) and kindly supplied by Dr. R. K. Gholson.

DNase Assay

DNase activity was determined by modification of the hyperchromicity assay of Kunitz (7) as described by Liao (13). One unit of enzyme is defined as the activity that causes the increase of one absorbance unit at 260 nm per minute per ml of assay medium at 25°C. Specific activity is designated as units of one milliliter of enzyme solution per absorbance of that solution at 280 nm (units/A_{280}).

Preparation of CM-Cellulose

The pre-swollen CM-cellulose was suspended in 4-5 volumes of distilled water and allowed to settle. The water was decanted, and the procedure was repeated. Five mM calcium acetate buffer, pH 4.7, 4-5 times volume was added, stirred gently with a glass rod and allowed to settle. The supernatant solution was decanted. This procedure was repeated several times until the pH of the suspension approached 4.7. The pH was determined by a pH meter with a glass electrode.

Preparation of Sephadex G-100

The dry Sephadex was allowed to swell in five times the final bed volume of water for 24 hrs. The supernatant solution was decanted and the gel was rinsed twice with the same volume of water, decanting slowly each time to remove the fine particles. The swollen gel was equilibrated with 0.1 M Tris-HCl, pH 7.0. After the gel was poured into the column it was washed with two columns of the same buffer.

Preparation of Con A-Agarose

Con A-Agarose suspension was washed with ten volumes of 50 mM Tris-HCl buffer, pH 7.0 containing 5 mM $CaCl_2$, 5 mM $MnCl_2$ and 0.15 M NaCl. After the column was packed, it was washed with one column volume of the buffer.

Preparation of Trypsin Inhibitor-Agarose

Trypsin inhibitor-Agarose gel was washed with 5 volumes of

distilled water several times. Gel was then equilibrated with 20 mM Tris-HCl buffer, pH 8.0, 5 mM $CaCl_2$.

Stability Test of DNase

Protease contamination in the DNase preparation was done as described by Otsuka et al. (11). DNase samples were incubated for 20 minutes at 37°C in 20 mM Tris-HCl buffer, pH 8. The inactivation of DNase by contaminating proteases was initiated by the chelation of Ca^{++} with 10 mM EDTA. DNase activity was assayed 2 minutes after EDTA addition to determine the initial activity and at suitable subsequent times.

Preparation of Blue Sepharose

Gel suspension was washed several times with 5-6 volumes of distilled water. It was then equilibrated with either 5 mM calcium acetate buffer pH 4.7 or 50 mM Tris-HCl, 5 mM $CaCl_2$ buffer pH 7.0. Gel was regenerated by washing with 3 M KCl, then with several column volumes of water and finally with starting buffer.

Preparation of DEAE Bio-Gel

Gel suspension was washed with 4-5 volumes of water, and then several column volumes of 0.1 M Tris-HCl pH 8.0 buffer until the pH of the suspension was 8.0, then washed with 20 mM Tris-HCl pH 8.0 buffer several times to equilibrate. The column was packed in the same buffer and was washed with at least two column volumes of the buffer.

Isoelectric Focusing

Isoelectric focusing was performed as described by Kim and Liao (66). A solution consisting of 1 ml of the monomer solution (24.25% (w/v) acrylamide and 0.75% (w/v) N,N'-methylene bis-acrylamide), 1 ml of 25% glycerol, 0.2 ml of the ampholyte solution (Bio-Lyte 4/10 or Bio-Lyte 3/10), 2.8 ml of H_2O, and 0.25 ml of 0.005% (w/v) riboflavin was pipetted into a gap formed between a glass plate and the plastic casting tray (Bio-Rad No. 170-4225). For a gel with the dimension of 125 x 50 x 0.8 mm, the glass plate was raised with two pieces of plastic rulers (0.8 mm thick). Polymerization occurred under fluorescent lights at 25°C for 1 hr. Samples (5-10 µ) were applied on a small piece of Whatman 3MM paper (3 x 5 mm) and placed on top of the gel. Gel attached to the glass plate, was placed upside down on two carbon electrodes, 10 cm apart. Samples were focused with voltage of 500 V at 4°C for 4 to 8 hrs.

