STRUCTURES OF DEOXYRIBONUCLEASE I FROM DIFFERENT MAMMALIAN SOURCES

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LIST OF ABBREVIATIONS

CM	-	Carboxymethyl
ConA	-	Concanavalin A
DEAE	-	Diethylaminoethyl
DNase	-	Deoxyribonuclease
RNase	-	Ribonuclease
SDS	-	Sodium dodecyl sulfate
PTH	-	Phenylthiohydantoin
Rcm	-	Reduced and S-carboxymethylated
TFA	-	Trifluoroacetic acid
HPLC	-	High performance liquid chromatography
CPase	-	Carboxypeptidase

CHAPTER I

INTRODUCTION

For a better understanding of the structure-function relationship of an enzyme, a comparison of the structures of the enzyme from various sources is essential. The present study concerns the investigation of the primary structures of pancreatic DNases from various sources. Bovine pancreatic DNase was first crystallized by Kunitz (1). Crystalline sample of bovine DNase is readily available. From this crystalline sample bovine DNase was purified to homogeneity by one step chromatography (2-4). The easily available pure form of the enzyme has attracted several investigators and hence has been studied most (5). The primary structure of bovine pancreatic DNase was determined in the laboratory of Moore and Stein (6, 7). The homogeneous form of pancreatic DNase from other animal sources has been very difficult to obtain, and therefore was not available for structural investigations. The difficulty was due to the proteases in pancreas (DNase is extremely sensitive to inactivation by proteases (2)). Although there have been a few successful methods for removing proteases (8-11), a scheme for purification of DNase with high yield was lacking. Described herein, purified DNases from ovine and porcine pancreas in substantial amounts was obtained via a new procedure. The amounts were sufficient for determining the complete amino acid sequences of ovine and porcine DNases. During the course of the sequence investigations of ovine and

porcine DNases, it was found that the published sequence of bovine DNase (7) has a gap of three amino acid residues between Arg-27 and Arg-28 when the sequences of DNase from the three species were aligned. Reinvestigation of the sequence around this region was therefore included in the study to correct the published sequence of bovine DNase (7).

CHAPTER II

LITERATURE REVIEW

DNase I was first discovered in pancreatic juice in 1903 (12). It was suggested that this enzyme is only found in the pancreas (13). Several investigators attempted to purify the enzyme (14-16). Kunitz (1) was able to obtain it in crystalline form from bovine pancreas. Almost as soon as crystalline bovine pancreatic DNase I was obtained doubts concerning its homogeneity arose. It was shown (17) that DNase I cocrystallizes with chymotrypsinogen B. Lindberg (18) passed the solution of commercial crystalline DNase I through a column of Sephadex G-100, removed contaminants of smaller molecular weight, and obtained a preparation of high purity. Purification of the homogenous enzyme from the commercially available crystalline DNase has been achieved by several workers (2-4). Salnikow et al. (3) in an attempt to purify bovine DNase on phosphocellulose column observed four active components of DNase (termed A, B, C, and D). The major component, DNase A, was used for detailed sequence analysis. Its primary structure is known (6, 7). It was also shown to be a glycoprotein with a neutral carbohydrate side chain (2, 19). DNase C differs from DNase A by a proline residue substituted for a histidine (20). The difference between DNase B and A, as well as between D and C is that the neutral carbohydrate side chain in A and C contains sialic acid and galactose in B and D (4).

Bovine DNase has been shown to be a single polypeptide of 257 amino

acid residues (7) with the carbohydrate side chain linked to Asn-18. It has two disulfide bonds (7). The disulfide bond connecting residues 98 and 101 could be selectively reduced in the presence of Ca^{2+} without loss of any activity (21). The carbohydrate composition of bovine DNase shows 2 residues of glucosamine and 5 mannose (19).

The DNase I class enzymes have been defined as those enzymes that cleave the substrate endonucleolytically to yield primarily 5'-phosphodi- and 5'-phosphooligo-nucleotides with a pH optimum near 7 and require divalent metal ions for activity (5). Kunitz (1) showed that the concentration of Mg^{2+} needed for activity is proportional to the DNA concentration. This has been confirmed subsequently by Wiberg (22) and Shack et al. (23). It was suggested that the substrate of DNase I is the Mg^{2+}-DNA complex (22, 23). Several other metal ions can also activate DNase I (24) although the highest activity is reached with Mg^{2+} plus Ca^{2+} or with Mn^{2+} alone (24). The activity of DNase I in the presence of both Mg^{2+} and Ca^{2+} is 4 to 5 fold greater than the sum of the activity of DNase I with Ca^{2+} alone plus Mg^{2+} alone (22). Two Ca^{2+} ions bind strongly to DNase I at pH 7.5 (25). One binding site becomes weak at pH 5.5 and the Ca^{2+} can be displaced by Mg^{2+} or Mn^{2+} (25). The other site can bind Ca^{2+} at pH 5.5 and 50 mM Mg^{2+} or Mn^{2+} can not displace it (25). Ca^{2+} binding to DNase produces a change in the circular dichroism and the ultraviolet spectrum of the protein (26, 27). Ca^{2+} binding to the protein is also required for the reformation of the disulfide bond in the reduced DNase (28), and to protect DNase from inactivation by chymotrypsin and trypsin (2). It was suggested that the essential role of Ca^{++} in DNase activity is to produce the structural change in DNase required for substrate binding

(29).

His-131, which is only alkylated by iodoacetate and not by iodoacetamide, has been proposed to be at the active site (30). DNase is inactivated by iodoacetate at pH 7.2 in the presence of Mn^{2+} or Cu^{2+} with the formation of one residue of 3-carboxymethylhistidine (30). Modification of Trp-155 by N-bromosuccinimide inactivates DNase (31). Nitration of DNase leads to loss of its activity (32). Ca^{2+} was not able to stabilize the nitrated enzyme towards chymotryptic digestion, thermal denaturation, or mercaptoethanol reduction of the essential disulfide linkage (32). Hence, nitrated residue Tyr-62 was suggested to be involved in Ca^{2+} binding (32). Liao et al. (33) by using tyrosine specific reagent p-nitrobenzenesulfonyl fluoride found that Tyr-172 and Tyr-145, but not Tyr-62 are crucial for activity. The reagent 2-nitro-5-thiocyanobenzoic acid inactivates Ca^{2+}-DNase, but not Ca^{2+}-free DNase (34). It was shown that this reagent modifies one threonine and three serine residues (35). The COOH-terminal residues of DNase, which are normally not available to carboxypeptidase action (36) become available when the enzyme is denatured with 0.005% SDS (37). It is thought that residues at the COOH-terminus are essential for folding of the enzyme into the active conformation.

CHAPTER III

MATERIALS AND METHODS

Materials

Frozen ovine and porcine pancreatic tissue were purchased from Pel-Freez. Bovine pancreatic DNase (DP grade) was obtained from Worthington, and was purified by the procedure as described (4). Carboxypeptidases, A and B, trypsin and chymotrypsin were also from Worthington. CM-Cellulose (CM-52) and DEAE-Cellulose were from Whatman. ConA-Agarose, Soybean trypsin inhibitor-Agarose, CNBr, pronase (protease from Streptomyces griseus), TFA, Sephadex G-100 were from Sigma Chemical Company. Bio-lyte 4/6 (40%), DEAE-Biogel A were from Bio Rad. Blue-Sepharose was from Pharmacia. The reagents and solvents of the Edman degradation procedure were "sequanal" grade of Pierce. Aminopeptidase M was from Rohm and Hans. All other reagents and chemicals were of reagent grade.

Methods

Amino Acid Analysis

Proteins and peptides were hydrolyzed in sealed, evacuated tubes in 0.2 ml 6 N HCl containing 0.2% phenol at 110^{o} C for 20-24 hr. Methionine and half-cystine were determined on the amino acid analyzer after oxidation of protein with performic acid and hydrolysis in 6 N

HCl (7). Tryptophan was determined on the amino acid analyzer after hydrolysis of protein in 6 N HCl containing 0.25% phenol, 0.5% thioglycolic acid, and 0.1% 3-(2-aminoethyl) indole hydrochloride. Amino acid analyses were performed on a nanomole scale with an analyzer (38), modified for use with a 2.8-mm bore column (39).

Unless otherwise stated, results are obtained from single amino acid analyses of 24 hr hydrolysate and expressed as the number of residues per mole. Values less than 0.2 residues are omitted; the integral number in parentheses in the tables are derived from the sequence (Figure 19, 29). Serine and threonine are corrected for 10% and 5% destruction, respectively. Half cystine and tryptophan were partially or completely destroyed. Values for Hsr (homoserine) are the sum of Hsr and its lactone. Yields of peptides have been calculated on the basis of the amount of parent peptide used for hydrolyses. Designations for peptides: CB, CNBr fragment; T, tryptic; C, chymotryptic; Th, thermolytic; (O), performic acid oxidized peptides; P, pronase digested peptides; A, mild acid hydrolysed peptides.

DNase Assay

The hyperchromicity assay of Kunitz (1) modified by Liao (4) was used to determine the DNase activity. One unit of the enzyme activity causes a change of one unit absorbance at 260 nm. Specific activity is expressed as units of enzyme activity in one ml of enzyme solution per absorbance unit of that solution at 280 nm (units/A_{280}).

Electrophoresis

SDS-gel electrophoresis was performed according to Weber and Osborn (40). Isoelectric focusing and DNase activity staining was

carried out as described by Kim and Liao (41). Isoelectric points were determined by comparing the relative locations of the unknown protein bands to the protein bands of known pIs (bovine DNases A, B, C, and D) (41).

End Group Analysis

The NH_2-terminal amino acid was determined by the dansylation procedure (42), and the dansyl amino acids were identified by thin layer chromatography on a polyamide sheet (43). The NH_2-terminal amino acid was also identified by the Edman degradation procedure (44). The anilinothiazolinone derivative was back hydrolyzed and determined on the amino acid analyzer (45). The COOH-terminus of the polypeptides was determined on the amino acid analyzer, by analyzing the amino acids released after hydrolysis with carboxypeptidase A in the presence of 0.005% SDS (37).

Cleavage of Peptide Bond with CNBr and Proteases

Proteins were cleaved at the methionine residues with CNBr in 88% formic acid for 24 hr at room temperature in the dark (7). Hydrolyses of paptides with trypsin, chymotrypsin, thermolysin, aminopeptidase M, carboxypeptidase A, carboxypeptidase B, and pronase were in 1% NH_4HCO_3 at 37° C for 24 hr unless otherwise stated. Mild acid hydrolysis for specific cleavage of peptide bonds at aspartic acid residues was performed in 0.2 ml of 0.003 N HCl pH 2.3 in a sealed evacuated tube at 110° C for 2 hr (46). Cleavage at the aspartyl-prolyl bond was achieved with 88% formic acid at 37° C for 24 hr (47).

Isolation of CNBr Fragments

The CNBr fragments were isolated by gel filtration on Sephadex G-100 column in 50% acetic acid (7).

Isolation of Small Peptides

Small peptides, resulting from protease digestion, were separated on a reverse phase column (Altex Ultrasphere C_{18} 5 μ ODS, 4.6 x 250 cm) using a HPLC system from Waters. The gradient used was 0-90% methanol in 0.1% TFA (33). The effluent was monitored at 214 and 280 nm. Peaks with absorbance at both wavelengths indicated tyrosine or tryptophan containing peptides. The effluent of peaks was collected manually, lyophilized and stored at -20° C. Due to a great number of peptides resulting from tryptic anc chymotryptic digests of the bigger CNBr fragments, the digests were fractionated on a Sephadex G-25 column prior to separation on HPLC.

Sequence Analysis

Manual Edman degradation was performed on the μ scale (48) using a coupling buffer which contained N-ethylmorpholine (6). Automated Edman degradation (49) was carried out in a Beckman 890C sequencer equipped with a cold trap. All runs used 0.1 M Quadrol, pH 9.0, as the coupling buffer and the Beckman Program 121078. Released residues, from both the manual and automated procedures, were converted to the PTH amino acid derivatives by heating in 0.2 ml 1 N HCl for 10 min at 80° C. PTH amino acids were identified according to the isocratic procedure of Black and Coon (50), with some modifications, on an Altex

Ultrasphere C_{18} 3 μ ODS HPLC column (4.6 x 7.5 mm). PTH amino acids were eluted with 15% methanol in 0.1 M ammonium acetate pH 4.5 for 10 min and then with 40% methanol in 0.1 M ammonium acetate pH, 4.5 at 30° C.

The approximate yields of the PTH derivatives were calculated from the peak height of high performance liquid chromatograms. Substractive Edman degradation was performed as described (6). The values of subtractive Edman degradation are expressed as the number of residues per molecule of peptide. Peptides are numbered in consecutive order from the NH_2 terminus, in accordance with the sequence for the enzyme in Figure 19 and 29. In the diagram of sequence, solid lines indicate residues that were positioned by sequence analysis of the given peptide; dashed lines indicate residues that were not sequenced in the given peptide but were in related peptide.

CHAPTER IV

RESULTS

Porcine Pancreatic DNase

Purification

Four hundred gm of frozen porcine pancreatic tissue were ground in a meat grinder and stirred in 600 ml of cold 10 mM calcium acetate, pH 4.7, for 1 hr. The solution was filtered through cheese cloth and the filtrate was centrifuged for 20 min. Solid ammonium sulfate was added to the supernatant to bring to 30% saturation and the mixture was allowed to sit for 1 hr. The mixture was centrifuged for 1 hr and the clear supernatant was subjected to 60% ammonium sulfate saturation. The resulting suspension solution was centrifuged for 20 min and the pellet was dissolved in 100 ml of 10 mM calcium acetate, pH 4.7. A saturated solution of barium chloride was added to this solution until no further precipitate was formed. This treatment removed excess SO_4^{2-} as insoluble $BaSO_4$, and shortened dialysis time. Removal of SO_4^{2-} prevented DNase from excessive inactivation during dialysis. The above solution was centrifuged for 5 min and the clear supernatant was dialyzed for 15 hr against 5 mM calcium acetate, pH 4.7. The dialysate was centrifuged for 2 min to remove any precipitate formed during dialysis. All of the above and following operations were performed at 4^{o} C unless otherwise stated. All the centrifugations were carried

out on a Sorvall centrifuge (Model RC-2B) at 27,000 x g.

The dialysate was loaded on a CM-Cellulose column previously equilibrated with 5 mM calcium acetate, pH 4.7. The DNase activity was eluted with a gradient of 10-30 mM calcium acetate, pH 4.7 (Figure 1).

The active fractions were pooled and the entire solution about 500 ml, was loaded directly on a Blue Sepharose column previously equilibrated with 5 mM calcium acetate, pH 4.7. The column was washed with 50 mM calcium acetate, pH 4.7 until the A_{280} of the effluent was near zero. The enzyme was eluted with 50 mM Tris-HCl, pH 7.0, containing 5 mM $CaCl_2$ (Figure 2).

The DNase activity recovered from the above step was chromatographed on a ConA-Agarose column in 50 mM Tris-HCl, pH 7.0, containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$. The enzyme was eluted with 10% methyl mannoside, 0.5 M NaCl, 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.0 (Figure 3). Active fractions were pooled and the solution was dialyzed against 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0 for 15 hr.

The dialysate was chromatographed on a DEAE-Biogel column in 20 mM Tris-HCl, pH 8.0, at room temperature. DNase was eluted with 100 mM Tris-HCl, pH 8.0 (Figure 4).

A summary of the purification steps is given in Table I. Although a procedure for the purification of ovine pancreatic DNase has been described (9), that method is not applicable to purification of porcine DNase. A drastic loss of activity resulted in first several steps and the enzyme remained non-homogenous after the last step. The enzymes, which have binding sites for nucleotides, have been found to interact with Cibacron Blue F3G-A (51). Porcine DNase also binds to Blue

Sepharose (Cibacron Blue F3G-A covalently bound to agarose) very strongly (Figure 2). The elution of DNase by changing the pH of the eluting buffer from 4.7 to 7.0 resulted in a high degree of purification (Table I). Bovine and ovine DNases bind to ConA (9). Porcine DNase also binds to ConA (Figure 3). Chromatography of porcine DNase on a ConA Agarose column not only resulted in a high degree of purification (Table I), but also removed proteases (9) as indicated by the stability of DNase during dialysis and DEAE-Biogel chromatography which were performed at rather low Ca^{2+} concentrations. The final step (DEAE-Biogel) of purification was performed to remove traces of other proteins.

SDS-Gel Electrophoresis and Isoelectric Focusing

The homogeneity of porcine DNase was verified by the single band on SDS-gel electrophoresis (Figure 5). However, when applied together on the same lane, porcine and bovine DNases did not comigrate (Figure 5B). From the mobility on the SDS-gel, the molecular weight of porcine DNase is estimated to be about 34,000, assuming that the molecular weight of bovine DNase is 31,000 (7).

Isoelectric focusing revealed 4 bands by protein staining, all of which showed activity when subjected to DNase activity staining (Figure 5A). Multiple forms of DNase have been shown in various species (3, 9, 52). Figure 5 clearly shows that porcine DNase also exists in multiple forms that differ in charge. The pI of the different forms is calculated to be 5.06, 4.96, 4.78, and 4.7.

End-group Analysis

Both dansylation and the Edman degradation procedures showed leucine at the amino terminus of porcine DNase. Thus, the NH_2-terminus is identical in bovine, ovine and porcine DNases (7, 9). After incubation with carboxypeptidase A at 37° C for 24 hr only alanine was released. Thus, in contrast to bovine and ovine DNases which have identical threonine at the COOH-terminus (7, 9), porcine DNase has alanine.

Amino Acid Analysis

The results of amino acid analyses of porcine DNase are shown in Table III. Like bovine and ovine DNases (7, 9), the porcine enzyme is a glycoprotein containing glucosamine with 4 half-cystine residues. However, it is higher in proline and lower in serine content compared to bovine and ovine DNases (7, 9).

Enzymatic Properties

The optimum pH of activity for bovine DNase is 7.0 and the enzyme requires divalent metal ions (5). When the effects of pH and divalent metal ions on the activity of bovine and porcine DNases were compared there were no significant differences between the two proteins (Figure 6). Like bovine DNase (24), porcine DNase is most active in the presence of both Mg^{2+} and Ca^{2+} or Mn^{2+} alone (Figure 6). Thus, both enzymes are very similar in pH optimum and divalent metal ion requirement.

Sequence Analysis

Intact porcine DNase was sequenced up to 17 cycles using the automated sequenator (Table IV). For further sequence analysis about 10 mg of protein was cleaved at the methionines by CNBr. The resulting 4 fragments, CB1, CB2, CB3 and CB4, were separated by gel filtration (Figure 7). The results of amino acid analyses of these fragments are given in Table III. The results of automated sequence analyses of the two largest fragments, CB2 and CB4, are given in Table IV. For further analyses, CB2 and CB4 along with Rcm DNase were digested with trypsin and their tryptic peptides were isolated (Figure 8-11, 16, 17). Small peptides were sequenced directly, large peptides were further fragmented with either chymotrypsin, thermolysin, or pronase, or by mild acid hydrolysis. The resulting small peptides were separated by HPLC and were used for sequence analyses. The results of sequence analyses of tryptic peptides are summarized in Tables V-XVIII. The peptides resulting from chymotryptic digestion of CB1, CB2 and CB4 were also isolated (Figures 12-15). Their compositions and sequence analyses are given in Tables XIX-XXVI. Thus, on the basis of all these data obtained, the sequence of peptide CB2 of 148 residues, and peptide CB4 as 96 residues were derived (see Figure 19). The residue numbers for each amino acid agree closely with those from the total amino acid analysis in Table III.

Porcine DNase has only three methionine residues (Table II) as compared to four residues each for bovine and ovine DNases (7, 9). Therefore, one expects to obtain four CNBr peptides from porcine DNase as described above (Figure 7). The order of the four peptides in the

intact protein is assigned as follows. Peptide CB4 does not contain homoserine (Table III), suggesting that it is the COOH-terminal peptide. This assignment was further substantiated by the result of the carboxypeptidase A action on CB4 which released alanine, identical to the amino acid released from the intact protein (see end group analysis above). Peptide CB1 is placed at the NH_2-terminus, as its amino acid composition (Table III) agrees with the sequence data for the intact protein (Table IV). Because the sequence data for the intact protein covered CB1 and extended to the NH_2-terminal end of CB2 (Table IV), CB1 must be followed immediately by CB2 in the intact protein. By difference, CB3 automatically finds its place in between CB2 and CB4. CB3 is a dipeptide (Table III) and its sequence is based on the specificity of CNBr cleavage. Thus, the complete amino acid sequence of porcine DNase consisting of 262 amino acid residues has been determined (Figure 19). It has leucine at the amino terminus and alanine at the carboxyl terminus which agrees with the end group analyses. The residue next to alanine at COOH-terminus is arginine (see Figure 19). This explains the release of only one residue of alanine upon carboxypeptidase A digestion of either porcine DNase or CB4, as carboxypeptidase A does not release arginine (53).

Porcine DNase has four half-cystine residues or two disulfide loops (Table II). The CNBr fragments were obtained from the intact protein without cleavage of the disulfide loops. The CB2 and CB4 each have two half-cystines (Table III). Hence, CB4 and CB2 each have one disulfide loop. The sequence analysis of peptide T2-A4 (Table XI) establishes the position of first disulfide loop, as connecting residues 101 and 104 (Figure 19). In CB4, the two half cystine-

residues are situated at positions 173 (Table IV and Figure 19) and 209 (Table XVI and Figure 19); hence, these two residues form a second disulfide loop.

Two tryptic peptides CB2-T1 (Table V) and CB2-T8 (Table XI) contain glucosamine. Thus, there are two carbohydrate side chains in the porcine DNase molecule. During sequence analysis of CB2-T1-Th1 (Table V) residues, Ser-1 and Ala-3 were identified as PTH amino acids. The fourth residue was identified as free Thr after three step Edman degradation. Since this peptide has one Asx in its molecule, the unidentified second residue has been assigned Asn with a carbohydrate side chain. The smallest glycopeptide obtained by pronase digest of T2 is T2-P2 (Table XI). The only possible glycosylation site in this peptide is second residue asparagine or fourth residue threonine. The corresponding glycopeptide T2-A4 obtained by mild acid hydrolysis does not have threonine. Hence, second residue asparagine is glycosylated. Thus, carbohydrate side chains have been positioned at Asn-18 and Asn-106 (Figure 19).

Ovine Pancreatic DNase I

Purification

Ovine pancreatic DNase was purified to homogeneity by the procedure as described (9), with some modifications. Blue Sepharose and DEAE Bio-Gel (Bio-Rad) chromatography were used in place of second CM-cellulose chromatography and gel filtration, respectively. The purity of the protein was verified by SDS gel electrophoresis.

Sequence Analyses

About 4 mg of ovine DNase was cleaved with CNBr as previously described in Materials and Methods section. Four fractions were obtained by gel filtration on Sephadex G-100 (Figure 20). Fractions I, II, and IV contained peptides CB2, CB4, and CB3, respectively. Fraction III contained peptides CB1 and CB5 which were separated by HPLC (Figure 21). The amino acid compositions of the five CNBr fragments are given in Table XXVII. The partial sequence analyses of intact protein, CB2 and CB4 by automated sequenator, and complete manual sequence analysis of CB5 is shown in Tables XXXVI and XXXVII, respectively.

Abe and Liao (54) have shown that ovine DNase and bovine DNase are immunologically identical. When the amino acid composition of purified ovine DNase is compared with that of bovine DNase A, these enzymes look very similar with only a few substitutions (9). They also have identical end groups (9). For these two closely related proteins, the approach to the complete derivation of amino acid sequence is to identify the altered amino acid residues in small peptides of ovine DNase. Small peptides derived from chemical or enzymatic cleavages of ovine DNase were analyzed. Based on the sequence homology regions of identical sequence between two proteins will result in identical amino acid composition while the altered amino acid residue can be identified when there is a difference in the amino acid compositions of peptides derived from the same region. When bovine or ovine DNase was cleaved with CNBr, both yield five fragments of the same size; thus, it is concluded that the position

of the methionines is the same in both proteins. The three smallest CNBr fragments of ovine DNase (CB1, CB3, and CB5) have identical sequence to those of bovine DNase (Tables XXVII, XXXVI, XXXVII). CB4 has only one amino acid difference as indicated by amino acid analysis (Table XXVII). The rest of the differences are in the CB2.

There are many possible cleavage sites in DNase for the three proteolytic enzymes (trypsin, chymotrypsin, and thermolysin). A great number of small peptides can be yielded which will result in complication of peptide isolation. Therefore, the isolated CNBr fragments were used for identification of altered amino acids. Although a partial sequence analyses of ovine DNase were done by Edman degradation (Table XXXVI, XXXVII), the rest of the sequence derived is based on homology to the sequence of bovine DNase and the amino acid compositions of the isolated small peptides of ovine DNase (Table XXVII-XXV). Figure 29 represents the complete sequence of ovine DNase. Assignment of the altered residues in ovine DNase at positions 83, 121, 127, and 132 are based on differences in the amino acid compositions of the small peptides between the two proteins. Asp-159 is based on the results of three steps of Edman degradation of peptide CB2-T12 (Table XXXVII). Because of the assignment of leucine at position 160, isoleucine is assigned at position 163 by difference in amino acid composition (Table XXXI). There is a genetic variant form with Val replacing Ile at position 163 (Table XXX, peptide T12'). Assignments of Ala-230 and Val-231 are based on the result of aminopeptidase M hydrolysis of paptide CB4-C4-Th3 (Table XXXIV); after 2 hr at 37^{o} C, only 1.0 residue of alanine was liberated as shown by amino acid analysis.

The CNBr fragments of bovine DNase were also subjected to peptide

analyses by HPLC (data not included). For peptides that are identical in the two proteins, the elution positions were also identical, suggesting that their sequences are likely to be identical. In other words, there are probably no internally substituted residues within the peptide. Thus, by amino acid composition alone one can conclude that the sequences of the same peptide from the two DNases are the same.

Two peptides, CB2-C1 and CB2-T1, contain glucosamine as shown by amino acid analyses (Tables XXIX, XXX). This places the carbohydrate side chain at the Asn-18 residue (also see Table XXXVI). Thus, bovine and ovine DNases have the same carbohydrate attachment site.

Isolation of Two Forms of Ovine DNase

Ovine DNase occurs in two forms which differ in charges (9, 41). To investigate the chemical difference between the two forms, their separation was accomplished by modification of the method described in the isolation of ovine DNase. After Blue-Sepharose chromatography the pooled active fractions were dialyzed against 20 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0, for 4 hr, and were loaded on a pasteur pipette containing soybean trypsin inhibitor Agarose (about one ml bed volume), equilibrated in 20 mM Tris-HCl, pH 8.0. DNase activity which passed through was found to be very stable and the contaminating proteases were all absorbed. The DNase fraction was dialyzed against 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0, for 15 hr. The dialysate was chromatographed on a DEAE-cellulose column (Figure 30) in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0. The column was eluted by a gradient of 2-10 mM $CaCl_2$ in the starting buffer. DNase activity appeared in two separate peaks. Each peak was pooled

separately. Complete separation of the two forms was verified by isoelectric focusing (Figure 31). The amino acid compositions of the two forms are very similar (Table XXXVIII). The two forms and their CNBr peptides were digested with trypsin, and the peptides were mapped on HPLC using a reverse phase column (data not shown). There was no apparent difference between the two maps for the two forms. Thus, it is concluded at this point that the difference in charge is not due to the difference in amino acid sequence. However, it is possible that they may differ in the carbohydrate side chain; for example, one of the forms may contain sulfated carbohydrate.

Bovine DNase I

The amino acid sequence of bovine DNase was derived in 1973 (6, 7). If one compares the sequences of porcine DNase (Figure 19) and ovine DNase (Figure 29) with that of bovine DNase (7), there is a tripeptide gap between residues 27 and 28 of the bovine DNase. To prove that the gap is actually due to the mistake in the first elucidation of the sequence of bovine DNase and that the gap should have been filled with a tripeptide, the following experiments were performed.

Bovine DNase was cleaved at aspartyl prolyl bond (residues 56 and 57 of the published sequence (7)) with 88% formic acid as described in the Materials and Methods and resulted in two fragments. The smaller fragment, residues 1 through 56, was isolated by HPLC (Figure 32A). This peptide was further cleaved at the aspartic acid residue 30 by mild acid hydrolysis (46) and the peptide residue 1 through 30 was isolated by HPLC (Figure 32B). The amino acid compositions of these two peptides (Table XXXIX) clearly indicate one extra residue each of

isoleucine, valine, and arginine in this region as compared to the compositions based on published sequence (7).

After CNBr treatment, the largest CNBr fragment of bovine DNase, namely CB2 (residues 17 through 161), was isolated as described (7). The result of 20 cycle sequence analysis of this fragment, on automated sequenator (Tablx XL), shows the position of the three extra amino acids to be in between the residues 27 and 28 of the published sequence (7). The final sequence derivation by Liao et al. (7) was partly based on the sequence of tryptic peptides. Due to the repeat sequence of Ile-Val-Arg-Ile-Val-Arg, the resulting two tryptic peptides from this region have the same composition and sequence. Since the overlapping chymotryptic peptide (Ile-Val-Arg-Ile-Val-Arg-Tyr) has never been isolated, the six residue repeating sequence (Ile-Val-Arg-Ile-Val-Arg) was considered as one three residue (Ile-Val-Arg) in the final derived sequence of bovine DNase (7). In the present investigation even after considerable efforts, a chymotryptic peptide (Ile-Val-Arg-Ile-Val-Arg-Arg-Tyr) to overlap this sequence could not be isolated, probably due to its highly positively charged (3 arginine) and hydrophobic nature (2 isoleucine and valine each). It should be noted that around this region the corresponding chymotryptic peptides in porcine and ovine DNases were also not isolated.

CHAPTER V

DISCUSSION

Glycoproteins with N-linked carbohydrate side chains normally contain Asn-X-Ser(Thr) sequences where asparagine is glycosylated (55). Bovine DNase has two regions with such a sequence; around Asn-18, which is glycosylated and around Asn-106, which is not glycosylated (Figure 33). Glycosylation of proteins occurs in the endoplasmic reticulum and golgi apparatus (55). The enzyme catalyzing disulfide exchange during folding of the proteins is also situated in the endoplasmic reticulum (56). Asn-106 of bovine DNase can be glycosylated by the oviduct membrane system only after S-carboxymethylation or S-aminoethylation (55). This suggests that, during post translational modifications, DNase is first locked by disulfide linkage and then glycosylated. Sugar side chains are mainly situated in the beta turn of the protein backbone (57). That beta turn conformation favors glycosylation at the asparagine residue has also been shown by Beeley (58). In addition, exposure of the tripeptide sequence, Asn-X-Ser(Thr) is needed so that it is accessible to the glycosyltransferase (55). However, porcine DNase is glycosylated at both sites, Asn-18 and Asn-106 (Figure 33). If it is assumed that post-translational modification procedure is the same for bovine and porcine DNases, then probably three substitutions, from bovine to porcine enzyme Ser-103 → Pro, Ser-105 → Thr and Ser-107 → Asn, at the vicinity of the Asn-106 are responsible for providing the

necessary conformation for glycosylation at Asn-106 of porcine DNase (see Figure 33). Especially proline, at position 103 of porcine DNase, can induce a beta turn structure and can neutralize the conformational hindrance brought by disulfide locking necessary for glycosylation. Ovine DNase, which does not have any substitution in this region, is devoid of a sugar side chain at Asn-106. Pig differs from beef and sheep in their digestive systems. Glycosylation of RNase has been found to be related to the type of digestive system of the animal (59). Species with coecal digestion, like pig, horse, and guinea pig produce RNase with large carbohydrate moieties attached to several positions (59). Ruminants, on the other hand, are found to contain more RNase activity than non-ruminants (60). Therefore, it was suggested that one of the function of RNase is to digest microbial nucleic acid (60). DNase also follows a similar pattern. Porcine DNase is not only more glycosylated, but also is less active (Table I) than bovine and ovine DNases. However, there is no significant difference in the amount of the enzyme in the procine pancreas as compared to bovine or ovine pancreas. Carbohydrate side chains not only alter the molecular weight of a protein but, also changes its conformation (61). DNases and RNases are consistently glycosylated. The amount of glycosylation depends upon digestive system of the animal in which the enzyme has to function. These observations clearly indicate that the carbohydrate side chains must have a definite role. However, in DNase and RNase, sugar side chain is not involved in the activity of the enzyme as removal of the carbohydrate side chains from both enzymes does not alter the activity (62).

Structurally, ovine DNase is very close to bovine DNase. There

are only 12 differences in the amino acid sequence between these two proteins (Figure 33). All changes have occurred due to single base changes in the genetic code (Table XLI). Except for the changes of His-121 to Pro, and Ala-132 to Pro, from bovine to ovine enzyme, all other changes are conservative. In other words, the degree of hydrophobicity, conformational preference of the back bone, and the bulk of the amino acids are retained. However, porcine DNase is structurally not as close to bovine as is ovine DNase. It differs from bovine DNase by as many as 52 amino acids in the primary sequence. With the exception of five changes, all others have occurred due to single base changes in the genetic code (Table XLI). Although the three dimensional structure of DNase is not known, it is probable that these three proteins have a very similar structure. Natural selection pressure mainly favors conservations of the three dimensional structure and permits only changes that do not disrupt the function of the protein (63). In bovine and porcine DNases, within the residues 74 to 132, 159 to 163, 178 to 191, 199 to 208 and 219 to 228, there are 31 differences, 33% of the amino acids are changed (Figure 33). This suggests that there are only certain regions in the molecule which allow changes. Conservative changes may not alter the structure of the protein, but changes in the regions in between residues 96-106, 121-124, 159-161 and 188-191 are not conservative in the sense of French et al. (64). These areas must be situated either outside or away from the regions which are involved in the conformation and function of the protein. It should also be noted that residues which have been changed between ovine and bovine are also changed in porcine, with 2 exceptions (Figure 33).

Porcine DNase, compared to ovine and bovine DNase, is longer by two amino acids (see Figure 33) at the COOH-terminus. Point mutation at the stop codon's first base changes it to the codon for arginine (see Table XLII), and translation hence continues until another termination codon is encountered.

TABLE XLII

COMPARISON OF THE COOH-TERMINUS OF PORCINE, BOVINE, AND OVINE DNases

Bovine and ovine DNases	260 -ThrStop ACXUGA
Porcine DNase	AAACGAGCXUGA -LysArgAlaStop

The reason for the less active nature of porcine DNase at this moment is unclear. Liao (33) has shown that residues at COOH-terminus of bovine DNase are essential for the active conformation of the enzyme. Bovine parotid DNase (54) and ovine and bovine pancreatic DNases (7, 9), have identical end groups, are equally active. Presence of positively charged lysine and arginine at the COOH terminus, Figure 19, of the porcine DNase may interfere with the substrate binding, thus lowering the activity.

Tyr-62, which has been reported to bind Ca^{2+}, essential for the

folding of the enzyme to the active conformation (31) in bovine DNase, is substituted by histidine in porcine DNase. This supports the observation of Liao et al. (33) that this residue is not crucial for activity of the enzyme. Finding of an identical repeat sequence of Ile-Val-Arg in bovine DNase and very conservative changes in ovine and porcine DNases in the same region indicates that this stretch might be important for enzyme function, that is, binding to the phosphoryl groups of double stranded DNA (Abe and Liao, unpublished results).

Functionally related proteins show marked similarity in their active site sequence (63). DNase and RNase are produced in the same tissue and have similar substrates. A comparison of active site sequence of these two enzymes (Table XLIII) shows marked homology.

TABLE XLIII

COMPARISON OF THE ACTIVE SITE SEQUENCE OF DNase AND RNase

Enzyme	Active site sequence
	119
Bovine RNase[a]	Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala
Porcine RNase[a]	Pro-Pro-Val-Pro-Val-His-Phe-Asp-Ala
Equine RNase[a]	Val-Pro-Val-His-Phe
	134
Bovine DNase[b]	Ala-Ile-Val-Ala-Leu-His-Ser-Ala-Pro
Ovine DNase	Ala-Ile-Val-Pro-Leu-His-Ser-Ala-Pro
Porcine DNase	Ala-Ile-Val-Pro-Leu-His-Ala-Ala-Pro

[a]From Dayhoff et al. (63).
[b]From Liao et al. (7).

Several common features between these two proteins have already been discussed. Thus it is reasonable to say that DNase and RNase belong to the same protein family and are as closely related as are trypsin and chymotrypsin.

CHAPTER VI

SUMMARY AND CONCLUSION

Porcine pancreatic DNase has been purified to homogeneity by ammonium sulfate fractionation, CM-Cellulose chromatography, Blue Sepharose chromatography, ConA Agarose chromatography and DEAE-Biogel chromatography. Like other DNases I, porcine DNase shows multiplicity. The pI values of the four bands are estimated to be 5.06, 4.96, 4.78, and 4.7. It is larger than ovine and bovine pancreatic DNases having a molecular weight of about 34,000. It has leucine at the amino terminus and alanine at the COOH-terminus. The complete amino acid sequence of porcine and ovine DNases has been determined. Sequence analyses were performed on intact proteins and their CNBr fragments. Porcine DNase is a single polypeptide of 262 amino acid residues with two disulfide loops. It is a glycoprotein with two carbohydrate side chains attached to Asn-18 and Asn-106. Porcine DNase is longer than the bovine and ovine enzymes by two amino acids. The amino acid sequence of ovine DNase, which has been shown to be very similar to bovine DNase (36) with only a few substitutions, has been derived from the amino acid composition of the small peptides obtained from the CNBr fragments and the known sequence of bovine DNase (7). To further substantiate the homology of sequences between ovine and bovine DNases, several stretches of ovine DNase were sequenced. Compared to bovine DNase, ovine DNase has 12 amino acid substitutions.

While comparing the sequences of ovine and porcine DNases with the reported sequence of bovine DNase (7), we found that the ovine and porcine enzymes have a tripeptide insertion in the sequence of bovine DNase between residues 27 and 28. On the basis of sequence analysis, of one of the CNBr fragments by the automated sequenator, and the amino acid analysis of two peptides obtained from this region, a tripeptide with the sequence of Ile-Val-Arg has been shown to exist in between residues 27 and 28 of the published sequence of bovine DNase (7). Therefore, there is no insertion in porcine or ovine DNases, instead, there is an apparent error in the published sequence of bovine DNase. Amino acid analysis of the two forms of ovine DNase, separated by DEAE Cellulose, do not show any difference. The cause of multiplicity in ovine and porcine DNases remains undetermined. The chemical analysis of the carbohydrate side chains of these two enzymes may provide some insight concerning this multiplicity.

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TABLE I

PURIFICATION SCHEME OF PORCINE DNase

Step	Total Activity	Activity Recovered	Specific Activity	Purification Fold
	Units x 10^3	%	Units/A_{280}	
1. Crude extract	75	100	1.7	1
2. 30-60% $(NH_4)_2SO_4$ fraction	50	67	5	3
3. CM Cellulose chromatography	31	41	20	12
4. Blue Sepharose chromatography	21	28	200	118
5. ConA agarose chromatography	12	16	470	227
6. DEAE Biogel chromatography	10	13	650	383

TABLE II

AMINO ACID ANALYSIS OF PORCINE DNase[a]

Aspartic Acid	31.4 (31)
Threonine	14.7 (15)
Serine	23.8 (24)
Glutamic Acid	21.4 (21)
Proline	15.2 (15)
Glycine	9.4 (9)
Alanine	23.2 (23)
Half-Cystine	3.5 (4)
Valine	21.7 (22)
Methionine	2.7 (3)
Isoleucine	11.6 (12)
Leucine	23.7 (24)
Tyrosine	11.8 (12)
Phenylalanine	12.9 (13)
Histidine	7.8 (8)
Lysine	7.1 (7)
Tryptophan	2.1 (3)
Arginine	15.1 (15)
Glucosamine	3.5

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

TABLE III

THE AMINO ACID COMPOSITIONS OF CNBr FRAGMENTS DERIVED FROM PORCINE DNase

Amino Acid	Peptide			
	CB1	CB2	CB3	CB4
Asx	1.1 (1)	23.4 (23)		8.5 (8)
Thr	1.9 (2)	4.8 (5)		8.3 (8)
Ser		12.3 (13)		9.5 (10)
Glx	1.1 (1)	13.9 (14)		7.1 (7)
Pro		8.2 (8)		6.7 (7)
Gly	1.0 (1)	4.5 (4)		3.9 (4)
Ala	2.0 (2)	9.5 (9)		11.8 (12)
1/2 Cys		1.2 (2)		1.5 (2)
Val		13.4 (14)		7.2 (8)
Ile	2.0 (2)	6.5 (7)		3.6 (4)
Leu	1.1 (1)	14.7 (15)	1.0 (1)	7.4 (7)
Tyr		8.5 (9)		2.7 (3)
Phe	1.9 (2)	6.3 (6)		4.6 (5)
Hsr	0.6 (1)	0.7 (1)	0.8 (1)	
His		3.7 (4)		2.5 (3)
Lys	1.0 (1)	5.1 (5)		1.4 (1)
Trp		0.0 (1)		0.3 (2)
Arg	2.2 (2)	8.4 (8)		5.5 (5)
GlcN		1.1		
Yield %	44	88	82	66

TABLE IV

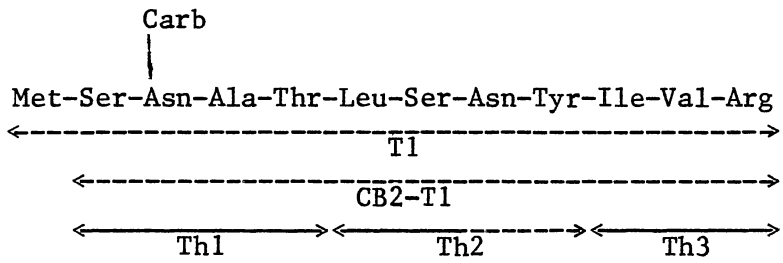
AUTOMATED EDMAN DEGRADATION OF PORCINE DNase
AND ITS CNBr PEPTIDES

Cycle	Porcine DNase		CB2		CB4	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Leu	26.7	Ser	10.5	Gly	26.7
2	Arg	10.0	Asn	—	Asp	25.2
3	Ile	24.2	Ala	30.1	Phe	21.3
4	Ala	22.5	Thr	5.6	Asn	20.1
5	Ala	22.2	Leu	26.5	Ala	19.2
6	Phe	19.8	Ser	3.2	Gly	18.0
7	Asn	18.4	Asn	16.2	Rcm	15.1
8	Ile	17.0	Tyr	17.1	Ser	2.5
9	Arg	8.1	Ile	10.6	Tyr	10.1
10	Thr	2.5	Val	8.4	Val	8.8
11	Phe	13.1	Arg	4.0		
12	Gly	10.2	Ile	10.2		
13	Glu	8.3	Leu	13.3		
14	Thr	1.2	Ser	3.2		
15	Lys	3.5	Arg	2.8		
16	Met	2.5	Tyr	5.9		
17	Ser	0.5	Asp	4.7		
18			Ile	3.0		
19			Ala	2.5		

TABLE V

SEQUENCE DETERMINATION OF PEPTIDE T1, CB2-T1
AND THERMOLYTIC PEPTIDES OF CB2-T1

Sequence (residues 16 through 27)[a]



Composition

Amino Acid	Peptide				
	T1	CB2-T1	Th1	Th2	Th3
Asx	2.2	2.1	0.9	1.1	
Thr	0.9	1.0	1.1		
Ser	1.9	1.8	0.9	1.0	
Ala	1.1	1.0	1.3		
Val	0.8	0.9			0.7
Met	0.5				
Leu	1.0	1.1		1.0	
Ile	0.9	0.8			0.7
Tyr	1.0	1.0		0.8	
Arg	1.1	1.1			1.1
GlcN	0.4	0.2	0.3		
Yield %	42	45	35	57	58

TABLE V (Continued)

Edman Degradation

Cycle	Th1		Th2		Th3	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Ser	0.5	Leu	5.6	Ile	4.8
2	—		Ser	0.3	Val	3.2
3	Ala	1.4			Arg	0.6

[a]Peptide Th1 is the only thermolytic glycopeptide (see composition above). First residue Ser and third residue Ala were identified as PTH amino acids by Edman degradation of Th1. This peptide released free Thr after three step Edman degradation. The position of the carbohydrate side chain hence has been assigned second Asn residue.