Amino Acid Analysis

The protein was hydrolyzed in 200 µl of 6 N HCl in evacuated tubes at 110°C (19). Amino acid analysis was performed on the nanomole scale with an analyzer (67) modified for use with a 2.8 mm bore column (68).

SDS Polyacrylamide Gel Electrophoresis

Slab gels were prepared in the apparatus described by Studier (69). The separating gel contained 12 ml 30% acrylamide, 0.8% N,N'-methylenebisacrylamide, 7.5 ml 1.5 mM Tris-HCl buffer, pH 8.8, 0.3 ml

10% SDS, 10.2 ml H_2O, 0.1 ml 10% ammonium persulfate and 10 µl TEMED. Stacking gel contained 1.66 ml 30% acrylamide, 0.8% N,N'-methylene-bisacrylamide, 1.26 ml 1 M Tris-HCl buffer, pH 6.8, 0.1 ml 10% SDS, 6.9 ml H_2O, 80 µl ammonium persulfate and 8 µl TEMED. Running buffer, pH 8.3 contained 50 mM Tris, 0.38 M glycine and 0.1% SDS. Fifteen to twenty units of enzyme was dissolved in 50 µl of the dissolving buffer (0.75 ml 60% glycerol, 0.1 ml 1 M Tris base, 0.1 ml 10% SDS, 50 µl bromophenol blue and 5 µl β-mercaptoethanol). Gels were stained with a 0.1% comassie brilliant blue R solution of 50% methanol and 10% acetic acid in water. Destaining was done in a solution of 5% methanol and 10% acetic acid in water.

Peptide Mapping

Peptide mapping was performed as described by Salnikow and Murphy (48). 0.3 mg DNase was digested with 5% trypsin and 1% chymotrypsin in a volume of 30 µl Of 0.5% ammonium bicarbonate for 3 hours at 37°C. The digest was subjected to high voltage paper electrophoresis on a sheet of Whatman No. 3MM paper (46 x 57 cm) at pH 6.5 and 76 volts per cm. Bromophenol-blue was used as marker dye. Electrophoresis for 30 minutes gave a migration distance of desired separation. It was air-dried and subjected to descending paper chromatography overnight in the solvent system 1-butanol-pyridine-acetic acid-water (75:50:15:60). Purple spots of peptides were developed overnight after a brief dipping of the chromatograms in 0.5% ninhydrin in acetone.

CHAPTER IV

RESULTS AND DISCUSSION

Purification of Ovine Pancreatic DNase

Initial Preparation

Two hundred grams of frozen ovine pancreas was minced with a meat grinder twice at 4°C and added to 400 ml of cold water containing 0.5 mM PMSF. The pH of the solution was brought to 3.0 with 0.5 N H_2SO_4. The solution was stirred for 1 hr at 4°C. The suspension was filtered through two layers of cheesecloth to remove tissue. Filtrate was centrifuged at 27,000 xg for 10 minutes at 4°C. This and all subsequent centrifugations were performed in a Sorvall (model RC-2B) centrifuge with an SS-34 rotor. The precipitate was discarded and the clear solution was subjected to $(NH_4)_2SO_4$ fractionation. Reagent grade solid salt was added to bring the clear solution to 0.2 $(NH_4)_2SO_4$ saturation. This solution was stirred for 5 minutes and then was allowed to stand for 5 minutes at 4°C. The precipitate was filtered through Whatman No. 1 filterpaper. The filtrate was brought to 0.5 $(NH_4)_2SO_4$ saturation and the solution was stirred for 5 minutes and then allowed to stand for 5 minutes at 4°C. The solution was centrifuged at 27,000 xg for 20 minutes at 4°C. The supernatant solution was discarded and the pellet was dissolved to 5 mM calcium acetate buffer, pH 4.7, 0.5 mM PMSF. DNase activity was determined as

described in Materials and Methods. The protein solution was dialyzed against water for 2 hr, and then against 5 mM calcium acetate for at least 12 hr. Dialysate was centrifuged at 27,000 xg for 5 minutes and the precipitate was discarded.