TABLE VI

SEQUENCE DETERMINATION OF PEPTIDE CB2-T2

Sequence (residues 28 through 31)

Ile-Leu-Ser-Arg

CB2-T2

Composition

Amino Acid	Peptide CB2-T2
Ser	0.9
Ile	1.0
Leu	1.0
Arg	1.1
Yield %	55

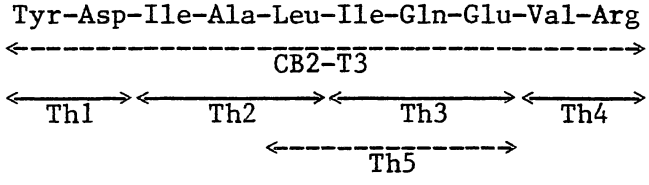
Subtractive Edman Degradation

	Ile	Leu	Ser	Arg
Step 1	0.2	1.1	0.8	1.2
Step 2	0.0	0.4	0.9	1.1
Step 3	0.0	0.1	0.1	1.0

TABLE VII

SEQUENCE DETERMINATION OF PEPTIDE CB2-T3 AND ITS THERMOLYTIC PEPTIDES

Sequence (residues 32 through 41)



Composition

Amino Acid	Peptide					
	CB2-T3	Th1	Th2	Th3	Th4	Th5
Asx	1.0	1.0				
Glx	2.1			2.0		1.9
Ala	1.0		0.9			
Val	0.9				1.0	
Ile	1.1		1.0	0.9		0.9
Leu	1.0		1.0			1.0
Tyr	0.9	0.8				
Arg	1.1				1.2	
Yield %	71	78	37	25	40	18

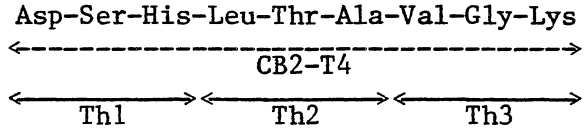
Edman Degradation

Cycle	Th1		Th2		Th3		Th4	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Tyr	10.5	Ile	12.2	Ile	9.9	Val	7.4
2	Asp	8.5	Ala	9.8	Gln	5.2	Arg	3.3
3			Leu	2.1	Glu	3.5		

TABLE VIII

SEQUENCE DETERMINATION OF PEPTIDE CB2-T4 AND ITS THERMOLYTIC PEPTIDES

Sequence (residues 42 through 50)



Composition

Amino Acid	Peptide			
	CB2-T4	Th1	Th2	Th3
Asx	1.1	1.1		
Thr	0.9		1.0	
Ser	1.0	0.9		
Gly	1.1			1.0
Ala	1.1		0.9	
Val	0.9			0.9
Leu	1.0		1.1	
His	0.9	1.0		
Lys	1.1			1.0
Yield %	60	65	73	84

TABLE VIII (Continued)

Substractive Edman Degradation

	Peptide Th1			Peptide Th2		
	Asx	Ser	His	Leu	Thr	Ala
Step 1	0.3	0.9	1.0	0.1	0.9	1.0
Step 2	0.0	0.4	1.1	0.0	0.0	1.0

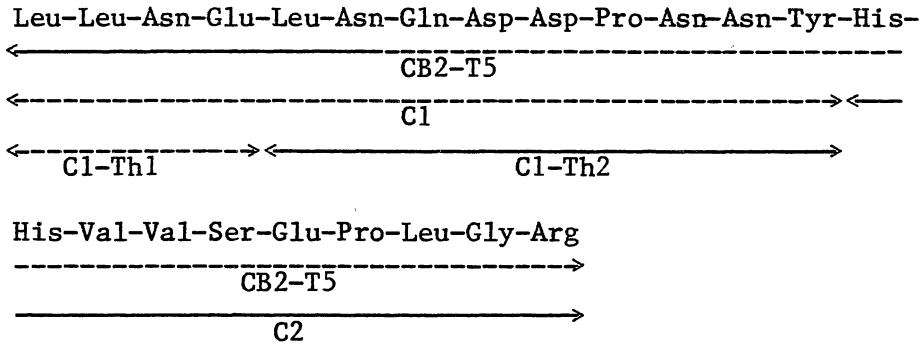
Edman Degradation

Cycle	Peptide Th3	
	PTH-	Yield n mole
1	Val	6.3
2	Gly	2.5
3	Lys	2.8

TABLE IX

SEQUENCE DETERMINATION OF PEPTIDE CB2-T5 AND PEPTIDES DERIVED FROM IT

Sequence (residues 51 through 73)[a]



Composition

Amino Acid	Peptide				
	CB2-T5	C1	C1-Th1	C1-Th2	C2
Asx	5.9	6.1	1.0	5.1	
Ser	0.9				1.0
Glx	3.0	2.0	0.9	0.9	1.1
Pro	2.0	1.0		1.1	1.0
Gly	1.0				1.2
Val	1.9				1.5
Leu	4.1	3.1	2.1	1.0	1.1
Tyr	0.9	0.8		0.9	
His	2.0				1.9
Arg	1.1				1.0
Yield %	72	68	75	69	84

TABLE IX (Continued)

Edman Degradation

Cycle	Peptide					
	CB2-T5		C1-Th2		C2	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Leu	15.1	Leu	4.5	His	-
2	Leu	8.5	Asn	2.6	His	-
3	Asn	4.8	Gln	1.3	Val	5.5
4	Glu	2.2	Asp	0.9	Val	2.3
5	Leu	1.3	Asp	0.6	Ser	0.3
6	Asn	0.5	Pro	+	Glu	0.6
7					Pro	0.8
8					Leu	0.2

Substractive Edman Degradation of Peptide C2

Step	His	Val	Ser	Glx	Pro	Leu	Gly	Arg
1	1.2	1.5	1.1	1.0	1.0	1.1	1.1	1.0
2	0.2	1.7	0.8	1.1	1.0	0.9	1.0	1.1

[a]Peptide Th2 released 1.0, Tyr, 0.5, Asn in 10 min and 1.0 Tyr, 1.3 Asn in two hr with carboxypeptidase A at room temperature. Peptide C2 with carboxypeptidase B released 1.0 Arg, 0.5 Gly and 0.2 Leu in two hr at room temperature.

TABLE X

SEQUENCE DETERMINATION OF PEPTIDE CB2-T6 AND PEPTIDE CB2-T7

Sequence of CB2-T6 (residues 75 through 77)

Ser-Thr-Tyr-Lys

CB2-T6

Composition

Amino Acid	CB2-T6	CB2-T7
Thr	1.0	
Ser	1.0	
Glx		0.9
Tyr	0.9	
Lys	1.1	
Arg		1.0
Yield %	61	89

Substractive Edman Degradation of CB2-T6

Amino Acid	Step 1	Step 2	Step 3
Ser	0.2	0.0	0.0
Thr	1.0	0.1	0.0
Tyr	1.0	1.0	0.0
Lys	1.1	1.0	1.0

Sequence of CB2-T7 (residues 79 and 80)

Glu-Arg

CB2-T7

Sequence of the peptide CB2-T7 is derived based on the specificity of tryptic cleavage.

TABLE XI

SEQUENCE DETERMINATION OF PEPTIDE T2, CB2-T8, CB2-T8a, CB2-T8b AND PEPTIDES DERIVED FROM T2

Sequence (residues 81 through 118)[a]

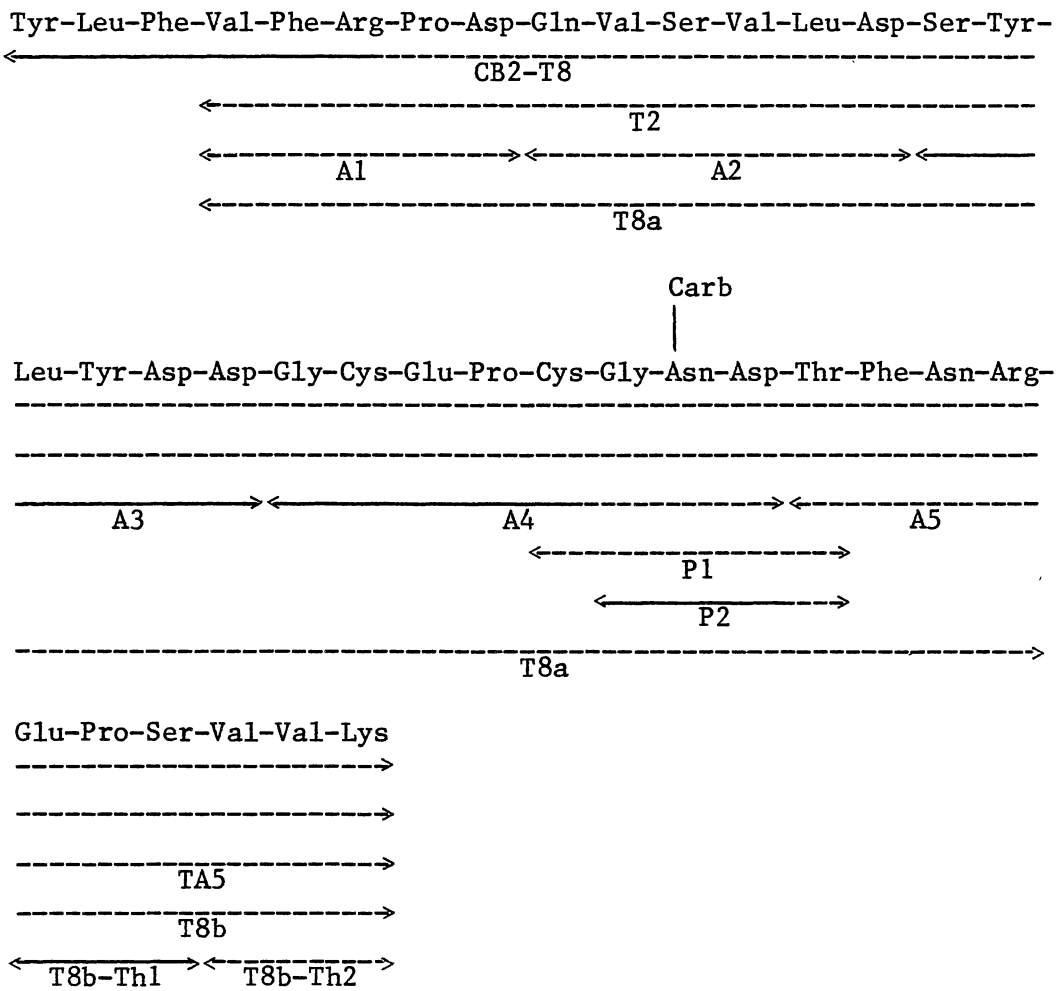


TABLE XI (Continued)

Composition

Amino Acid	Peptide								
	CB2-T8	T2	T8a	T8b	T8b Th1	T8b Th2	A1	A2	A3
Asx	6.8	7.2	6.7				1.1	0.9	1.9
Thr	1.0	0.9	0.9						
Ser	2.7	2.8	2.0	0.9	0.8			1.0	1.1
Glx	3.0	3.1	2.3	1.0	1.0			1.0	
Pro	3.3	3.2	2.3	1.0	1.0		1.1		
Gly	2.2	2.0	2.0						
1/2 Cys	0.7	0.8	0.5						
Val	4.5	4.7	3.0	1.8		1.7	1.0	1.9	
Leu	2.9	2.0	2.1					1.3	1.1
Tyr	2.7	2.1	1.9						2.0
Phe	2.9	2.0	1.9				0.8		
Lys	1.0	0.8		1.0		1.0			
Arg	2.0	2.3	2.0				1.0		
GlcN	0.4	0.5	0.5						
Yield %	15	55	32	28	62	59	35	21	22

Amino Acid	Peptide			
	A4	A5	P1	P2
Asx	2.0	1.2	2.0	2.1
Thr		0.9	0.8	0.9
Ser		0.9		
Glx	1.0	1.0		
Pro	0.9	1.2		
Gly	2.1		1.1	1.0
1/2 Cys	0.8		0.5	
Val		1.6		
Phe		0.9		
Lys		1.0		
Arg		1.1		
GlcN	1.1		1.2	1.9
Yield %	30	31	16	31

TABLE XI (Continued)

Edman Degradation

Cycle	Peptide					
	CB2-T8		A3		A4	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Tyr	4.5	Ser	1.5	Gly	2.4
2	Leu	2.6	Tyr	3.8	Rcm	1.5
3	Phe	1.5	Leu	1.4	Glu	0.4
4	Val	0.5	Tyr	0.8	Pro	0.2
5	Phe	0.3	Asp	0.2	Rcm	0.2
6	Arg	+	Asp	+		

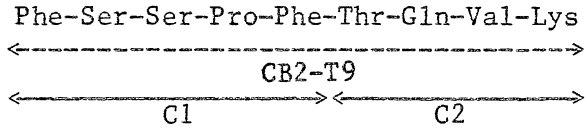
Cycle	Peptide					
	P2		T8b-Th1		T8b-Th2	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Gly	2.4	Glu	2.9	Val	3.7
2	-		Pro	0.7	Val	2.2
3	Asp	+	Ser	0.1	Lys	0.4

[a]Pronase digest was performed to establish the position of the carbohydrate side chain, hence, only 2 peptides having carbohydrate, obtained by this digest, are reported. Since A4 has carbohydrate but does not have threonine (see above), the position of the carbohydrate has been assigned to Asn-106 (Figure 19).

TABLE XII

SEQUENCE DETERMINATION OF PEPTIDE CB2-T9 AND ITS CHYMOTRYPTIC PEPTIDES

Sequence (residues 118 through 126)



Composition

Amino Acid	Peptide		
	CB2-T9	C1	C2
Thr	0.9		1.0
Ser	1.8	1.7	
Glu	1.1		1.0
Pro	1.0	1.0	
Val	1.0		0.9
Phe	2.0	1.9	
Lys	1.1		1.0
Yield %	73	83	78

Edman Degradation of C2

Cycle	PTH-	Yield (n mole)
1	Thr	3.5
2	Gln	5.6
3	Val	2.7
4	Lys	0.3

TABLE XII (Continued)

Substractive Edman Degradation of C1

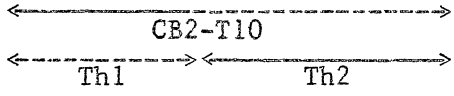
	Phe	Ser	Pro
Step 1	1.3	1.9	1.0
Step 2	1.0	1.2	1.0
Step 3	1.1	0.3	1.0
Step 4	Free 1.3 n mole Phe		

TABLE XIII

SEQUENCE DETERMINATION OF PEPTIDE CB2-T10 AND ITS THERMOLYTIC PEPTIDES

Sequence (residues 127 through 133)

Glu-Phe-Ala-Ile-Val-Pro-Leu



Composition

Amino Acid	Peptide		
	CB2-T10	Th1	Th2
Glu	1.1	1.1	
Pro	0.9		1.2
Ala	1.0	0.9	
Val	0.9		0.7
Ile	0.9		0.8
Leu	1.0		1.2
Phe	1.0	1.0	
Yield %	41	73	66

Edman Degradation

Cycle	Peptide			
	CB2-T10		Th2	
	PTH-	Yield n mole	PTH-	Yield n mole
1	Glu	6.5	Ile	12.5
2	Phe	4.0	Val	6.2
3	Ala	1.5	Pro	1.7
4			Leu	0.4

TABLE XIV

SEQUENCE DETERMINATION OF PEPTIDE CB2-T11 AND CB2-T12

Sequence of CB2-T11 (residues 134 through 155)

His-Ala-Ala-Pro-Ser-Asp-Ala-Ala-Ala-Glu-Ile-Asp-Ser-
<----------CB2-T11----------

Leu-Tyr-Asp-Val-Tyr-Leu-Asn-Val-Arg
<----------

Sequence of T12 (residues 156 through 163)

Gln-Lys-Trp-Asp-Leu-Gln-Asp-Ile-Hsr
<----------CB2-T12----------

Composition

Amino Acid	Peptide	
	CB2-T11	CB2-T12
Asp	4.0	1.9
Ser	1.8	
Glu	1.0	1.9
Pro	1.0	
Ala	5.1	
Val	1.9	
Ile	1.0	1.2
Leu	2.0	1.1
Tyr	1.9	
His	1.0	
Lys		1.0
Trp		0.2
Arg	1.1	
Hsr		0.8
Yield %	35	41

TABLE XIV (Continued)

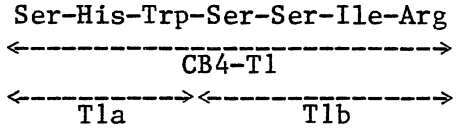
Edman Degradation of T12

Cycle	PTH-	Yield (n mole)
1	Gln	0.3
2	Lys	0.4
3	Trp	+

TABLE XV

SEQUENCE DETERMINATION OF PAPTIDE CB4-T1 AND CB4-T2

Sequence of CB4-T1 (residues 179 through 185)[a]



Composition

Amino Acid	Peptide			
	CB4-T1	CB4-T1a	CB4-T1b	CB4-T2[b]
Ser	2.7	0.8	1.7	
Ile	1.0		1.0	
Leu				1.0
His	1.0	1.0		
Trp	0.0	0.0		
Arg	0.9		1.1	1.0
Yield %	44	27	33	83

Edman Degradation of CB4-T1

Cycle	Amino Acid	Yield (n mole)
1	Ser	0.5
2	-	-
3	Trp	0.2
6	Ile	0.3

TABLE XV (Continued)

Substractive Edman Degradation of CB4-T1

	Ser	His	Trp	Ile	Arg
Step 1	1.8	0.7	0.0	1.0	1.1
Step 2	1.6	0.3	0.0	1.1	0.9
Step 3	1.7	0.0	0.0	1.0	1.1
Step 4	0.8	0.0	0.0	0.9	1.0
Step 5	0.0	0.0	0.0	0.8	1.0

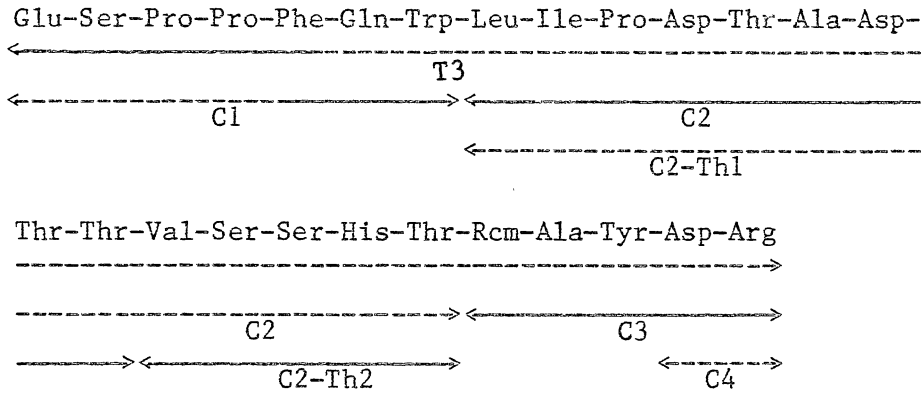
[a]Only residues 1, 3 and 6 were identified as PTH-amino acids, rest by Substractive Edman, Arginine by difference.

[b]CB4-T2 is a dipeptide. Its sequence is derived on the basis of specificity of tryptic cleavage.

TABLE XVI

SEQUENCE DETERMINATION OF PEPTIDE T3[a]

Sequence (residues 188 through 213)



Composition

Amino Acid	Peptide						
	T3	C1	C2	C3	C4	C2-Th1	C2-Th2
Asx	3.1		2.0	1.0	1.0	1.9	
Thr	3.8		3.9			2.7	1.2
Ser	2.6	1.1	1.9				1.8
Glx	2.1	2.0					
Pro	3.0	1.9	0.9			1.3	
Ala	2.1		1.0	1.1		0.9	
Rcm	0.9			0.3			
Val	1.0		1.0				0.9
Ile	1.0		1.2			0.9	
Leu	1.2		1.3			1.2	
Tyr	1.1			1.0			
Phe	1.1	1.0					
His	1.0		0.8				1.0
Trp	0.4	0.0					
Arg	0.9			0.9	1.0		
Yield %	72	81	78	59	24	65	40

TABLE XVI (Continued)

Edman Degradation

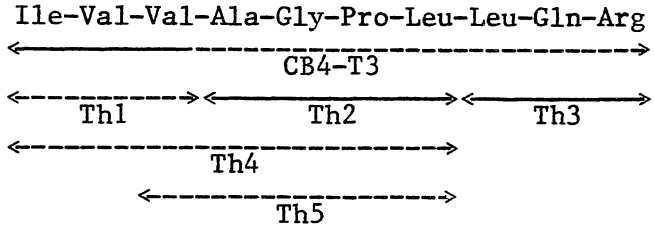
Cycle	Peptide							
	T3		C2		C3		C2-Th2	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Glu	5.6	Leu	6.7	Rcm	6.5	Val	2.2
2	Ser	2.6	Ile	3.5	Ala	4.5	Ser	0.5
3	Pro	0.4	Pro	0.8	Tyr	1.2	Ser	0.6
4	Pro	0.2	Asp	0.7	Asp	0.5	His	+
5	Phe	+	Thr	0.2	Arg	+		
6			Ala	0.2				
7			Asp	+				

[a]CPase hydrolysis.
C1 released 1.0, Trp, and 0.6 Gln in 2 hr at room temperature with CPase B. With CPase A it released 1.0 Trp, 0.8 Gln, and 0.7 Phe under similar conditions.
C2-Th1 with CPase A released 1.5 residues of Thr in two hr at room temperature.
C2 released 1.0 Thr, 0.8 His, 0.6 Ser in 10 min at room temperature with CPase A.

TABLE XVII

SEQUENCE DETERMINATION OF PEPTIDE CB4-T3

Sequence (residues 214 through 223)



Composition

Amino Acid	Peptide					
	CB4-T3	Th1	Th2	Th3	Th4	Th5
Glx	1.0			1.0		
Pro	1.0		0.8		1.1	1.0
Gly	1.1		1.2		1.0	0.8
Ala	1.0		1.0		1.0	1.0
Val	1.8	1.8			1.9	0.9
Ile	0.8	1.0			0.8	
Leu	2.0		1.1	1.0	1.1	1.0
Arg	1.1			0.9		
Yield %	69	12	21	43	63	17

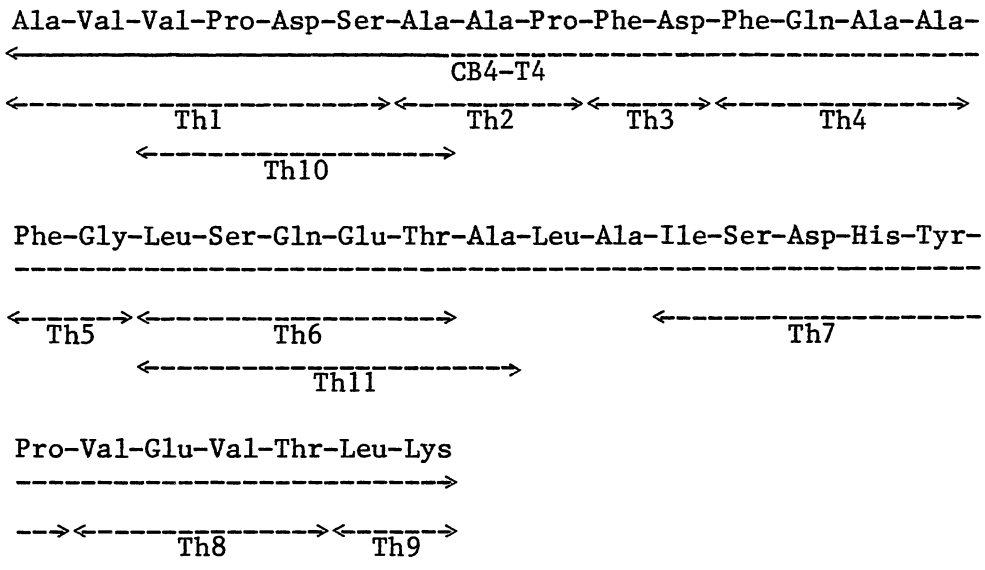
Edman Degradation

Cycle	Peptide					
	CB5-T3		Th2		Th3	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Ile	6.7	Ala	3.6	Leu	8.1
2	Val	2.5	Gly	1.3	Gln	4.1
3	Val	3.2	Pro	0.4	Arg	0.3
4			Leu	0.2		

TABLE XVIII

SEQUENCE DETERMINATION OF PEPTIDE CB4-T4, ITS THERMOLYTIC PEPTIDES AND CB4-T5

Sequence of CB4-T4 (residues 224 through 260)



Edman Degradation of CB4-T4

Cycle	PTH-	Yield (n mole)
1	Ala	5.5
2	Val	2.5
3	Val	1.3
4	Pro	0.5
5	Asp	0.7
6	Ser	+
7	Ala	+

TABLE XVIII (Continued)

Composition of CB4-T4

Amino Acid	Peptide											
	CB4-T4	Th1	Th2	Th3	Th4	Th5	Th6	Th7	Th8	Th9	Th10	Th11
Asx	3.2	1.2		1.0				1.1			1.0	
Thr	2.0						0.9		1.1			0.9
Ser	2.7	1.0					1.2	0.8			0.9	1.0
Glx	3.7				1.0		1.8		1.0			1.9
Pro	3.1	1.1	0.9					1.0			1.0	
Gly	1.3					1.1						
Ala	6.7	1.0	1.9		2.1						1.2	0.9
Val	3.5	1.7							1.9		0.8	
Ile	1.0							1.1				
Leu	2.7						1.2			1.2		1.1
Tyr	1.0							0.9				
Phe	2.7			0.9	1.1	0.9						
His	1.0							1.0				
Lys	1.0									0.9		
Arg												
Yield %	80	40	17	26	40	39	41	50	45	43	14	21

Sequence of CB4-T5 (residues 261 and 262)[a]

Arg-Ala
←CB4-T5→

Composition of CB4-T5

Ala	1.0
Arg	1.0
Yield %	74

[a]For sequence of CB4-T5, refer to CB4-C11 (Figure 19).

TABLE XIX

THE AMINO ACID COMPOSITIONS OF CHYMOTRYPTIC PEPTIDES OF PORCINE CB1

Amino Acid	Peptide		
	CB1-C1	CB1-C2	CB1-C3
Asx		1.1	
Thr		0.9	0.9
Glu			1.0
Gly			1.0
Ala	2.1		
Ile	1.0	1.0	
Leu	1.0		
Phe	0.9	1.0	
Lys			1.1
Arg	1.1	1.0	
Hsr			0.8
Yield %	79	82	68

TABLE XX

THE AMINO ACID COMPOSITIONS OF CHYMOTRYPTIC PEPTIDES OF PORCINE CB2

Amino Acid	Peptide																		
	C1	C2	C3	C4	C4a	C5	C5a	C5b	C6	C6a	C6b	C7	C8	C9	C10	C11	C12	C14	C15
Asx	2.0		2.1	6.1	5.8							1.1	5.2	1.0				1.1	1.7
Thr	0.9				1.1	0.9		0.9					1.0			1.0			
Ser	1.9	0.9	0.9			1.8	0.9	0.8				0.8	0.9	0.9	1.9				
Glx			2.0	2.3	2.2	1.0	1.2		0.9	1.0		1.0	1.0	1.0		2.1		1.0	1.0
Pro				0.8	0.9	0.9	1.0					0.9	0.9	1.1	1.0		0.9		
Gly				1.0		1.0		1.0					2.2						
Ala	1.0		1.0														1.0		
Val			0.9	1.1		1.5	1.6					1.9		1.7		0.9	0.9	0.8	
½Cys													0.8						
Ile			1.9														0.9		1.0
Leu	1.1		2.0	3.2	2.2	1.0	1.0		1.1		1.1	1.1	1.0				1.2	1.0	1.0
Tyr	0.9	1.0		1.0	0.9	0.9		0.9	0.9	1.0			1.9						
Phe									0.9		1.0		1.0	1.0	1.0	0.9			
His			1.0			1.8	2.0												
Lys				1.1					1.0	1.1				1.0		1.0		1.0	
Arg		1.1	1.1			1.0		1.0	1.1	1.1				1.1					
GlcN	0.8												1.2						
Trp																		0.1	
Hsr																			0.9
Yield %	67	92	78	40	52	25	68	42	17	70	62	64	69	68	80	71	71	55	53

TABLE XXI

THE AMINO ACID COMPOSITION OF CHYMOTRYPTIC PEPTIDES OF PORCINE CB4

Amino Acid	Peptide										
	C1	C2	C2a	C3	C4	C5	C6	C8	C9	C10	C11
Asx	2.0					2.1	1.1	1.1		1.0	
Thr		1.9				3.9			0.9	0.9	
Ser	0.9	1.0	0.8	1.7	0.9	1.8			0.8	0.9	
Glx					2.0			1.0	1.9	1.1	
Pro					2.1	1.0	0.9			0.9	
Gly	2.1						1.0		1.1		
Ala	1.1					1.0	1.1	2.0	1.0	1.0	0.9
½Cys	0.5										
Val		1.0				0.9	1.8			2.1	
Ile				0.9		1.1	0.7			0.9	
Leu				1.0		1.0	1.0		2.0	1.0	
Tyr	1.1									0.9	
Phe	1.0				1.0			1.9			
His		1.1	1.0			0.9				0.8	
Trp		0.2	0.0		0.0						
Lys											1.0
Arg				1.1	1.0		1.1				1.1
Yield %	67	57	23	44	56	53	38	68	67	74	91

TABLE XXII

SEQUENCE DETERMINATION OF SOME CHYMOTRYPTIC PEPTIDES

Peptide CB2-C2

Sequence (residues 30 through 32)

Ser-Arg-Tyr

←——→

CB2-C2

Composition See Table XX

Substractive Edman Degradation of CB2-C2

	Ser	Arg	Tyr
Step 1	0.2	1.0	0.9
Step 2	0.0	0.4	1.0

Peptide CB2-C4

Sequence (residues 46 through 63)

Thr-Ala-Val-Gly-Lys-Leu-Leu-Asn-Glu-Leu-Asn-Gln-Asp-Pro-Asn-Asn-Tyr

<----------CB2-C4----------→

<----------CB2-C4a----------→

Composition See Table XX

Peptide CB2-C5

Sequence (residues 64 through 76)

His-His-Val-Val-Ser-Glu-Pro-Leu-Gly-Arg-Ser-Thr-Tyr

<----------CB2-C5----------→

<----------CB2-C5a----------→ <----------CB2-C5b----------→

Composition See Table XX

TABLE XXII (Continued)

Edman Degradation of CB2-C5b

Cycle	PTH-	Yield (n mole)
1	Gly	0.7
2	Arg	+

Peptide CB2-C6

Sequence (residues 77 through 82)

Lys-Glu-Arg-Tyr-Leu-Phe

CB2-C6

CB2-C6a CB2-C6b

Composition See Table XX

Edman Degradation

Cycle	CB2-C6		CB2-C6b	
	PTH-	Yield n mole	PTH-	Yield n mole
1	Lys	2.7	Leu	1.5
2	Glu	1.6	Phe	0.5
3	Arg	0.5		
4	Tyr	1.0		

Peptide CB2-C8

Sequence (Residues 93 through 109)

Carb.

Asp-Ser-Tyr-Leu-Tyr-Asp-Asp-Gly-Cys-Glu-Pro-Cys-Gly-Asn-Asp-Thr-Phe

CB2-C8

Composition See Table XX

TABLE XXII (Continued)

Edman Degradation

Cycle	PTH-	Yield (n mole)
1	Asp	2.8
2	Ser	0.1

CPase A One residue of Phe in 2 hr, 37°C.

Peptide CB2-C9

Sequence (residues 110 through 118)

Asn-Arg-Glu-Pro-Ser-Val-Val-Lys-Phe

←——————————————→
CB2-C9

Composition See Table XX

Edman Degradation:

Cycle	PTH-	Yield (n mole)
1	Leu	3.5
2	Asn	2.6
3	Val	2.2
4	Arg	0.4
5	Gln	+

Peptide CB2-C14

Sequence (residues 152 through 158)

Leu-Asn-Val-Arg-Gln-Lys-Try

←——————————→
CB2-C14

Composition See Table XX

TABLE XXII (Continued)

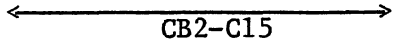
Edman Degradation:

Cycle	PTH-	Yield (n mole)
1	Leu	3.5
2	Asn	2.6
3	Val	2.2
4	Arg	0.4
5	Gln	+

Peptide CB2-C15

Sequence (residues 159 through 164)

Asp-Leu-Gln-Asp-Ile-Hsr



Composition See Table XX

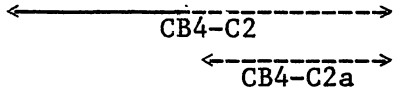
Edman Degradation of CB2-C15

Cycle	PTH-	Yield (n mole)
1	Asp	2.5
2	Leu	2.0
3	Gln	0.5
4	Asp	0.3
5	Ile	+

Peptide CB4-C2

Sequence (residues 176 through 181)

Val-Thr-Thr-Ser-His-Trp



Composition See Table XXI

TABLE XXII (Continued)

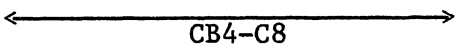
Edman Degradation of CB4-C2

Cycle	PTH-	Yield (n mole)
1	Val	3.4
2	Thr	1.5
3	Thr	0.2

Peptide CB4-C8

Sequence (residue 234 through 239)

Asp-Phe-Gln-Ala-Ala-Ala-Phe



CB4-C8

Composition See Table XXI

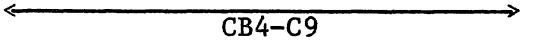
Edman Degradation of CB4-CB

Cycle	PTH-	Yield (n mole)
1	Asp	5.3
2	Phe	3.2
3	Gln	2.0
4	Ala	1.5
5	Ala	1.7
6	Ala	0.5

Peptide CB4-C9

Sequence (residues 240 through 247)

Gly-Leu-Ser-Gln-Glu-Thr-Ala-Leu



CB4-C9

Composition See Table XXI

TABLE XXII (Continued)

Edman Degradation of CB4-C9

Cycle	PTH-	Yield (n mole)
1	Gly	14.0
2	Leu	9.5
3	Ser	1.5
4	Gln	7.5
5	Glu	2.5
6	Thr	0.5
7	Ala	0.5
8	Leu	(1.5 n mole free Leu after 7 step Edman)

Peptide CB4-C11

Sequence (residues 260 through 262)

Lys-Arg-Ala
⟷
CB4-C11

Composition See Table XXI

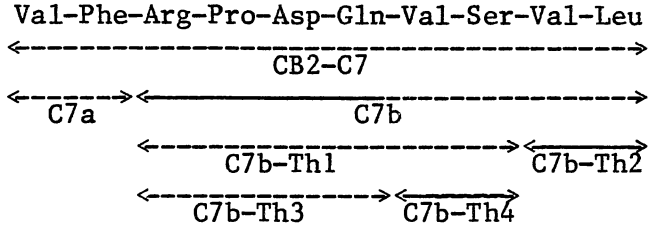
Edman Degradation

Cycle	PTH-	Yield (n mole)
1	Lys	3.5
2	Arg	0.5
3	Ala	0.7

TABLE XXIII

SEQUENCE DETERMINATION OF PEPTIDE CB2-C7, CB2-C7a, CB2-C7b AND THERMOLYTIC PEPTIDES OF C7b

Sequence (residues 83 through 92)



Composition

Amino Acid	Peptide						
	CB2-C7	C7a	C7b	C7b-Th1	C7b-Th2	C7b-Th3	C7b-Th4
Asx	1.2		1.1	1.2		1.1	
Ser	0.8		1.1	1.1			0.9
Pro	0.9		1.0	0.8		0.9	
Gly	1.0		1.0	1.1		1.0	
Val	2.7	1.0	1.0	0.9	0.9		1.0
Leu	1.1		1.1		1.0		
Phe	1.0	1.0					
Arg	1.1		1.0	1.1		1.0	
Yield %	16	72	77	47	78	41	35

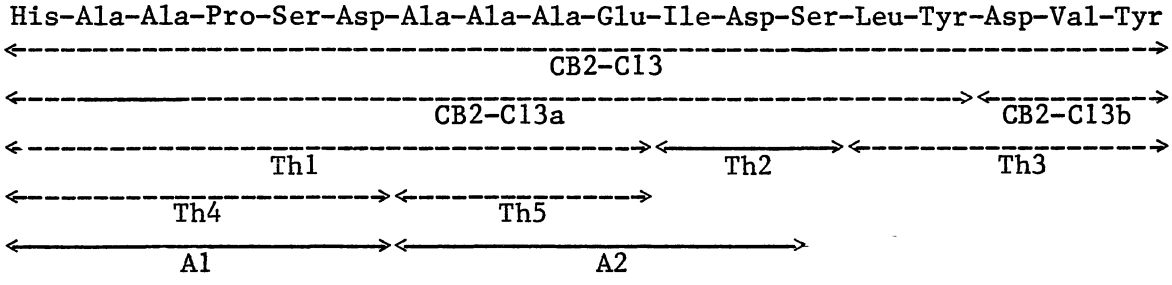
Edman Degradation

Cycle	Peptide					
	C7b		C7b-Th2		C7b-Th4	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Arg	2.4	Val	10.2	Val	5.5
2	Pro	1.4	Leu	7.5	Ser	1.2
3	Asp	1.0				
4	Gln	+				

TABLE XXIV

SEQUENCE DETERMINATION OF PEPTIDE CB2-C13, CB2-C13a, CB2-C13b, AND PEPTIDES DERIVED FROM CB2-C13

Sequence (residues 134 through 151)



Composition

Amino Acid	Peptide									
	CB2-C13	CB2-C13a	CB2-C13b	Th1	Th2	Th3	Th4	Th5	A1	A2
Asx	3.2	2.1	1.1	1.3	1.1	1.1	1.2		1.1	1.0
Ser	2.1	1.9		1.2	0.9		1.1		1.0	
Glx	1.2	1.1		1.2				1.1		0.9
Pro	0.9	1.0		0.9			0.8		0.9	
Ala	4.7	4.9		4.7			2.1	3.0	1.9	2.8
Val	1.0		0.9			1.0				
Ile	1.0	1.1			1.0					
Leu	1.1	1.2				1.0				1.3
Tyr	1.8	1.0	1.0			1.8				
His	1.0	0.9		0.9			0.9		1.1	
Yield %	48	16	25	35	58	44	21	25	33	40

TABLE XXIV (Continued)

Edman Degradation:

Cycle	Peptide									
	CB2-C13		Th2		Th3		A1		A2	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	His	-	Ile	4.7	Leu	4.5	His	-	Ala	4.5
2	Ala	3.5	Asp	1.4	Tyr	6.5	Ala	5.2	Ala	3.0
3	Ala	1.5	Ser	0.4	Asp	3.5	Ala	2.5	Ala	3.5
4					Val	1.5	Pro	0.3	Glu	0.8
5					Tyr	0.7	Ser	+	Ile	0.2
6							Asp	+		

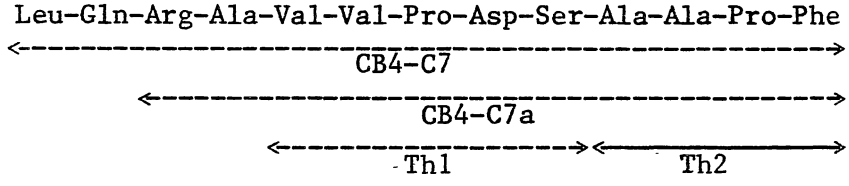
Substrative Edman Degradation of A1

	His	Ala	Pro	Ser	Asx
Step 1	0.3	2.2	1.0	0.9	1.0
Step 2	0.0	1.1	1.0	1.0	1.1

TABLE XXV

SEQUENCE DETERMINATION OF CB4-C7, CB4-C7a AND THERMOLYTIC PEPTIDES FROM CB4-C7a

Sequence (residues 221 through 233)



Composition

Amino Acid	Peptide			
	CB4-C7	CB4-C7a	Th1	Th2
Asx	1.1	1.1	1.0	
Ser		1.0	0.9	
Glx	1.0			
Pro	1.9	2.0	1.0	1.0
Ala	2.8	2.9		2.1
Val	1.7	1.6	1.8	
Leu	1.1			
Phe	0.9	0.9		1.0
Arg	1.0	1.1		
Yield %	41	27	73	85

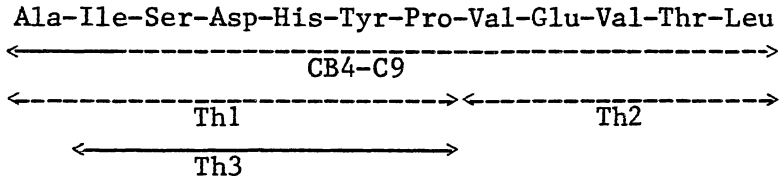
Edman Degradation of Th2

Cycle	PTH-	Yield (n mole)
1	Ala	3.5
2	Ala	2.2
3	Pro	0.7
4	Phe	0.2

TABLE XXVI

SEQUENCE DETERMINATION OF PEPTIDE CB4-C9 AND ITS THERMOLYTIC PEPTIDES

Sequence (residues 248 through 259)



Composition:

Amino Acid	Peptide			
	CB4-C9	Th1	Th2	Th3
Asx	1.1	1.1		1.0
Thr	0.9		0.9	
Ser	0.9	0.8		1.0
Glx	1.1		1.0	
Pro	1.0	0.9		1.1
Ala	1.0	1.1		
Val	1.9		2.0	
Ile	1.0	1.0		1.0
Leu	1.1		1.1	
Tyr	1.0	0.9		1.0
His	0.9	1.0		0.9
Yield %	77	32	66	41

TABLE XXVI (Continued)

Edman Degradation

Cycle	Peptide					
	CB4-C9		Th2		Th3	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Ala	1.3	Val	6.5	Ile	7.4
2	Ile	0.3	Glu	4.2	Ser	0.3
3			Val	1.6	Asp	4.2
			Thr	0.5	His	+
			Leu	+	Tyr	0.2
					Pro	+

TABLE XXVII

THE AMINO ACID COMPOSITIONS OF CNBr PEPTIDES OF OVINE DNase

Amino Acid	Peptide				
	CB1	CB2	CB3	CB4	CB5
Asp	1.0(1)	22.4(23)		9.2(9)	1.2(1)
Thr	2.0(2)	2.5(3)		6.2(7)	1.7(2)
Ser		16.4(17)		13.3(14)	1.0(1)
Glx	1.0(1)	11.4(11)		4.7(5)	1.0(1)
Pro		7.5(7)		2.7(3)	1.1(1)
Gly	1.0(1)	4.4(4)		4.2(4)	
Ala	1.9(2)	8.8(9)		7.8(8)	1.9(2)
½Cys		0.1(2)		1.4(2)	
Val		14.5(16)		5.7(6)	1.9(2)
Ile	1.8(2)	5.7(7)		2.8(3)	1.1(1)
Leu	1.0(1)	14.3(14)	1.0(1)	5.1(5)	2.1(2)
Tyr		9.4(10)		2.9(3)	0.9(1)
Phe	1.9(2)	4.7(5)		3.7(4)	
His		2.7(3)			1.0(1)
Lys	1.9(2)	7.3(7)			
Trp		0.0(1)		0.0(2)	
Arg	0.9(1)	8.4(8)		3.2(3)	
Hsr	1.0(1)	0.8(1)	0.9(1)	1.0(1)	

TABLE XXVIII

THE AMINO ACID COMPOSITION OF TRYPTIC AND CHYMOTRYPTIC PEPTIDES OF OVINE CB1

Amino Acid	Peptide				
	CB1-C1	CB1-C2	CB1-T1	CB1-T1a	CB1-T2
Asx		1.3	1.3		
Thr		1.1	0.9		2.3
Glx					0.9
Gly					0.9
Ala	1.9		1.9	2.1	
Ile	1.0	1.0	2.0	1.0	
Leu	1.0				
Phe	0.9	0.8	0.8	0.9	0.8
Lys	1.1				0.9
Arg		0.9	1.1		

TABLE XXIX

THE AMINO ACID COMPOSITIONS OF CHYMOTRYPTIC PEPTIDES OF OVINE CB2

Amino Acid	Peptide									
	CB2-C1[a]	CB2-C2	CB2-C3	CB2-C4	CB2-C4a	CB2-C5	CB2-C5a	CB2-C6	CB2-C7	CB2-C8
Asx	1.0			2.3	1.0	5.9	5.9			1.0
Thr	1.0									
Ser	0.9	1.8		0.8		0.9	0.9		1.0	0.9
Glx				1.7		1.0	1.1		1.1	
Pro						1.1	0.7		1.0	
Gly						1.1				1.0
Ala	0.9			0.8	0.7	1.0				
Val				1.0		2.1			1.7	
Ile				1.9	1.2					
Leu	1.1			2.0	1.1	3.0	2.4		1.1	
Tyr		1.0	1.0			0.9	0.9	1.0		1.0
His				0.8				1.0		
Lys						1.0				
Arg			2.0	1.0						1.1
½Cys										
Phe										

TABLE XXIX (Continued)

Amino Acid	Peptide									
	CB2-C9	CB2-C10	CB2-C11	CB2-C12	CB2-C13	CB2-C14	CB2-C15	CB2-C16	CB2-C16a	CB2-C16b
Asx				1.0		1.0	4.1			
Thr						1.1				
Ser					0.9		1.8	1.0	1.0	
Glu	1.0						2.0	1.2	1.1	
Pro				0.9				1.0	1.0	
Gly							1.9			
Ala								1.1	0.9	
Val			1.0		2.0			2.0	1.8	
Ile										
Leu		1.0			1.1					
Tyr	0.9					1.0	1.0			
His										
Lys	1.0				0.9			1.3		1.0
Arg	1.1			1.1				1.3		
½Cys							0.8			
Phe		0.9	0.9				1.0	1.0		0.9

TABLE XXIX (Continued)

Amino Acid	Peptide								
	CB2-C17	CB2-C17a	CB2-C17b	CB2-C18	CB2-C18a	CB2-C18b	CB2-C19	CB2-C20	CB2-C21
Asx				2.2	2.1		1.0	1.0	2.9
Thr	0.9	0.8	0.9						
Ser	3.2	2.8	2.7	2.7	1.8	0.9			
Glu				1.1	1.1			1.9	
Pro	1.0	1.0	0.9	1.0	1.0				
Gly									
Ala				2.8	2.9				
Val	0.9	1.1		1.2	1.0		0.9	1.1	
Ile				1.1	1.1				0.9
Leu				1.0		1.1		1.0	1.1
Tyr				0.9		1.1	1.0		
His				1.0	1.0				
Lys	2.1	1.3	1.4					1.0	
Arg									
½Cys									
Phe									
Trp								0.1	
Hsr									1.0

TABLE XXX

THE AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF OVINE CB2

Amino Acid	Peptide							
	CB2-T1[a]	CB2-T2	CB2-T3	CB2-T4	CB2-T5	CB2-T6	CB2-T7	CB2-T8
Asp	1.2		1.2	1.1	6.2	1.0	1.1	5.3
Thr	0.9							0.8
Ser	2.8			0.9	2.0	0.8		3.7
Glx			1.9		2.1			2.2
Pro					1.9		0.9	
Gly				1.1	1.2			2.2
Ala	1.1		0.9	1.0				
½Cys								1.1
Val	0.9		1.0	2.1	1.8		0.9	2.2
Ile	1.0	1.1	2.1					
Leu	1.2	1.0	1.2	1.0	3.9			1.3
Tyr	1.1		0.9		2.0	1.0		2.3
Phe							0.8	1.0
His				1.0	1.0			
Lys				1.1		1.0	1.0	
Arg	1.1	1.0	1.9		1.0		1.0	1.1

TABLE XXX (Continued)

Amino Acid	Peptide						
	CB2-T9	CB2-T10	CB2-T11	CB2-T11a	CB2-T11b	CB2-T12	CB2-T12'
Asp			4.1		4.1	2.8	3.0
Thr		0.9					
Ser		2.6	2.6		2.7		
Glx	1.0		2.9		3.0		
Pro	1.1	1.0	1.7	1.0	0.8		
Ala	1.0		4.8	2.0	3.1		
Val	1.7		3.7	0.9	3.0		0.8
Ile			2.2	1.1	1.4	1.2	
Leu			3.1	1.4	2.3	1.3	0.9
Tyr			2.0		2.0		
Phe		1.0	0.9	0.9			
His			1.0		1.0		
Lys	1.1	1.3	1.1		1.1		
Trp						0.1	0.1
Hsr						0.9	1.0

[a]Glucosamine is also present in this peptide.