CM-Cellulose Chromatography

The dialysate was loaded on a CM-cellulose column (Figure 1) previously equilibrated with 5 mM calcium acetate, pH 4.7. The enzyme was eluted in a gradient from 10 to 30 mM calcium acetate, pH 4.7. Absorbance at 280 nm and the enzyme activity were measured and active fractions were pooled for further purification.

Blue Sepharose Chromatography

The active pooled fraction (420 ml) was loaded on a Blue Sepharose column (1.5 x 10 cm), washed with 5 mM calcium acetate buffer, pH 4.7, until the A_{280} of the effluent solution was zero, then with one column volume, 50 mM Tris-HCl, 5 mM $CaCl_2$ buffer, pH 7.0. The enzyme was eluted with 1 M NaCl in the same buffer. The active fractions were pooled together and dialyzed against water for 2 hr at $4^{o}C$.

Con A-Agarose Chromatography

A Con A-Agarose column (Figure 2) previously equilibrated with 50 mM Tris-HCl buffer pH 7.0 containing 5 mM $CaCl_2$, 5 mM $MnCl_2$ and 0.15 M NaCl was loaded with the active fractions from Blue Sepharose. The column was washed with the starting buffer until A^{280} was zero, then with one column volume of starting buffer without $MnCl_2$. The enzyme was then eluted with 10% α-methylmannoside in 50 mM Tris-HCl buffer,

pH 7.0 containing 5 mM $CaCl_2$, 0.5 M NaCl.

The active pooled fraction from Con A-Agarose chromatography was subjected to a second CM-cellulose chromatography followed by gel filtration on Sephadex G-100. In both steps no further purification was observed.

Second Blue Sepharose Chromatography

A 4 ml bed volume Blue Sepharose column previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.0 containing 5 mM $CaCl_2$ was loaded with the enzyme active fraction. Elution was performed with 1 M NaCl in the starting buffer. The active pooled fraction was dialyzed against water for 2 hr and then against 20 mM Tris-HCl buffer pH 8.0 containing 5 mM $CaCl_2$ for 12 hr at $4^{o}C$.

Soybean Trypsin Inhibitor-Agarose Chromatography

The equilibrated sample was loaded on a soybeam trypsin inhibitor-Agarose column (1 ml bed volume). The column was eluted with starting buffer 20 mM Tris-HCl pH 8.0 containing 5 mM $CaCl_2$. The enzyme activity was in the void volume. The stability of DNase in Ca^{++} free media of the different preparations was measured as described in Materials and Methods. The highly stable preparation of the soybean trypsin inhibitor-Agarose chromatographed DNase has been obtained (Table II).

DEAE Bio-Gel Chromatography

The active fraction from trypsin inhibitor-Agarose was diluted 5

times with 20 mM Tris-HCl buffer, pH 8.0 to lower the Ca^{++} concentration to less than 1 mM. It was then loaded on a DEAE Bio-Gel column (Figure 3) equilibrated with 20 mM Tris-HCl buffer pH 8.0, washed with 2-3 column volumes of the starting buffer and then with 2 to 3 column volumes of 0.1 M Tris-HCl buffer, pH 8. The enzyme was eluted with 2 mM $CaCl_2$ in 0.1 M Tris-HCl buffer, pH 8.0. The enzyme activity and A_{280} of each fraction was measured, and the active fractions were pooled. This final preparation of DNase showed a high specific activity (Table I), a single band on SDS gel electrophoresis (Figure 4a) and two very close bands on isoelectric focusing (Figure 4b). The overall purification steps are summarized in Table I. All the above chromatographic experiments were performed at $4^{o}C$ except soybean trypsin inhibitor-Agarose, and DEAE Bio-Gel chromatography which were performed at room temperature.

Peptide Mapping

Peptide maps of ovine DNase and bovine DNase show that these two proteins have almost identical structures. However, there were at least 4 distinct spots in the ovine DNase map which were not found in that of bovine (Figure 6) indicating that there are only a few amino acid differences between the two proteins. This conclusion is further supported by the results from amino acid analysis (Table III), and the findings by Wadano et al. (15).