TABLE XXXI

THE AMINO ACID COMPOSITIONS OF THERMOLYTIC PEPTIDES OF PEPTIDES CB2-T5, CB2-T11a AND CB2-T12

Amino Acid	Peptide										
	CB2-T5 -Th1	CB2-T5 -Th1a	CB2-T5 -Th1b	CB2-T5 -Th1c	CB2-T5 -Th1d	CB2-T5 -Th2	CB2-T5 -Th3	CB2-T11a -Th1	CB2-T11a -Th2	CB2-T12 -Th1	CB2-T12 -Th2
Asp	6.1	6.1		2.1	4.1					1.0	2.2
Ser	0.8	0.9			0.9	0.8					
Glu	1.0	1.0			1.0	1.0					
Pro	1.0	0.9			1.0	1.0			1.1		
Gly							1.1				
Val						1.7			0.9		
Ala								2.0			
Leu	3.1	2.8		1.0	1.0		0.9		1.1		1.0
Tyr	1.8		1.7								
His	0.9		1.1								
Arg							1.0				
Ile									0.8		
Phe								1.0		1.0	
Trp										0.3	

TABLE XXXII

THE AMINO ACID COMPOSITIONS OF CHYMOTRYPTIC PEPTIDES OF OVINE CB4

Amino Acid	Peptide							
	CB4-C1	CB4-C2	CB4-C3	CB4-C4	CB4-C5	CB4-C6	(o)CB4 -C1	(o)CB4 -C1
Asp					1.1	1.0	2.7	1.8
Thr	1.0		1.7					
Ser	2.3	2.0	2.2	3.0		0.8	1.3	0.8
Glx	1.3			1.0	1.1	1.2		0.8
Pro				2.1				
Gly				1.0		0.9	1.0	1.0
Ala				1.1	1.9		1.0	2.0
$CySO_3^-$							0.8	0.9
Val	1.2			2.8				1.6
Ile		0.9						1.0
Leu		1.0				1.0		2.4
Tyr					1.0		0.9	0.9
Phe			1.0	1.0	1.0		0.8	
Trp	0.8							
Arg		1.0	1.1					
Hsr						1.0		

TABLE XXXIII

THE AMINO ACID COMPOSITIONS OF THERMOLYTIC PEPTIDES OF OVINE CB4

Amino Acid	Peptide								
	CB4 -Th1	CB4 -Th1a	CB4 -Th1b	CB4 -Th2	CB4 -Th3	CB4 -Th4	CB4 -Th4a	CB4 -Th5	CB4 -Th5a
Asp	5.3	3.3	1.7		1.0	1.4			
Thr	3.5	1.6	2.1						
Ser	3.2	2.3	0.9						
Glx				1.1				1.1	1.0
Pro	1.0		0.9			1.0	1.1		
Ala	2.8	1.8	1.2		1.1	1.0	0.9	1.0	
½Cys	0.3	0.2							
Val						0.9	1.0		
Ile	1.0		0.9						
Leu	1.2		0.8						
Tyr	0.7	1.0			0.9				
Phe	1.1	1.0		1.0		1.1		0.9	0.9
Trp				0.4					
Arg					1.0				

TABLE XXXIV

THE AMINO ACID COMPOSITIONS OF THERMOLYTIC PEPTIDES OF OVINE CB4-C4

Amino Acid	Peptide		
	CB4-C4-Th1	CB4-C4-Th2	CB4-C4-Th3
Ser	1.9	0.9	
Glx	1.1		
Pro		1.1	1.1
Gly		1.2	
Ala			1.2
Val		1.7	1.1
Phe			1.1

TABLE XXXV

THE AMINO ACID COMPOSITIONS OF THERMOLYTIC PEPTIDES OF OVINE CB5

Amino Acid	Peptide			
	CB5-Th1	CB5-Th2	CB5-Th3	CB5-Th4
Asp		1.0		
Thr			1.1	0.9
Ser		0.8		
Glx			1.0	
Pro		1.0		
Ala	2.1			
Val			1.9	
Ile		1.2		
Leu	1.1			1.0
Tyr		1.0		
His		1.0		

TABLE XXXVI

AUTOMATED EDMAN DEGRADATION OF OVINE DNase, OVINE CB2 AND CB4

Cycle	Peptide					
	Ovine DNase		Ovine CB2[a]		Ovine CB4	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Leu	18.6	Ser	8.5	Gly	27.5
2	Lys	18.2	-	-	Asp	28.3
3	Ile	10.7	Ala	32.5	Phe	25.2
4	Ala	13.3	Thr	3.5	Asn	20.0
5	Ala	14.1	Leu	30.1	Ala	21.2
6	Phe	10.2	Ser	+	Asp	18.3
7	Asn	4.0	Ser	4.2	Rcm	18.0
8	Ile	5.3	Tyr	28.3	Ser	5.2
9	Arg	1.1	Ile	25.3	Tyr	16.1
10	Thr	+	Val	23.7	Val	15.2
11	Phe	0.7	Arg	12.2	Thr	10.1
12	Gly	0.3	Ile	19.5	Ser	4.0
13	Glu	0.2	Leu	18.6	Ser	2.5
14	Thr	+	Arg	7.3	Gln	7.5
15	Lys	0.2	Arg	10.5	Trp	5.0
16	Met	0.1	Tyr	14.4	Ser	2.2
17	Ser	+	Asp	13.7	Ser	0.8
18			Ile	12.7	Ile	11.2
19			Ala	11.6	Arg	6.2
20			Leu	10.5	Leu	10.3
21			Ile	9.7	Arg	4.1
22			Glu	9.3	Thr	1.2
23			Gln	7.3	Ser	0.7
24			Val	8.3	Ser	0.5
25			Arg	2.1	Thr	0.8
26			Asp	7.3	Phe	2.1
27			Ser	+	Gln	1.7
28			His	+	Trp	0.2
29			Leu	4.2	Leu	0.3
30			Val	3.9	Ile	0.2
31			Ala	3.1	Pro	+

TABLE XXXVI (Continued)

Cycle	Peptide					
	Ovine DNase		Ovine CB2[a]		Ovine CB4	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
32			Val	2.5		
33			Gly	1.7		
34			Lys	1.6		
35			Leu	1.2		
36			Leu	0.8		
37			Asp	0.6		
38			Asp	0.3		
39			Leu	0.3		
40			Asn	0.1		
41			Gln	0.1		
42			Asp	0.05		

[a]Second residue Asn could not be identified due to carbohydrate side chain.

TABLE XXXVII

EDMAN DEGRADATION OF THERMOLYTIC PEPTIDES OF OVINE CB5, AND TRYPTIC PEPTIDES, CB2-T5, AND CB2-T12, OF OVINE CB2

Cycle	Peptide											
	CB5-Th1		CB5-Th2		CB5-Th3		CB5-Th4		CB5-T5		CB2-T12	
	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole	PTH-	Yield n mole
1	Ala	5.4	Ile	6.7	Val	4.8	Leu	2.5	Leu	5.8	Trp	0.8
2	Leu	2.3	Ser	0.3	Glu	3.2	Thr	0.4	Leu	4.3	Asp	1.1
3			Asp	3.2	Val	1.1			Asp	2.1	Leu	0.4
4			His	+	Thr	0.2			Asp	0.7		
5			Tyr	0.5					Leu	0.2		
6			Pro	0.2					Asn	0.1		

TABLE XXXVIII

THE AMINO ACID COMPOSITIONS OF TWO FORMS OF OVINE DNase

Amino Acid	Form	
	Pool I	Pool II
Asx	33.5	32.6
Thr	13.5	13.9
Ser	30.6	31.2
Glx	18.6	17.7
Pro	11.3	10.6
Gly	11.7	10.9
Ala	21.3	20.7
½Cys[a]	4.0	4.0
Val	23.1	22.7
Met	3.1	3.5
Ile	11.7	12.2
Leu	23.5	24.1
Tyr	22.1	21.9
Phe	10.7	11.4
His	3.9	4.3
Trp[a]	3.0	3.0
Arg	12.9	13.3

[a]Trp and ½Cys were not determined and have been assumed to be the same in both forms. Their values were obtained from the sequence derived (Figure 29).

TABLE XXXIX

AMINO ACID COMPOSITIONS OF PEPTIDE-1 AND PEPTIDE-2 OF BOVINE DNase

Amino Acid	Peptide[a]			
	Peptide-1	Theoretical[b]	Peptide-2	Theoretical
Asx	7.9 (8)	8	2.8 (3)	3
Thr	2.7 (3)	3	2.7 (3)	3
Ser	3.1 (3)	3	1.2 (1)	1
Glx	4.2 (4)	4	1.3 (1)	1
Gly	2.1 (2)	2	1.2 (1)	1
Ala	5.0 (5)	5	4.1 (4)	4
Val[c]	5.9 (6)	5	1.9 (2)	1
Met	0.8 (1)	1	0.5 (1)	1
Ile[c]	5.8 (6)	5	3.9 (4)	3
Leu	7.0 (7)	7	2.0 (2)	2
Tyr	3.2 (3)	3	2.0 (2)	2
Phe	2.1 (2)	2	1.9 (2)	2
His	1.1 (1)	1		
Lys	3.0 (3)	3	2.0 (2)	2
Arg	5.0 (5)	4	4.1 (4)	3
Yield %	47		40	

[a]Refer to Figure 32 for isolation and designation of peptides. The underlined numbers show the differences between the observed and theoretical values.

[b]Values from Liao et al. (7).

[c]Values for Ile and Val were obtained from the 72 hr hydrolysate.

TABLE XL

AUTOMATED EDMAN DEGRADATION OF BOVINE CB2

Cycle	PTH-	Yield (n mole)
1	Ser	+
2[a]	-	-
3	Ala	20.2
4	Thr	5.3
5	Leu	16.9
6	Ala	15.2
7	Ser	0.3
8	Tyr	12.0
9	Ile	11.1
10	Val	10.0
11	Arg	2.1
12	Ile	9.0
13	Val	7.2
14	Arg	3.8
15	Arg	6.5
16	Tyr	5.3
17	Asp	5.0
18	Ile	3.7
19	Val	3.0
20	Leu	2.1

[a]This residue Asn-18 (7) could not be identified due to carbohydrate side chain.

TABLE XLI

SUMMARY OF THE SUBSTITUTIONS IN THE PRIMARY STRUCTURES OF BOVINE, OVINE AND PORCINE DNases AND THEIR CORRESPONDING GENETIC CODES

Residue No.	In Bovine DNase	In Ovine DNase[a]	In Porcine DNase
2	Lys (AAA,G)		Arg (AGA,G)
22	Ala (GCX)	Ser (UCX)	Ser (UCX)
23	Ser (AGU)		Asn (AAU)
29	Val (GUX)	Leu (CUX)	Leu (CUX)
30	Arg (CGX)		Ser (AGU)
35	Val (GUX)	Ala (GCX)	Ala (GCX)
46	Val (GUX)		Thr (ACX)
53	Asp (GAU)		Asn (AAU)
54	Tyr (UAU)	Asp (GAU)	Gln (CAA)
62	Thr (ACX)	Ser (UCX)	Asn (AAU)
65	Tyr (UAU)		His (CAU)
74	Asn (AAU)		Ser (AGU)
75	Ser (AGU)		Thr (ACU)
83	Leu (CUX)	Val (GUX)	Val (GUX)
88	Lys (AAA)		Gln (CAA)
94	Thr (ACX)		Ser (AGU)
96	Gln (CAA)		Leu (CUX)
103	Ser (UCX)		Pro (CCX)
108	Ser (AGU)		Asn (AAU)
114	Ala (GCX)		Ser (UCX)
121	His (CAU)	Pro (CCX)	Pro (CCX)
122	Ser (UCX)		Phe (UUX)
124	Lys (AAA)		Gln (CAA)
127	Glu (GAA)	Ala (GCX)	
132	Ala (GCX)	Pro (CCX)	Pro (CCX)
135	Ser (UCX)		Ala (GCX)
141	Val (GUX)		Ala (GCX)
155	Gln (CAA)		Arg (CGA)
159	His (CAU)	Asp (GAU)	Asp (GAU)
161	Asn (AAU)		Gln (CAU)

TABLE XLI (Continued)

Residue No.	In Bovine DNase	In Ovine DNase[a]	In Porcine DNase
163	Val (GUX)	Ile (AUU)	Ile (AUU)
172	Asp (GAU)		Gly (GGU)
178	Ser (AGU)		Thr (ACU)
180	Gln (CAA)		His (CAU)
188	Thr (ACX)		Glu (GAA)
190	Ser (UCX)		Pro (CCX)
191	Thr (ACX)		Pro (CCX)
198	Ser (UCX)		Thr (ACX)
203	Ala (GCX)		Val (GUX)
207	Thr (ACX)		His (CAU)
208	Asn (AAU)		Thr (ACX)
219	Ser (UCX)		Pro (CCX)
223	Ser (UCX)		Arg (CGX)
224	Ser (UCX)		Ala (GCX)
227	Gly (GGX)		Pro (CCX)
228	Pro (CCX)		Asp (GAU)
231	Ala (GCX)	Val (GUX)	Ala (GCX)
239	Tyr (UAU)		Phe (UUU)
243	Asn (AAU)		Gln (CAA)
245	Met (AUG)		Thr (ACX)
260	Thr (ACX)		Lys (AAA)

[a]Residues other than indicated are same as in bovine DNase.

Figure 1. CM-cellulose Chromatography of Porcine DNase. DNase activity which was fractionated between 30-60% ammonium sulfate saturation, was dialyzed against 5 mM calcium acetate, pH 4.7 and loaded on a 2x30 cm CM-cellulose column equilibrated in the same buffer. DNase was eluted by the gradient from 7 chambers: 150 ml each of 10, 12, 15, 20, 22, 25, and 30 mM of calcium acetate, pH 4.7. Bar indicates the pooled fraction.

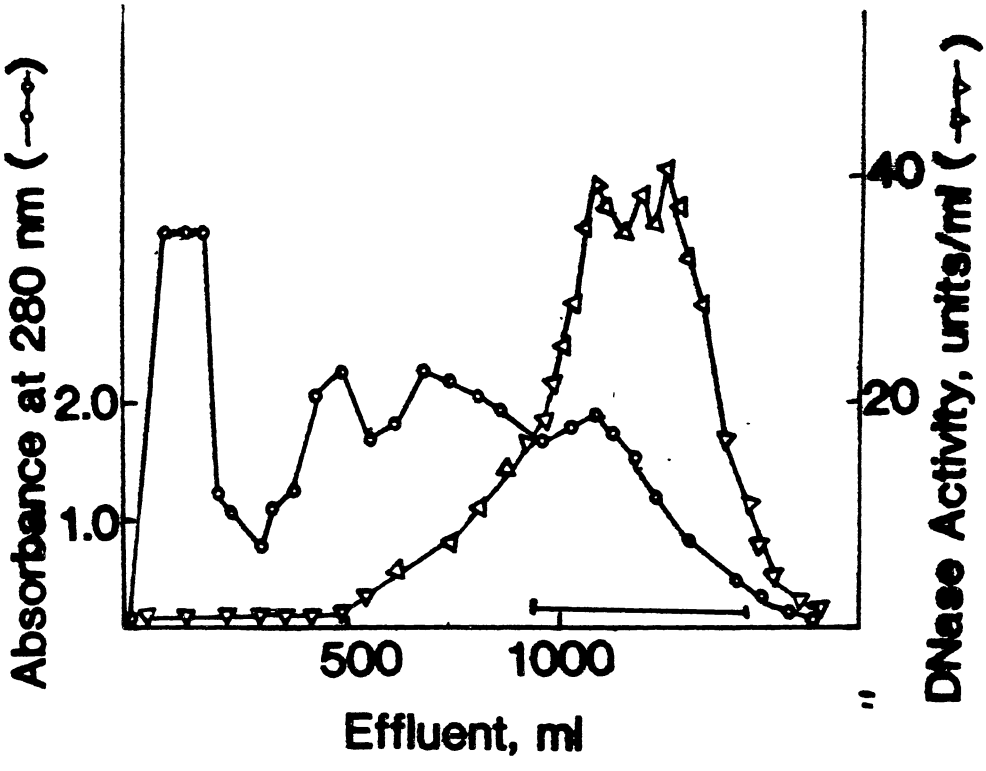

Absorbance at 280 nm (—o—)
2.0
1.0
500
1000
Effluent, ml
40
20
DNase Activity, units/ml (—▽—)

Figure 2. Chromatography of Porcine DNase on Blue Sepharose Column. About 600 ml pooled fraction, having DNase activity, was loaded directly on a Blue Sepharose column (2x10 cm) equilibrated in 5 mM calcium acetate, pH 4.7. Column was washed, extensively to remove nonspecifically bound materials, with 50 mM of the equilibrating buffer until the A_{280} of the effluent approached zero. DNase was eluted with 50 mM Tris-HCl, 5 mM $CaCl_2$ pH 7.0, at the point indicated by arrow. Fractions indicated by bar with DNase activity were pooled.

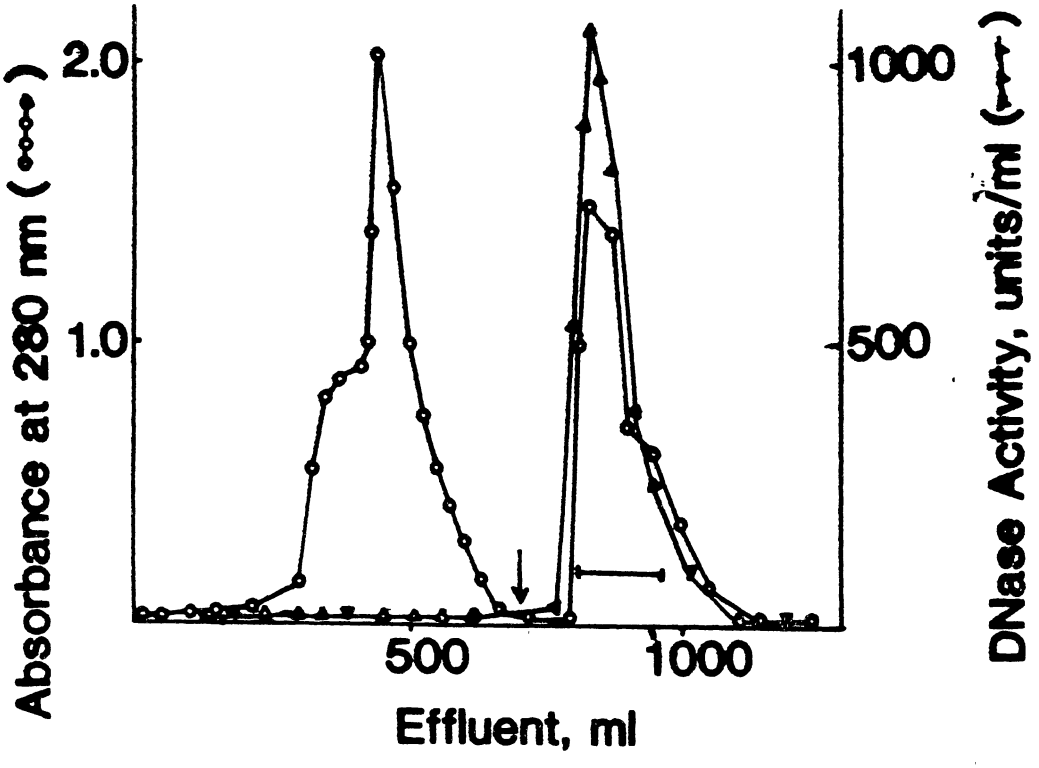

Absorbance at 280 nm (•—•)
2.0
1.0
500
1000
Effluent, ml
1000
500
DNase Activity, units/ml (▲—▲)

Figure 3. ConA Agarose Chromatography of Porcine DNase. Active fraction from Figure 2 was directly loaded on a ConA Agarose column (0.9x10 cm) equilibrated in 50 mM Tris-HCl, 5 mM $CaCl_2$, 5 mM $MnCl_2$, pH 7.0. Column was washed with several column volumes of the equilibrating buffer and DNase was eluted with 10% α methylmannoside in 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8 as shown by arrow. Active fractions were pooled as indicated by bar.

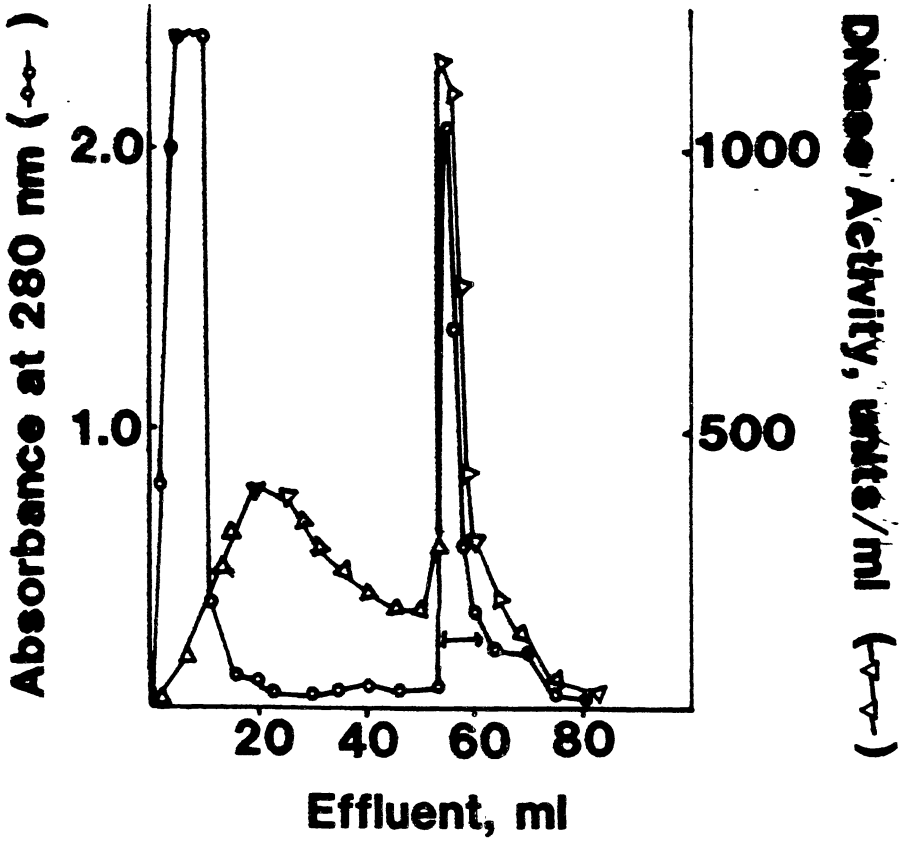

Absorbance at 280 nm (-o-)
2.0
1.0
1000
500
DNase Activity, units/ml (-▽-)
20
40
60
80
Effluent, ml

Figure 4. DEAE-Biogel Chromatography of Porcine DNase. DNase activity from Figure 3 was dialyzed against 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 8, and loaded onto a DEAE-Biogel column (0.8x10 cm) equilibrated in 20 mM Tris-HCl, pH 8.0. Column was washed with 2-3 column volumes of equilibrating buffer and DNase was eluted with 100 mM Tris-HCl, pH 8.0. Active fractions, as shown by bars, were pooled, dialyzed against water and lyophilized.

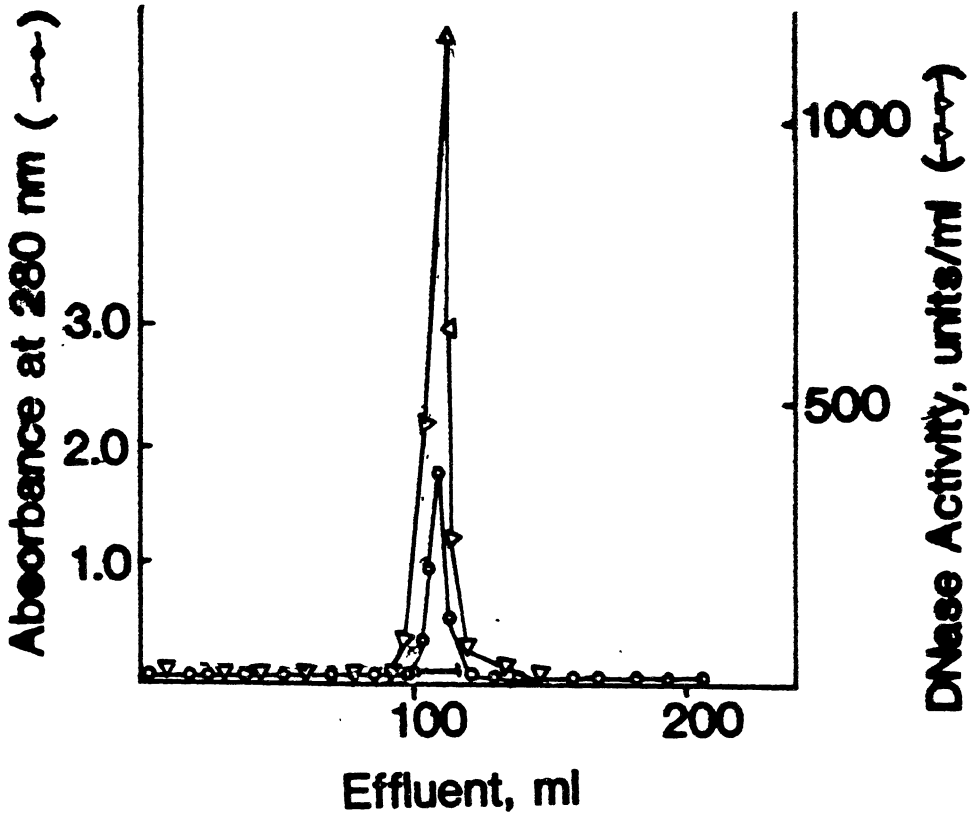

Absorbance at 280 nm (—o—)
3.0
2.0
1.0
100
200
Effluent, ml
1000
500
DNase Activity, units/ml (—▽—)

Figure 5. (A) Isoelectric Focusing and (B) SDS Gel Electrophoresis of Porcine DNase. Four μgm of porcine DNase was focused at 4°C for 4 hr (ampholine 4-6). One gel was stained with Coomassie Blue (a) and another was subjected to DNase activity staining (b). Ten μgm of pancreatic DNases (a) porcine, (b) porcine and bovine, and (c) bovine were electrophoresed in 10% gel. See text for detail.

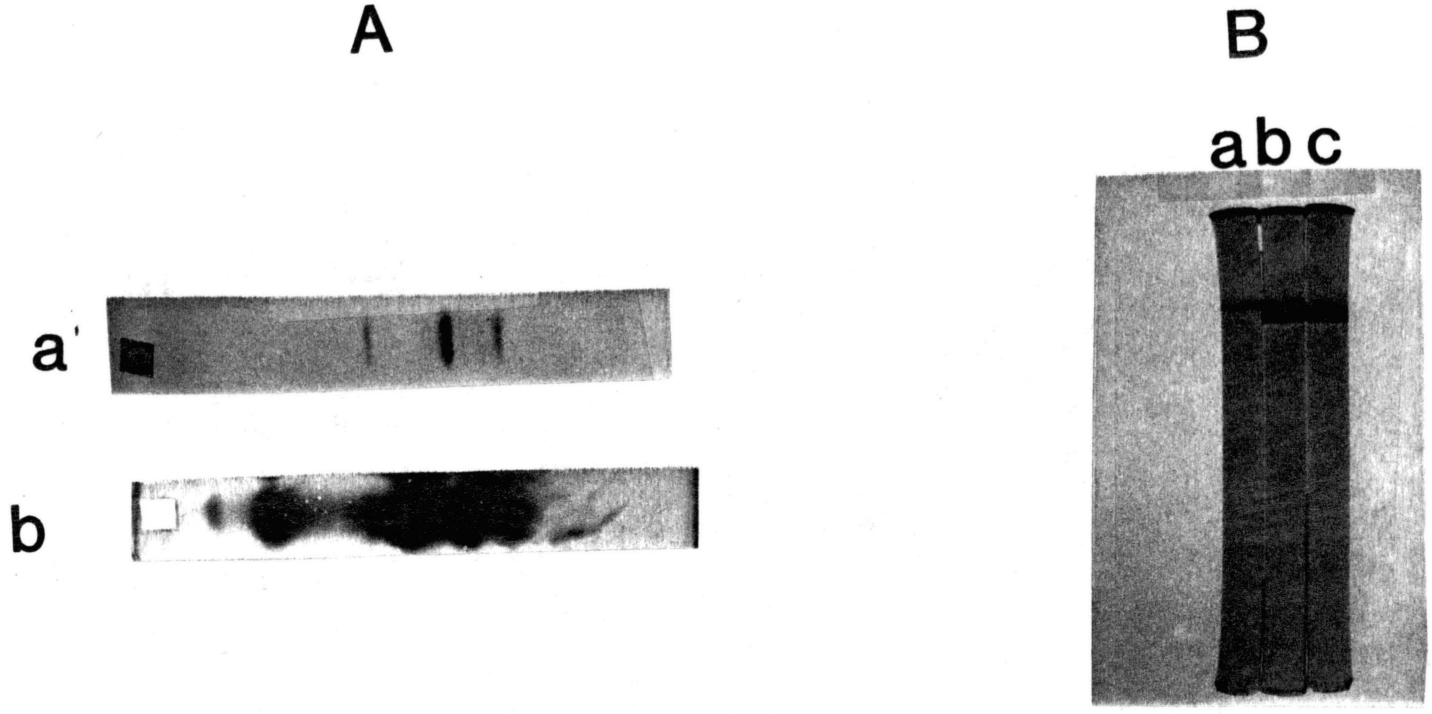

A
a
b
B
a b c

Figure 6. Effect of Divalent Metal Ions and pH on the Activity of Porcine DNase. DNase activity was assayed in 0.1 M Tris acetate. The concentration of metal ions was 10 mM. DNase activity in 10 mM Mn^{++} at pH 7.0 has been taken as 100%. Open circles in Mg^{++} plus Ca^{++}, filled in circles in Mn^{++}, triangles in Mg^{++} and rectangles in Ca^{++}.

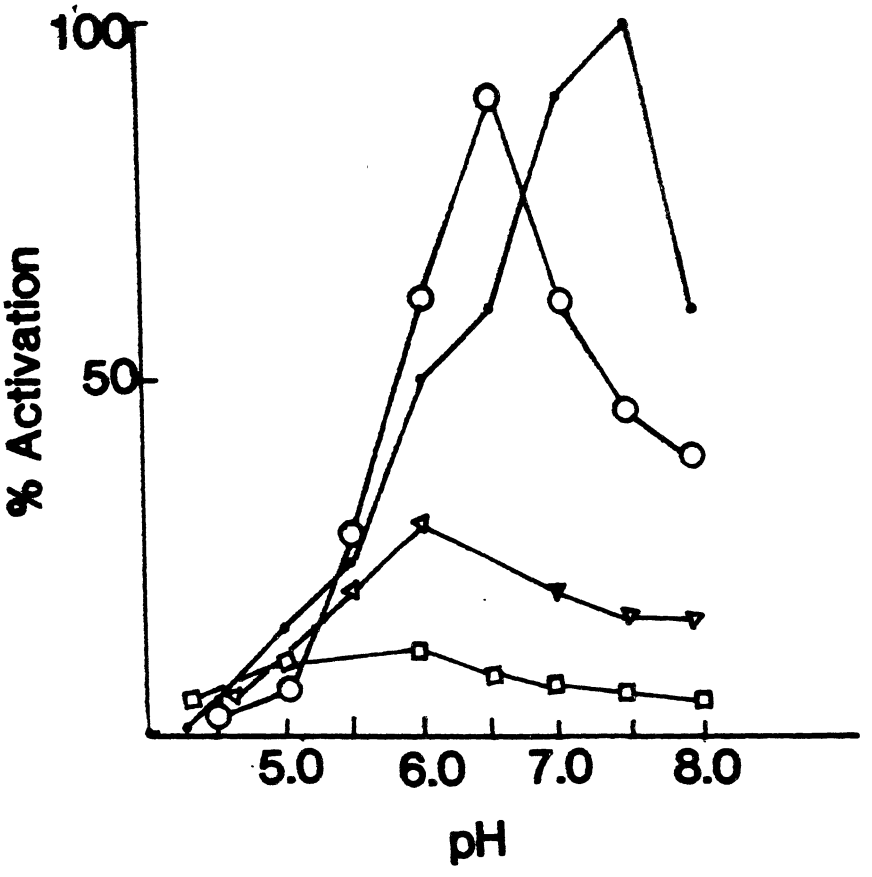

100
50
% Activation
5.0
6.0
7.0
8.0
pH

Figure 7. Gel Filtration of CNBr Fragments of Porcine DNase. About 10 mg of DNase was treated with CNBr, and incubated at room temperature for 24 hr. Mixture was loaded on a 1x150 cm Sephadex G-100 column. Elution was performed with 50% acetic acid at room temperature. Each fraction was 1 ml. Pooled fractions are indicated by bars. Peptides CB2, CB4, CB1 and CB3 were recovered from pool I, pool II, pool III and pool IV, respectively

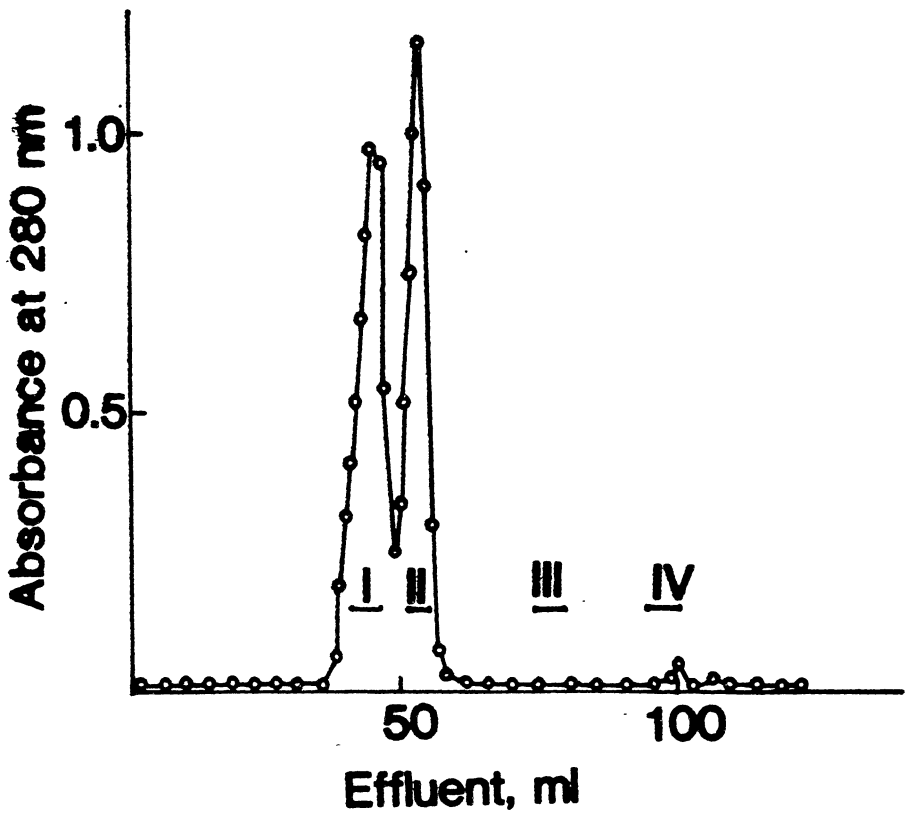

Absorbance at 280 nm
1.0
0.5
I
II
III
IV
50
100
Effluent, ml

Figure 8. Gel Filtration of Tryptic Digest of Porcine CB2. One fourth of Peak I (CB2) from Figure 7 was digested with trypsin and the digest was chromatographed on a 0.7x50 cm Sephadex G-25 (superfine) column. Column was eluted with 0.1% TFA. One ml fractions were collected.

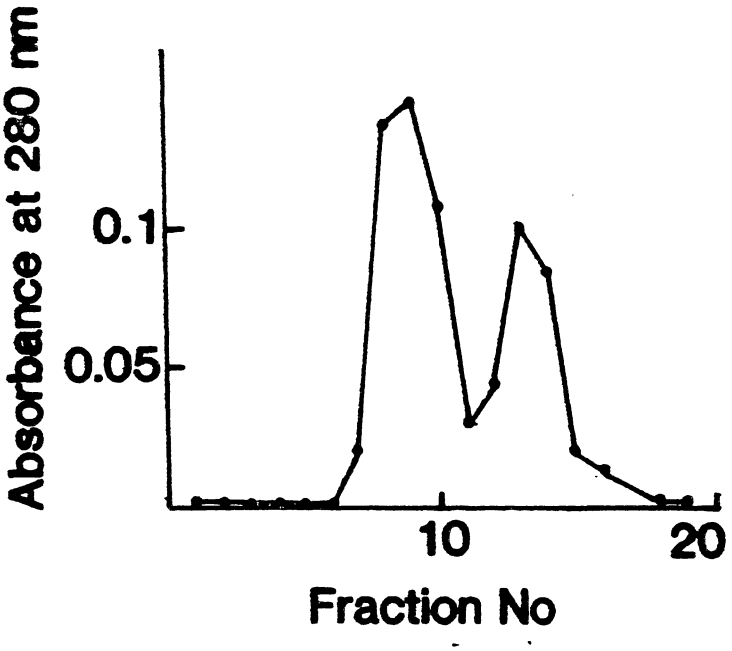

Absorbance at 280 nm
0.1
0.05
10
20
Fraction No

Figure 9. Separation of Peptides T1, T8, T8a, T4, T5 and T11 of Porcine CB2. (A) Fraction 8, (B) Fraction 10, from Figure 8 were injected onto an Altex Ultrasphere, ODS, 5 micron (0.46x25 cm) HPLC column, and eluted with a gradient 40 ml each of 0-90% methanol in 0.1% TFA at room temperature. Flow rate was 1 ml/min. Peaks as indicated were collected and lyophilized.

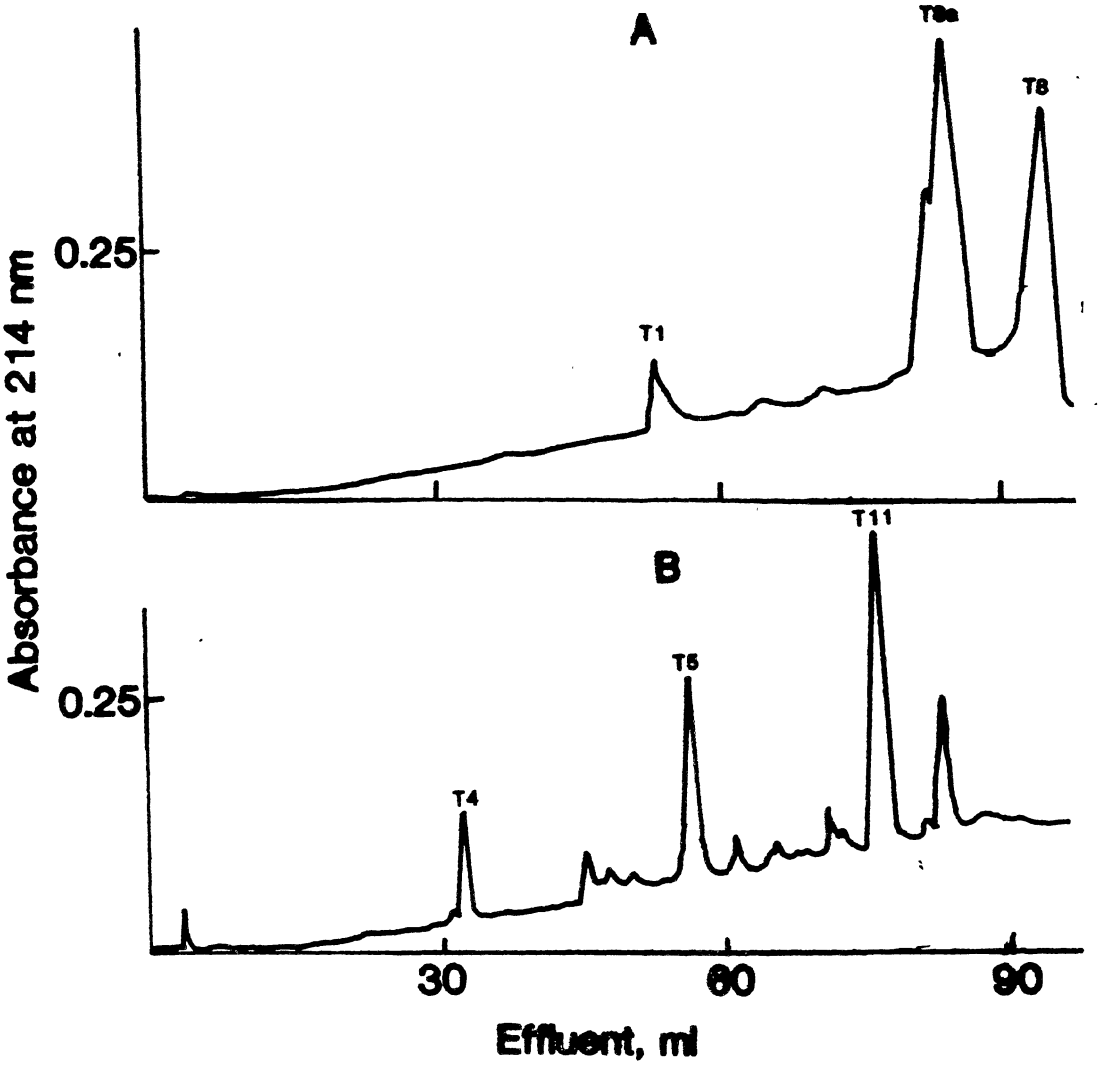

A
T8a
T8
T1
0.25
B
T11
T5
T4
0.25
Absorbance at 214 nm
30
60
90
Effluent, ml

Figure 10. Separation of Peptides T2, T3, T6, T7, T8b, T9, T10, and T12 of Porcine CB2. (A) Fraction 15 and (B) Fraction 13 from Figure 8 were chromatographed on HPLC. Conditions same as in Figure 9.

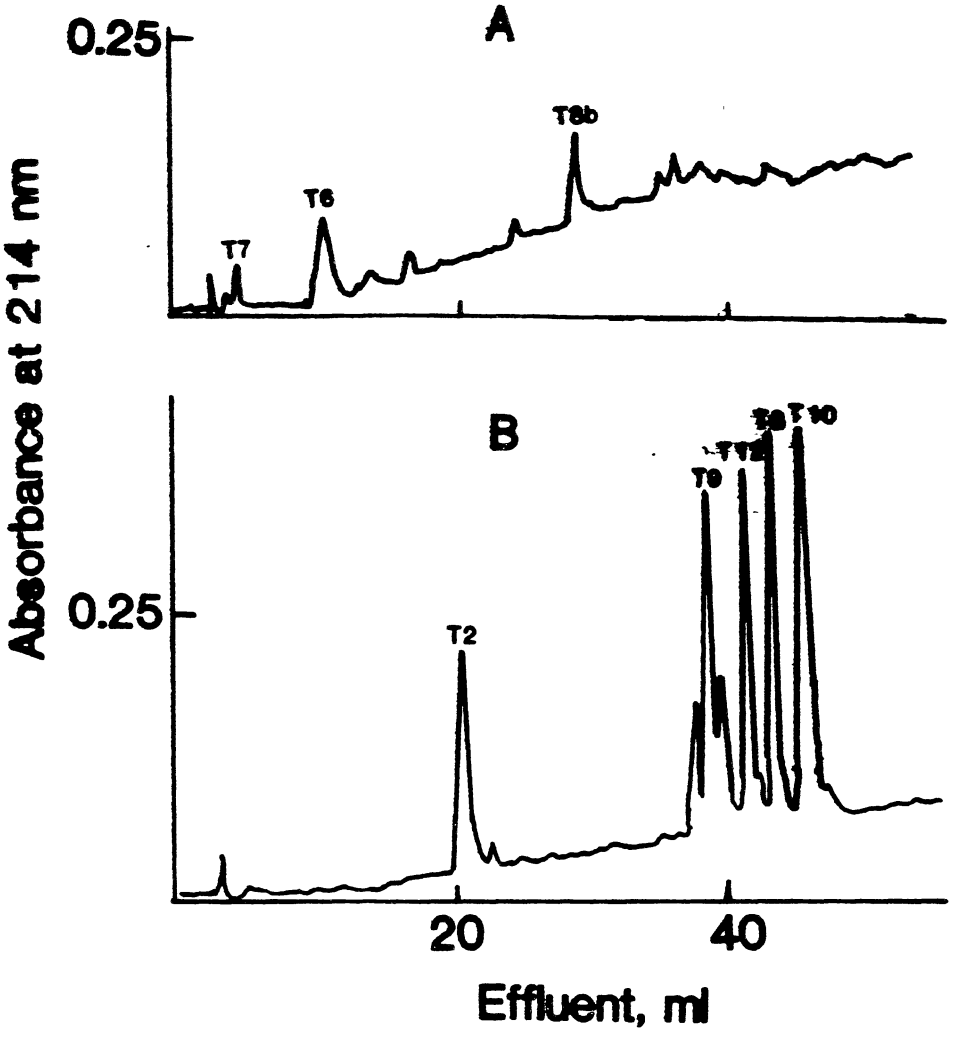

A
0.25
T8b
T6
T7
B
T10
T9
T2
0.25
20
40
Effluent, ml
Absorbance at 214 nm

Figure 11. Separation of Peptides T1-T5 of Porcine CB4. One fourth of Pool II (CB4) from Figure 7 was digested with trypsin for 15 hr. Resulting peptides were isolated by HPLC. Conditions same as in Figure 9.