Discussion

Calcium is known to protect DNase from inactivation by proteases (16). Instability of DNase in the absence of Ca^{++} made its purification

a formidable problem. All enzyme activity was lost when one of the enzyme preparations was chromatographed on phosphocellulose because of the absence of Ca^{++} in the eluting buffer, although phosphocellulose has been used successfully in the separation of the four forms of bovine DNase (48). The highest loss of activity normally occurred during the $(NH_4)_2SO_4$ fractionation, especially in the 50% saturation step (Table I) perhaps due to the removal of Ca^{++} by sulfate. It is essential to work fast during the initial steps when the enzyme has no protection. The $(NH_4)_2SO_4$ step cannot be omitted since it was found that without this step the enzyme will not bind to Con A-Agarose, probably because by $(NH_4)_2SO_4$ fractionation many glycoprotein impurities were removed.

The first CM-cellulose chromatography step is important. It not only gives 16 fold purification over previous steps but also shows two enzyme activity peaks, indicating multiple forms of the protein. Significant loss of activity in this step is during dialysis. The second CM-cellulose chromatography does not give any further purification over previous Con A-Agarose step. A protein band very closely associated with DNase was found on SDS gel as a major contaminant after Con A-Agarose chromatography. Neither a second CM-cellulose nor Sephadex G-100 could remove it even though the gel filtration column used was 150 cm long (data not included). This led to the use of DEAE Bio-Gel chromatography. However, the instability of the enzyme in the absence of Ca^{++} during chromatography led us to use trypsin inhibitor-Agarose to remove the proteases completely (Table II).

Blue Sepharose chromatography is a step for concentration of the

enzyme. Concentration by lyophilization led to the loss of activity partially due to the formation of insoluble materials. However, this step did not give any significant purification.

Con A-Agarose affinity chromatography is a vital step in the purification. Five fold purification was achieved over the previous step. Most of the proteases were removed at this stage, since they are not glycoproteins. Once the proteases were removed completely by trypsin-inhibitor-Agarose, the enzyme was purified to complete homogeneity by Ca^{++} affinity elution on DEAE Bio-Gel.

CHAPTER V

SUMMARY AND CONCLUSION

Amino acid analysis and peptide mapping show that ovine and bovine pancreatic DNases are structurally very similar. Results of amino acid analysis agree with the finding of Wadano et al. (15); there are only a few amino acid substitutions in the primary sequence. Exact numbers and locations of substitutions are still to be determined. The enzyme exhibits multiplicity, as shown by CM-cellulose chromatography and isoelectric focusing; it will be interesting to find out any differences in the structure that give rise to multiple forms. Further studies are needed but substantial amounts of the pure enzyme are required. The previously reported method of purification is not reproducible. The new approach for purification which includes $(NH_4)_2SO_4$ fractionation, CM-cellulose, Blue Sepharose, Con A-Agarose, trypsin inhibitor-Agarose, and DEAE Bio-Gel was proven effective. As previously shown, Con A-Agarose not only gives a high degree of purification but also removes most of the protease contamination. However, the complete removal of proteases requires trypsin inhibitor-Agarose chromatography. Finally, the enzyme can be purified to complete homogeneity by Ca^{++} affinity elution on DEAE-Bio-Gel. Although Blue Sepharose does not result in purification it does serve as a means to concentrate the enzyme.

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APPENDIX

TABLE I

SUMMARY OF PURIFICATION OF OVINE DNASE

Purification Step	Total Activity Units x 10^3	Activity Recovered %	Specific Activity Units/A_{280}	Purification x-fold
1. Crude extract	99	100	1.5	1
2. $(NH_4)_2SO_4$ Precipitation	51	51	7.0	5
3. CM-Cellulose Chromatography	21	21.2	118	79
4. Con A-Agarose	12	12	600	400
5. DEAE Bio-Gel	3.0	3.0	900	600

TABLE II

INSTABILITY OF DNASE IN THE ABSENCE OF CALCIUM

Stage of DNase Purification	Half-Life at pH 8.0 10 mM EDTA
Crude Extract	< 1 minute
CM-Cellulose	< 1 minute
Con A-Agarose	24 hours
Trypsin Inhibitor Agarose	>100 hours

TABLE III

AMINO ACID COMPOSITION OF OVINE DNASE

	Ovine		Bovine DNase A
	Observed[a]	Reported[c]	
Aspartic Acid	32.4 (32)	33	32
Threonine	14.2 (14)	14	15
Serine	31.3 (31)	31	30
Glutamic Acid	19.1 (19)	18	19
Proline	11.6 (11)	11	9
Glycine	10.8 (11)	10	9
Alanine	20.9 (21)	21	22
Half-Cystine	nd (4)[b]	4	4
Valine	24.1 (24)	24	24
Methionine	4.2 (4)	4	4
Isolucine	11.8 (12)	12	11
Leucine	22.1 (22)	22	23
Tyrosine	14.2 (14)	14	15
Phenylalanine	9.8 (10)	11	11
Histidine	3.0 (4)	4	6
Lysine	8.6 (9)	9	9
Tryptophan	nd (3)[b]	3	3
Arginine	11.9 (12)	12	11

[a]Results are expressed as calculated number of residues per enzyme molecules based on the average of three analyses. The possible number of residues is in parentheses.

[b]Not determined; residues are assumed from reported value.

[c]Taken from Wadano et al. (15).

Figure 1. Chromatography of Ovine DNase on CM-Cellulose Column, 1.5 x 42 cm. Elution was performed with 650 ml each of 0.01 and 0.03 M calcium acetate buffer, pH 4.7. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity; bars indicate pooled activity fraction; solid lines indicate calcium acetate concentration.