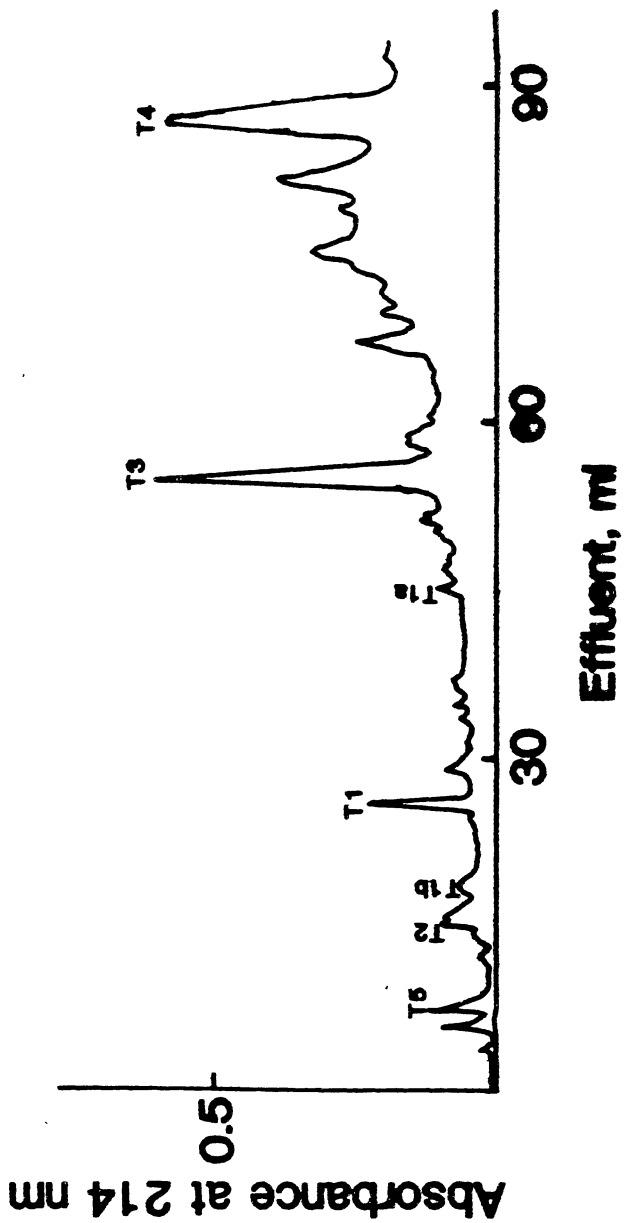

Absorbance at 214 nm
0.5
Effluent, ml
30
60
90
T5
T2
T1b
T1
T1a
T3
T4

Figure 12. Gel Filtration of Chymotryptic Digest of Porcine CB2. One fourth of Pool I (CB2) from Figure 7 was digested with chymotrypsin and the digest was chromatographed on a Sephadex G-25 (superfine) column. Conditions same as in Figure 8.

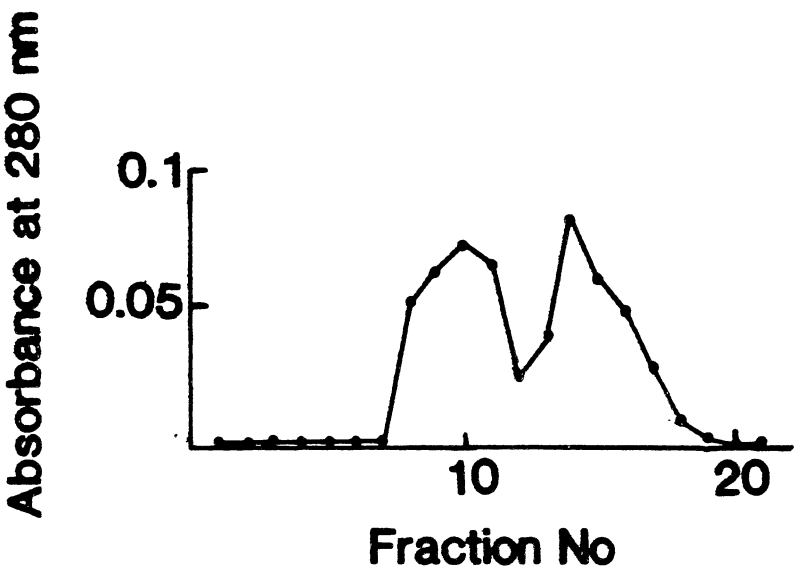

Absorbance at 280 nm
0.1
0.05
10
20
Fraction No

Figure 13. Separation of Peptides C1, C3, C4, C4b, C5, C5a, C7, C7b, C8, C9, C12, C13, and C15 of Porcine CB2 by HPLC. (A) Fraction 10, and (B) Fraction 12 from Figure 12 were injected into the HPLC column. Conditions same as in Figure 9.

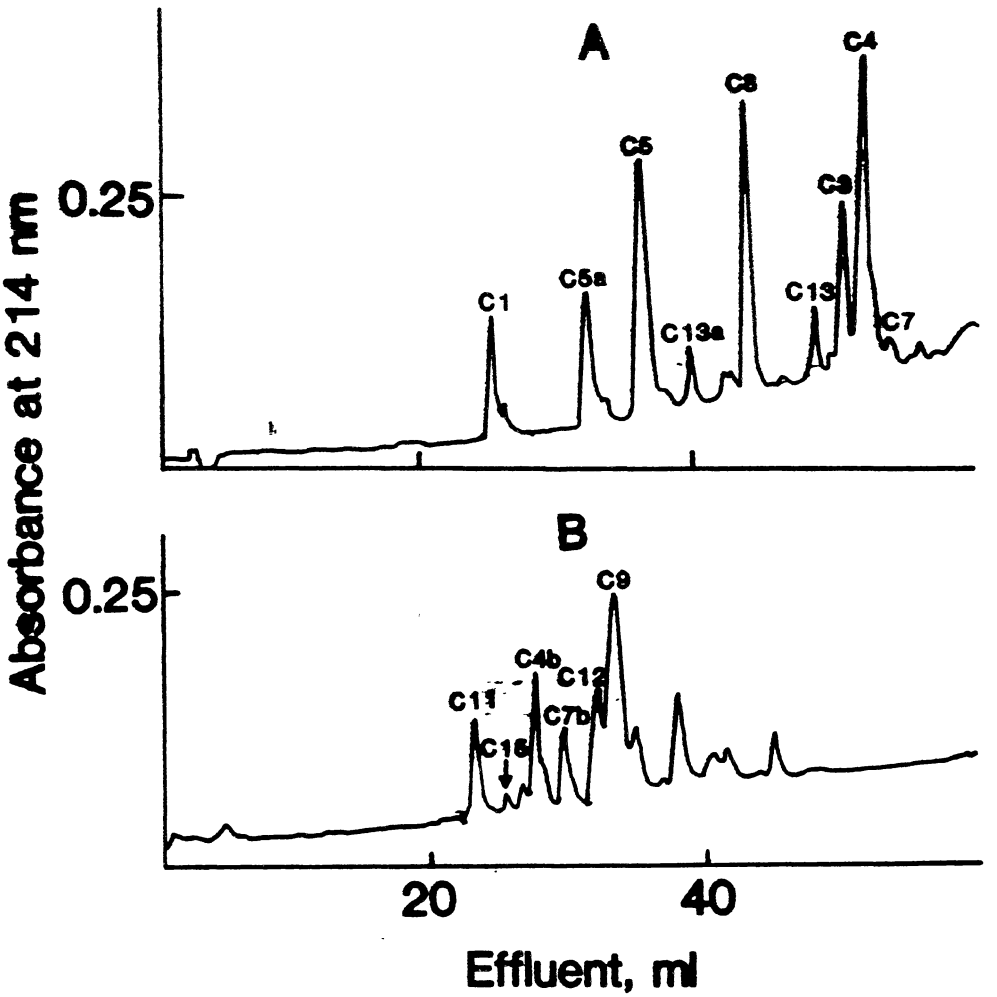

A
B
C1
C5a
C5
C13a
C8
C9
C13
C4
C7
C9
C4b
C12
C11
C7b
C15
0.25
0.25
Absorbance at 214 nm
20
40
Effluent, ml

Figure 14. Separation of Peptides C2, C5b, C6, C6a, C7a, C9, C10, C13b, and C14 of Porcine CB2 by HPLC. (A) Fraction 14 and (B) Fraction 16 from Figure 12 were used for isolation. Conditions were the same as in Figure 9.

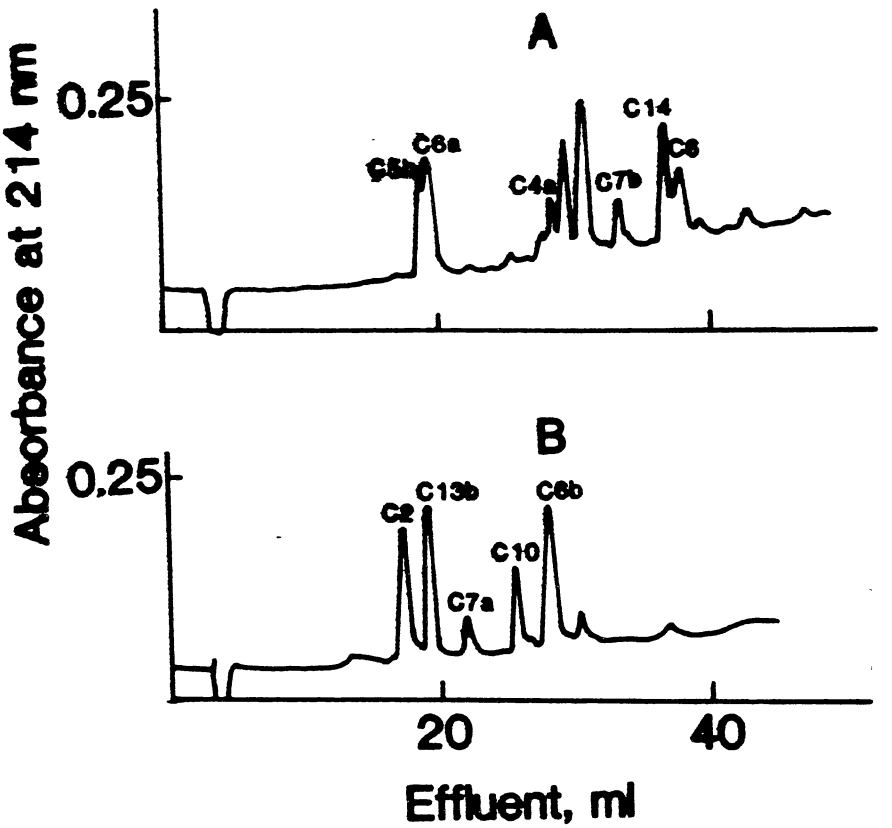

A
0.25
C14
C6a
C5b
C4a
C7b
C6
B
0.25
C2
C13b
C6b
C10
C7a
20
40
Effluent, ml
Absorbance at 214 nm

Figure 15. Elution Profile of Chymotryptic Digest of Porcine CB4. 1 ml of CB4 from Figure 7 was digested with chymotrypsin for 15 hr. Resulting peptides were chromatographed by HPLC. Conditions were the same as in Figure 9.

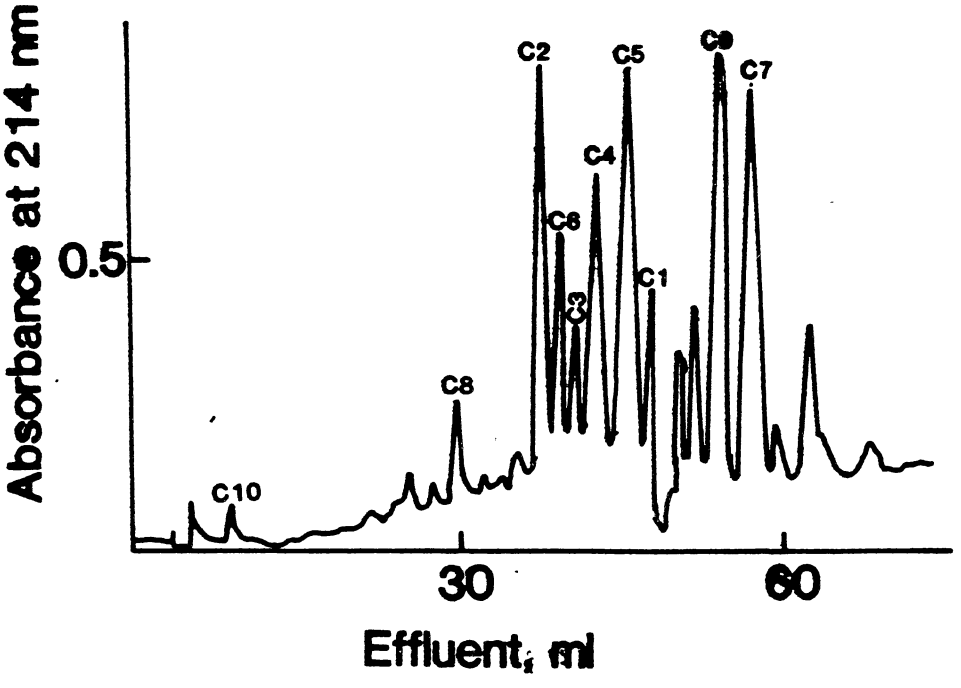

Absorbance at 214 nm
0.5
C10
C8
C2
C6
C3
C4
C5
C1
C9
C7
30
60
Effluent, ml

Figure 16. Gel Filtration of the Tryptic Digest of Rcm Porcine DNase. About 5 mg of DNase was digested with trypsin. Conditions were the same as in Figure 8.

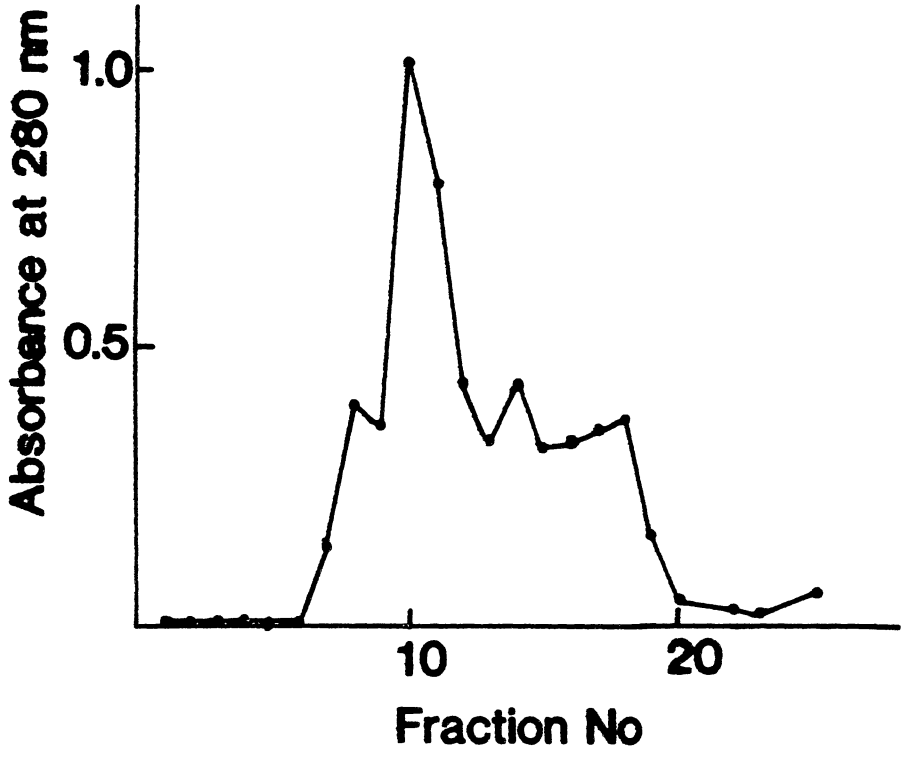

Absorbance at 280 nm
1.0
0.5
10
20
Fraction No

Figure 17. Separation of Peptides T1, T2, and T3 Derived from the Intact Porcine DNase by HPLC. (A) Fraction 8 and (B) Fraction 11, from Figure 16 were chromatographed. Conditions were the same as in Figure 9.

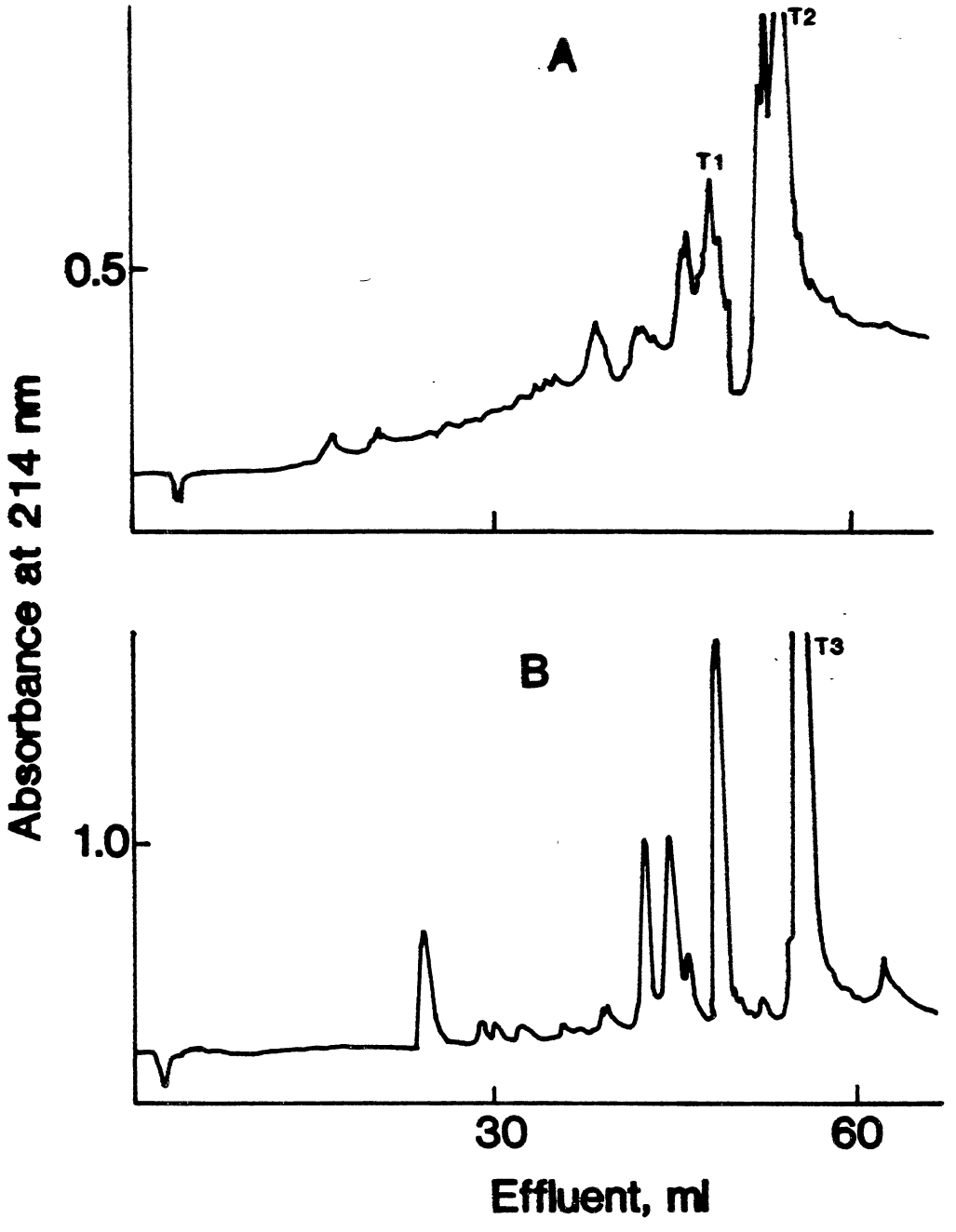

A
T1
T2
0.5
B
T3
1.0
Absorbance at 214 nm
30
60
Effluent, ml

Figure 18. Separation of Chymotryptic Peptides Derived from Porcine CB1 by HPLC. CB1 (pool III) from Figure 7 was digested with chymotrypsin and chromatographed. Conditions were the same as in Figure 9.

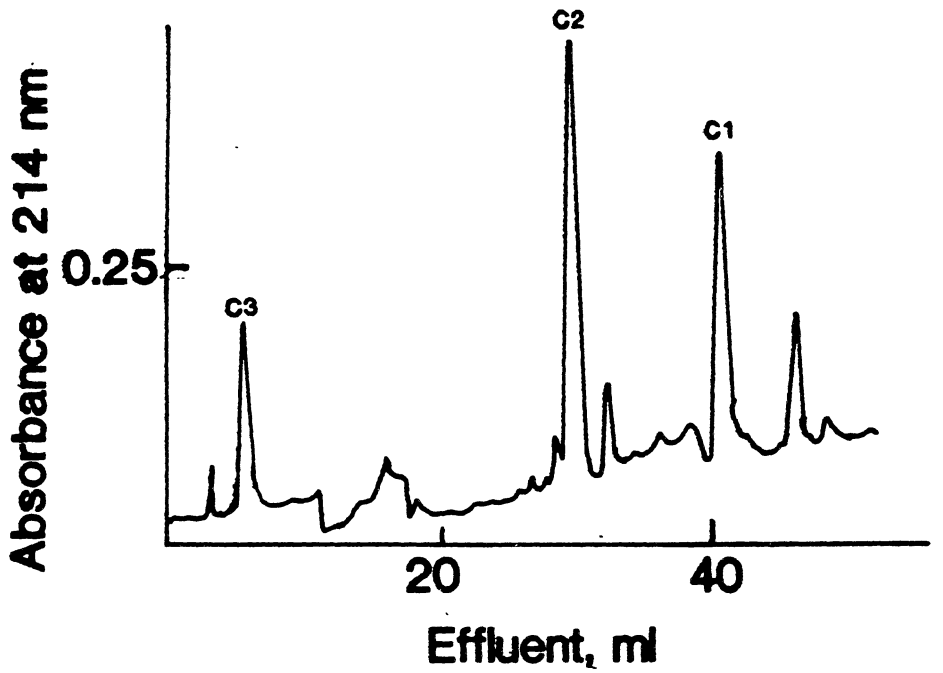

C2
C1
C3
Absorbance at 214 nm
0.25
20
40
Effluent, ml

Figure 19. Complete Amino Acid Sequence of Porcine DNase. Location of major peptides used for sequence analysis are indicated. Sequences of the residues identified are indicated by heavy lines. Products of limited protease digestion that subsequently generated additional fragments from internal cleavage with the same enzyme are not shown as separate fragments. Ordering of chymotryptic peptides CB4-C7 through CB4-C9 is based on thermolytic peptides CB4-T4-Th3, CB4-T4-Th5 (Table XVIII).