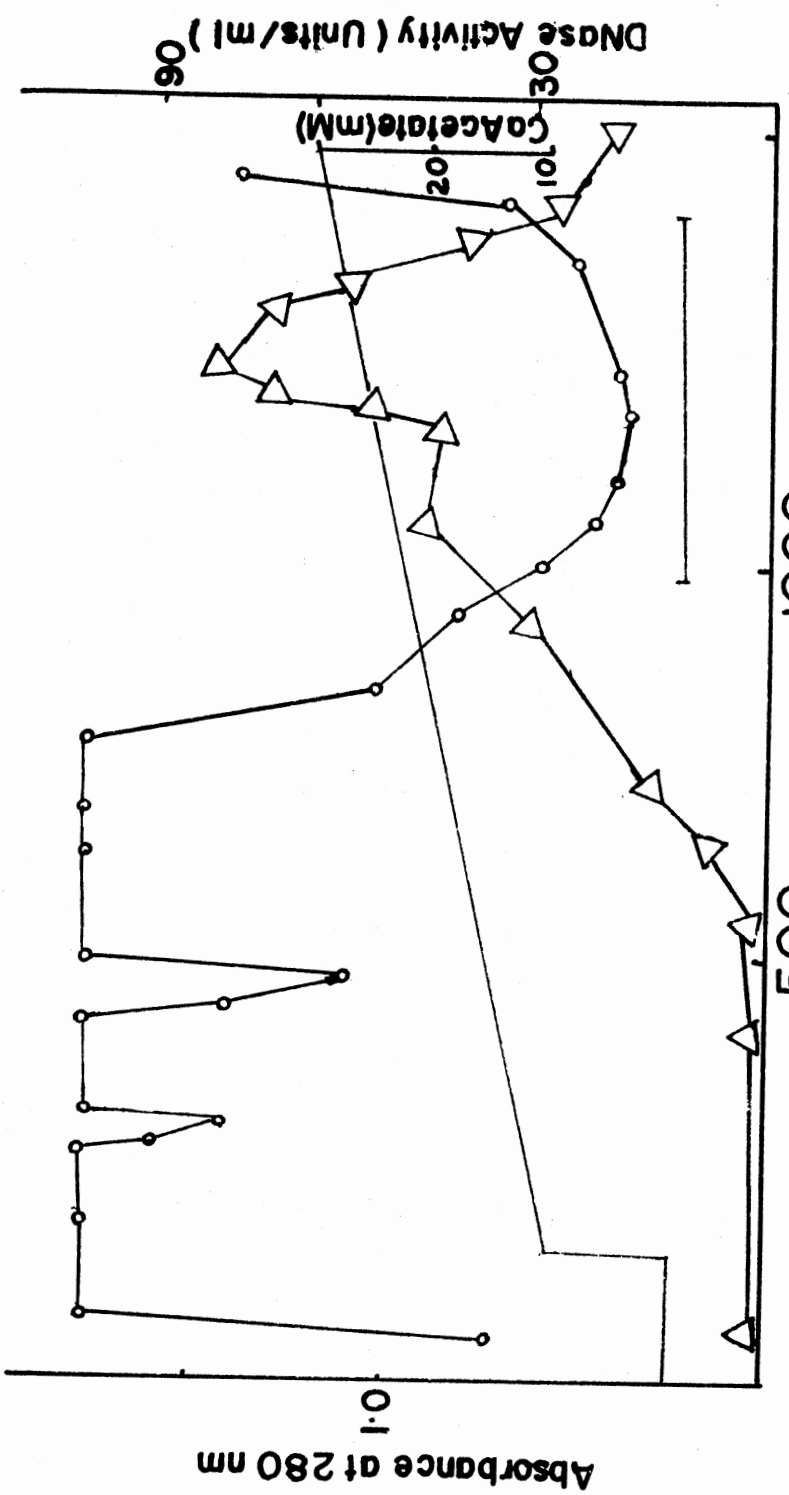

Absorbance at 280 nm
0.1
DNase Activity (Units/ml)
90
30
Ca Acetate(mM)
20
10
500
1000
EFLUENT VOLUME, ml

Figure 2. Chromatography of the Active Fraction from CM-Cellulose on a Con-A Agarose Column, 2 x 12 cm. The column was equilibrated with 50 mM Tris-HCl, pH 7.0, 5 mM $CaCl_2$, 5 mM $MnCl_2$ and 0.15 M NaCl. After sample loading, the column was washed with 50 mM Tris-HCl, pH 7.0, 5 mM $CaCl_2$ and 0.5 M NaCl. DNase was eluted with α-methylmannoside (10%) in the same buffer at the point indicated by an arrow. Open circles indicate absorbance at 280 nm; trianbles indicate DNase activity. Bars indicate pooled active fractions.

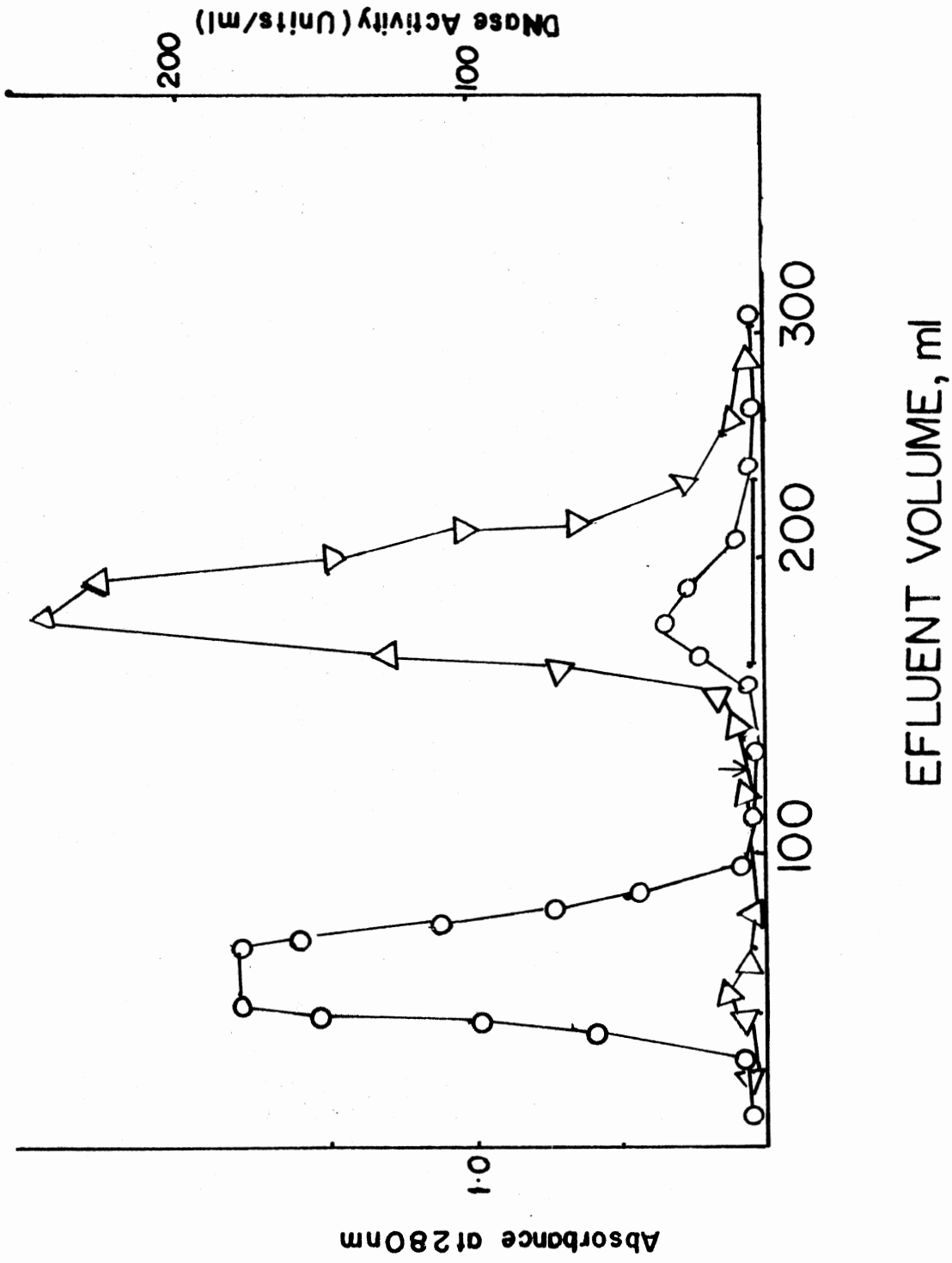
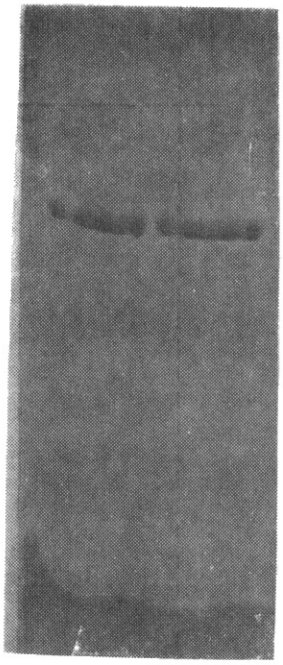

Absorbance at 280nm
1.0
DNase Activity (Units/ml)
100
200
100
200
300
EFLUENT VOLUME, ml

Figure 3. Chromatography of the Active Fraction from Con A-Agarose Chromatography on DEAE-Bio-Gel Column, 1 x 20 cm. The column was equilibrated with 20 mM Tris-HCl, pH 8.0 buffer and after loading DNase was eluted with 0.1 M Tris-HCl, pH 8.0, 2 mM $CaCl_2$ as indicated by the arrow. Open circles indicate absorbance at 280 nm; triangles indicate DNase activity. Activity and absorbance were not measured during the initial loading and washing.

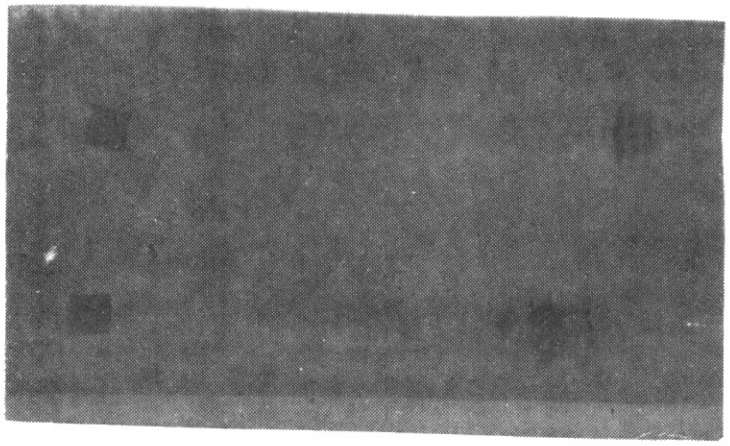
Absorbance at 280 nm
1·0
0·5
DNase Activity (Units/ml)
400
200
25
50
EFLUENT VOLUME, ml

Figure 4. a) SDS Polyacrylamide Gel Electrophoresis and b) Isoelectric Focusing of Ovine (A) and Bovine (B) DNases. Bovine DNase was purified from Worthington DP grade DNase by DEAE-Cellulose Chromatography.

A B



a

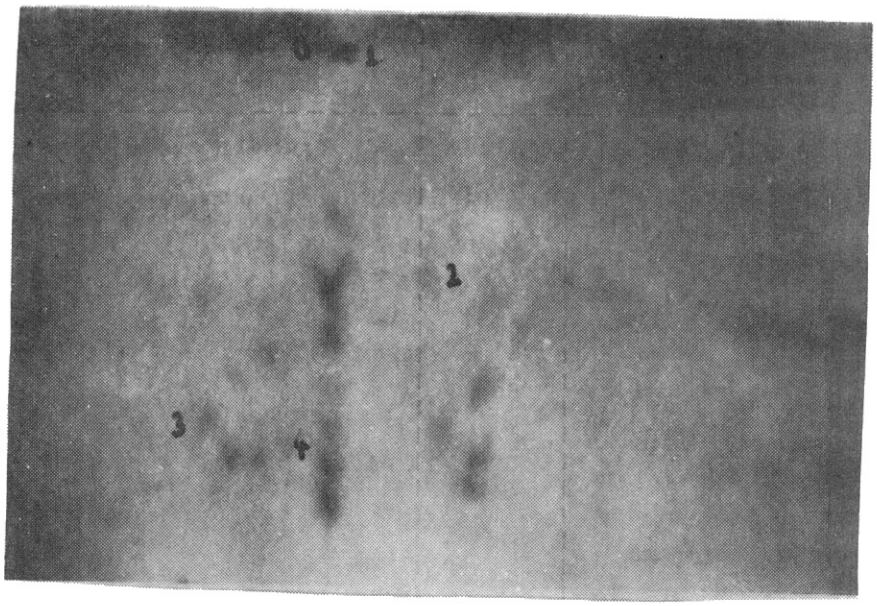


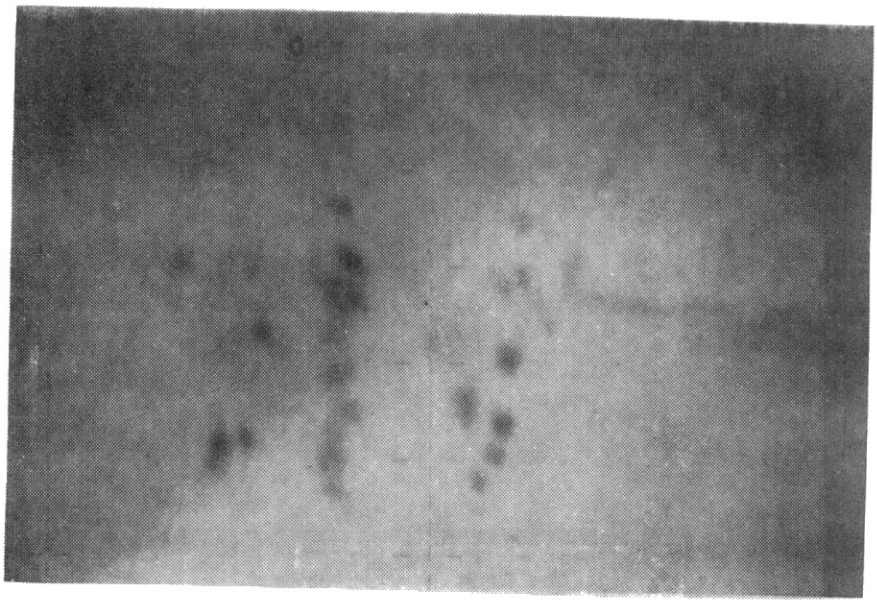
B

b

Figure 5. Peptide Maps of Ovine (A) and Bovine (B) DNases. Electrophoresis was at pH 6.5, 76 volts per cm. O, origin; anode to the right, chromatography:descending.

A





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