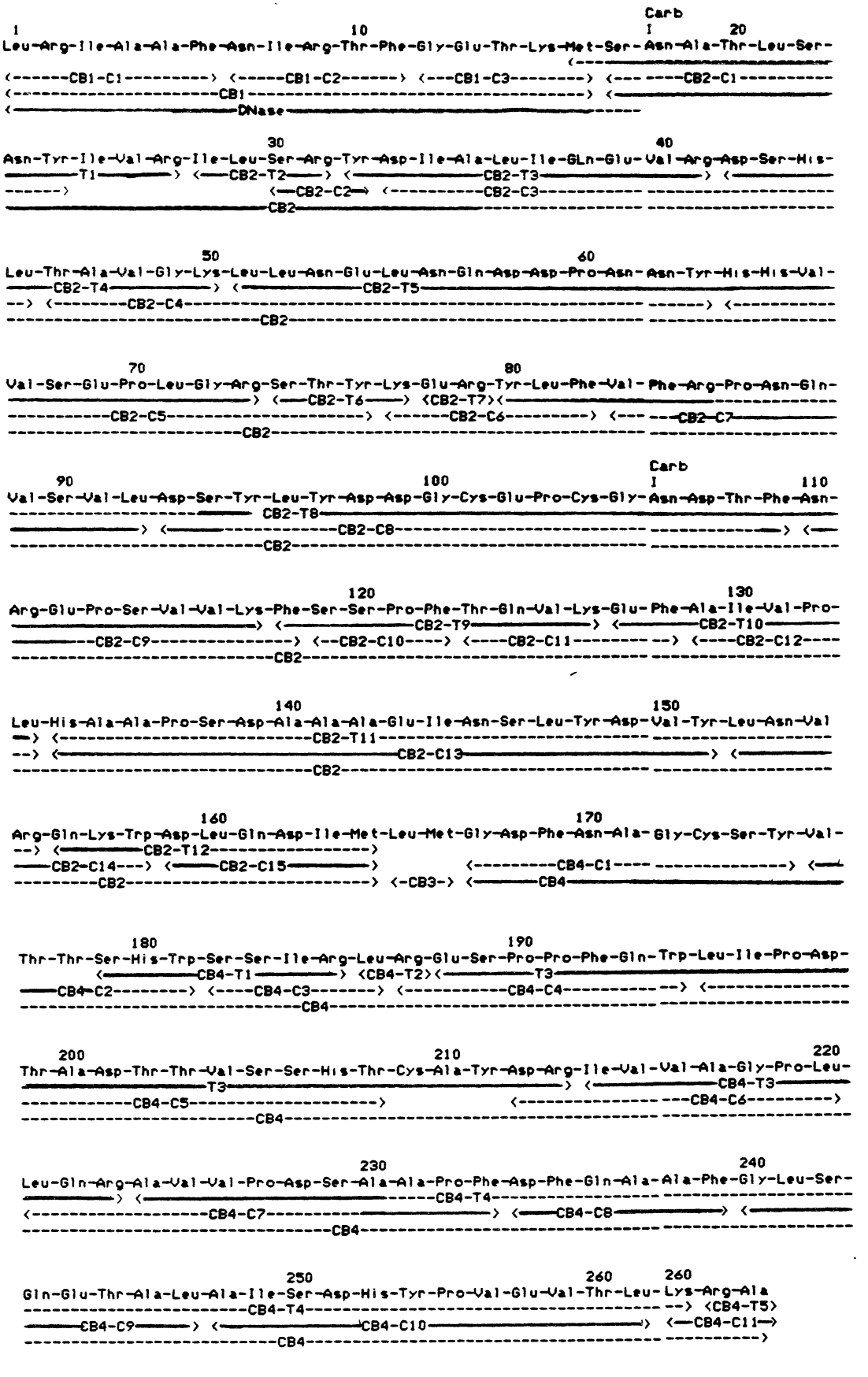

Carb
1 10 20
Leu-Arg-Ile-Ala-Ala-Phe-Asn-Ile-Arg-Thr-Phe-Gly-Glu-Thr-Lys-Met-Ser-Asn-Ala-Thr-Leu-Ser-
CB1-C1
CB1-C2
CB1-C3
CB2-C1
CB1
DNase
30 40
Asn-Tyr-Ile-Val-Arg-Ile-Leu-Ser-Arg-Tyr-Asp-Ile-Ala-Leu-Ile-Gln-Glu-Val-Arg-Asp-Ser-His-
T1
CB2-T2
CB2-T3
CB2-C2
CB2-C3
CB2
50 60
Leu-Thr-Ala-Val-Gly-Lys-Leu-Leu-Asn-Glu-Leu-Asn-Gln-Asp-Asp-Pro-Asn-Asn-Tyr-His-His-Val-
CB2-T4
CB2-T5
CB2-C4
CB2
70 80
Val-Ser-Glu-Pro-Leu-Gly-Arg-Ser-Thr-Tyr-Lys-Glu-Arg-Tyr-Leu-Phe-Val-Phe-Arg-Pro-Asn-Gln-
CB2-T6
CB2-T7
CB2-C5
CB2-C6
CB2-C7
CB2
Carb
90 100 110
Val-Ser-Val-Leu-Asp-Ser-Tyr-Leu-Tyr-Asp-Asp-Gly-Cys-Glu-Pro-Cys-Gly-Asn-Asp-Thr-Phe-Asn-
CB2-T8
CB2-C8
CB2
120 130
Arg-Glu-Pro-Ser-Val-Val-Lys-Phe-Ser-Ser-Pro-Phe-Thr-Gln-Val-Lys-Glu-Phe-Ala-Ile-Val-Pro-
CB2-T9
CB2-T10
CB2-C9
CB2-C10
CB2-C11
CB2-C12
CB2
140 150
Leu-His-Ala-Ala-Pro-Ser-Asp-Ala-Ala-Ala-Glu-Ile-Asn-Ser-Leu-Tyr-Asp-Val-Tyr-Leu-Asn-Val
CB2-T11
CB2-C13
CB2
160 170
Arg-Gln-Lys-Trp-Asp-Leu-Gln-Asp-Ile-Met-Leu-Met-Gly-Asp-Phe-Asn-Ala-Gly-Cys-Ser-Tyr-Val-
CB2-T12
CB2-C14
CB2-C15
CB4-C1
CB2
CB3
CB4
180 190
Thr-Thr-Ser-His-Trp-Ser-Ser-Ile-Arg-Leu-Arg-Glu-Ser-Pro-Pro-Phe-Gln-Trp-Leu-Ile-Pro-Asp-
CB4-T1
CB4-T2
T3
CB4-C2
CB4-C3
CB4-C4
CB4
200 210 220
Thr-Ala-Asp-Thr-Thr-Val-Ser-Ser-His-Thr-Cys-Ala-Tyr-Asp-Arg-Ile-Val-Val-Ala-Gly-Pro-Leu-
T3
CB4-T3
CB4-C5
CB4-C6
CB4
230 240
Leu-Gln-Arg-Ala-Val-Val-Pro-Asp-Ser-Ala-Ala-Pro-Phe-Asp-Phe-Gln-Ala-Ala-Phe-Gly-Leu-Ser-
CB4-T4
CB4-C7
CB4-C8
CB4
250 260 260
Gln-Glu-Thr-Ala-Leu-Ala-Ile-Ser-Asp-His-Tyr-Pro-Val-Glu-Val-Thr-Leu-Lys-Arg-Ala
CB4-T4
CB4-T5
CB4-C9
CB4-C10
CB4-C11
CB4

Figure 20. Gel Filtration of CNBr Fragments of Ovine DNase. About 4 mg of DNase was used for cleavage with CNBr and loaded on a Sephadex G-100 column (1.0x56 cm). Conditions were the same as in Figure 7. Bars indicate the pooled fractions.

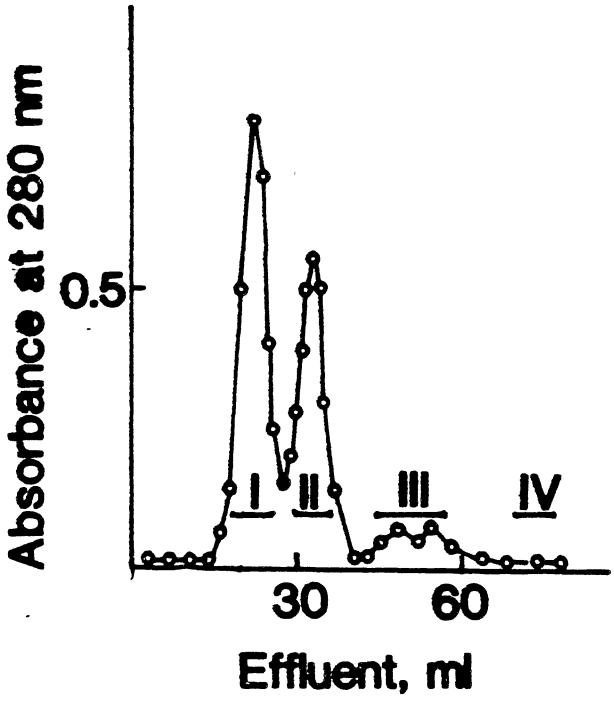

Absorbance at 280 nm
0.5
I
II
III
IV
30
60
Effluent, ml

Figure 21. Separation of Ovine CB1 and CB5 by HPLC. Pool III from Figure 17 containing CB1 and CB5, was chromatographed. Conditions were the same as in Figure 9.

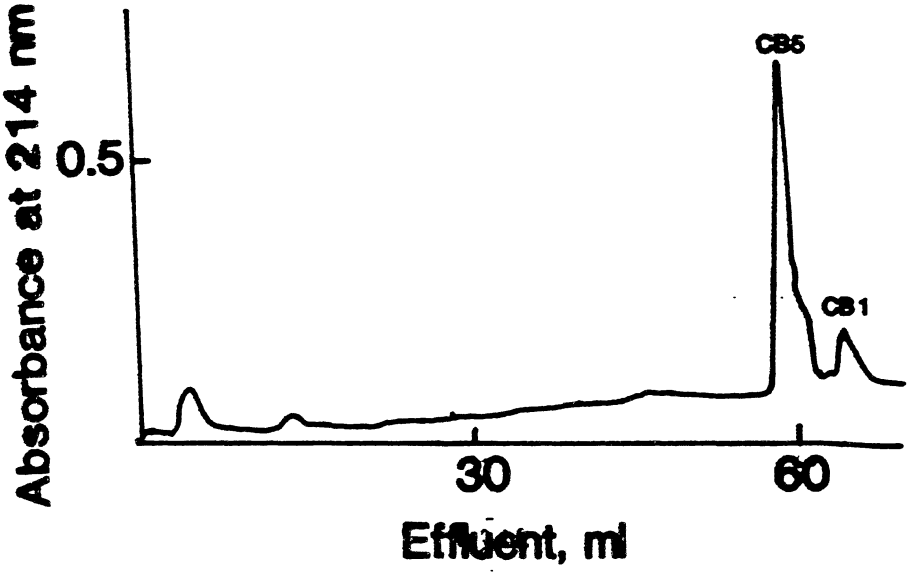

Absorbance at 214 nm
0.5
CB5
CB1
30
60
Effluent, ml

Figure 22. Separation of Tryptic Peptides of Ovine CB2 by HPLC. Tryptic digest of CB2 (Figure 17) was chromatographed. Conditions were the same as in Figure 9.

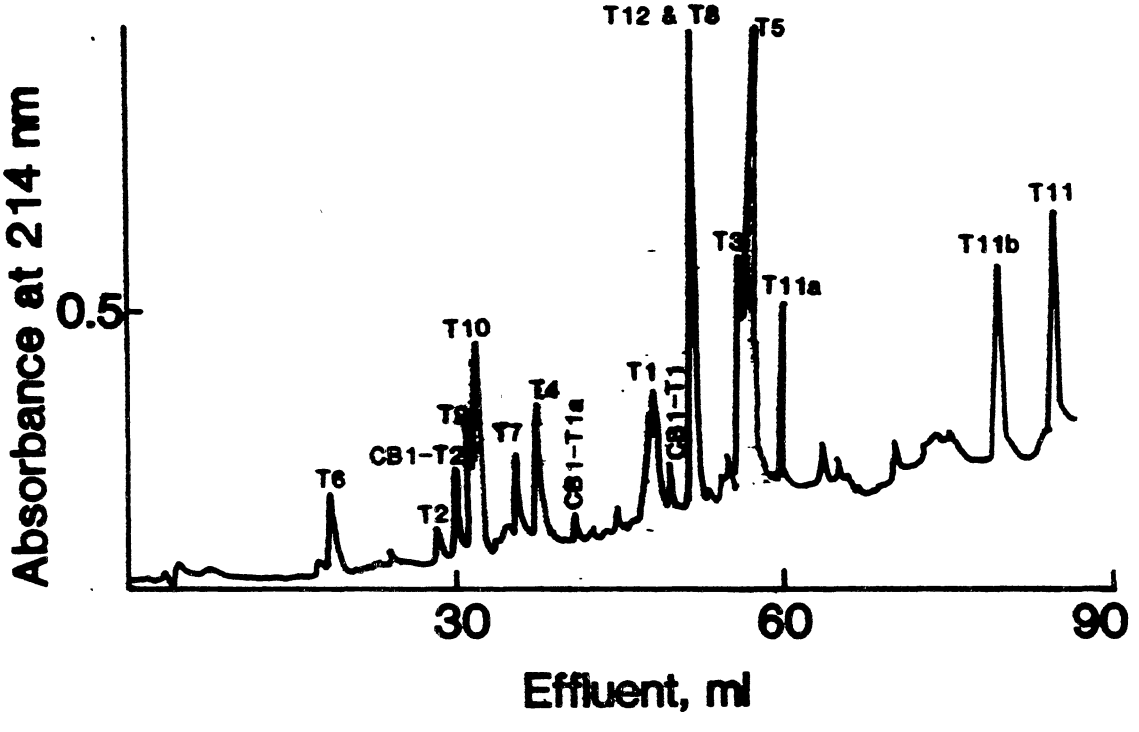
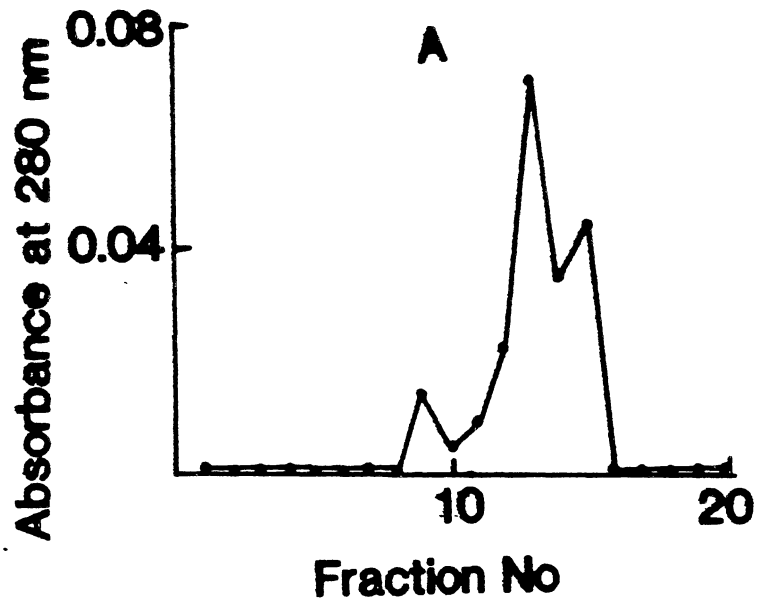

Absorbance at 214 nm
0.5
T12 & T8
T5
T11
T11b
T3
T11a
T10
T1
T4
T9
T7
CB1-T2
CB1-T1a
CB1-T1
T6
T2
30
60
90
Effluent, ml

Figure 23. (A) Gel Filtration of Chymotryptic Digest of Ovine CB2. Conditions were the same as in Figure 8. (B) Isolation of peptide CB1-C1 of ovine DNase from fraction 8 from (A). Conditions were the same as in Figure 9.


A
0.08
0.04
Absorbance at 280 nm
10
20
Fraction No

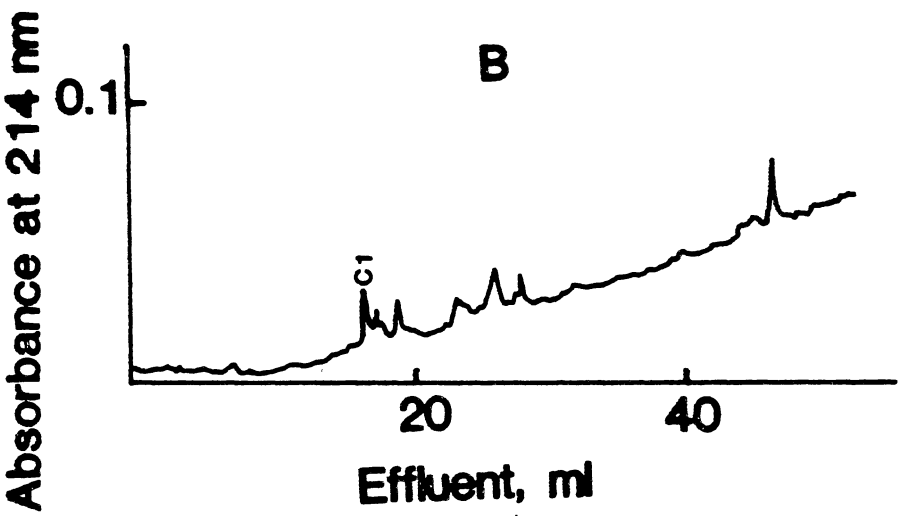

B
0.1
Absorbance at 214 nm
C1
20
40
Effluent, ml

Figure 24. Isolation of Chymotryptic Peptides of Ovine CB2 by HPLC. Conditions were the same as in Figure 9. (A) Fraction 10 and (B) Fraction 11 from Figure 23(a) were chromatographed.

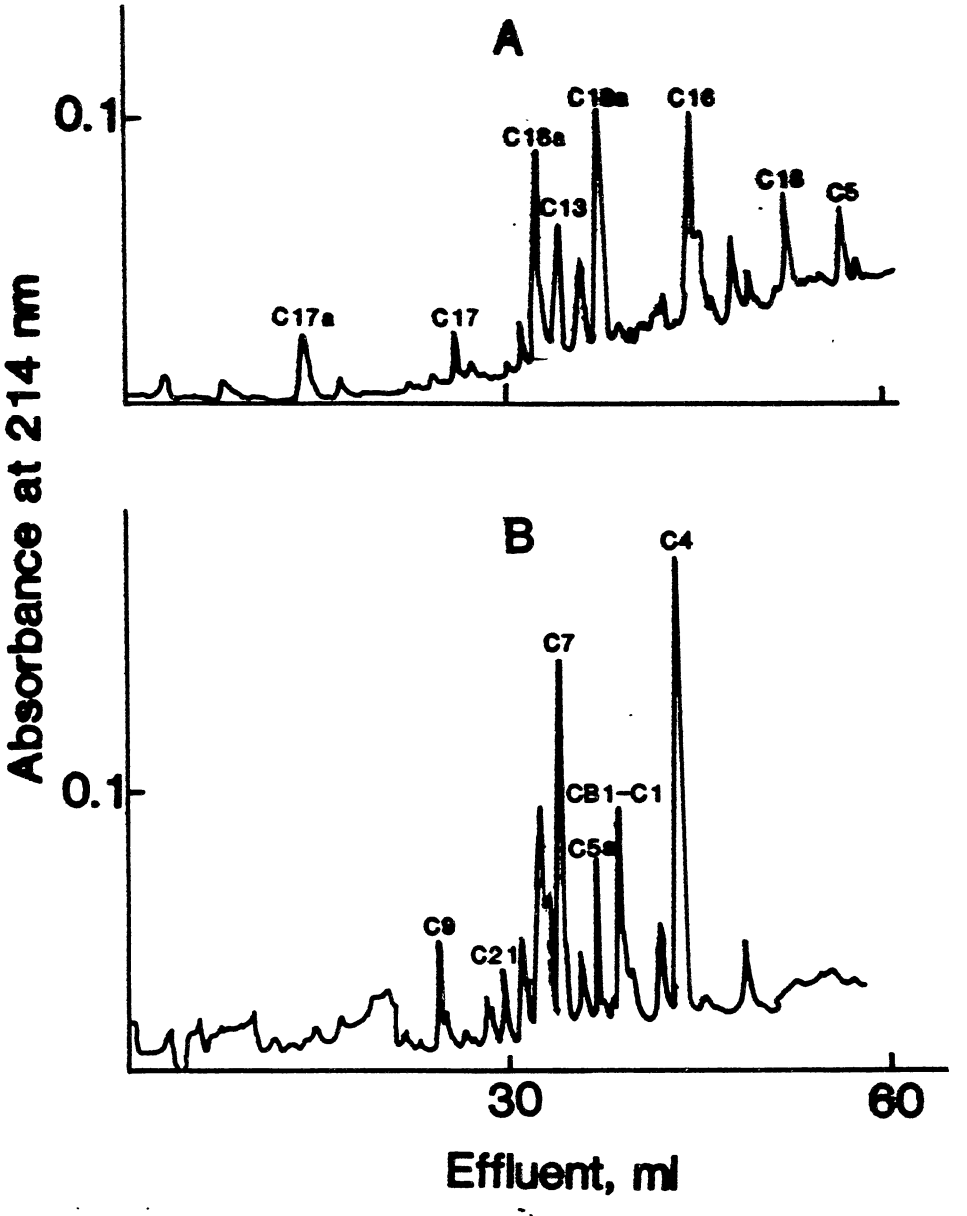

A
0.1
C18a
C16
C18
C5
C13
C17a
C17
B
C4
C7
0.1
CB1–C1
C5a
C9
C21
30
60
Absorbance at 214 nm
Effluent, ml

Figure 25. Isolation of Chymotryptic Peptides of Ovine CB2 by HPLC. (A) Fraction 13 and (B) Fraction 15 from Figure 23a were chromatographed. Conditions were the same as in Figure 9.

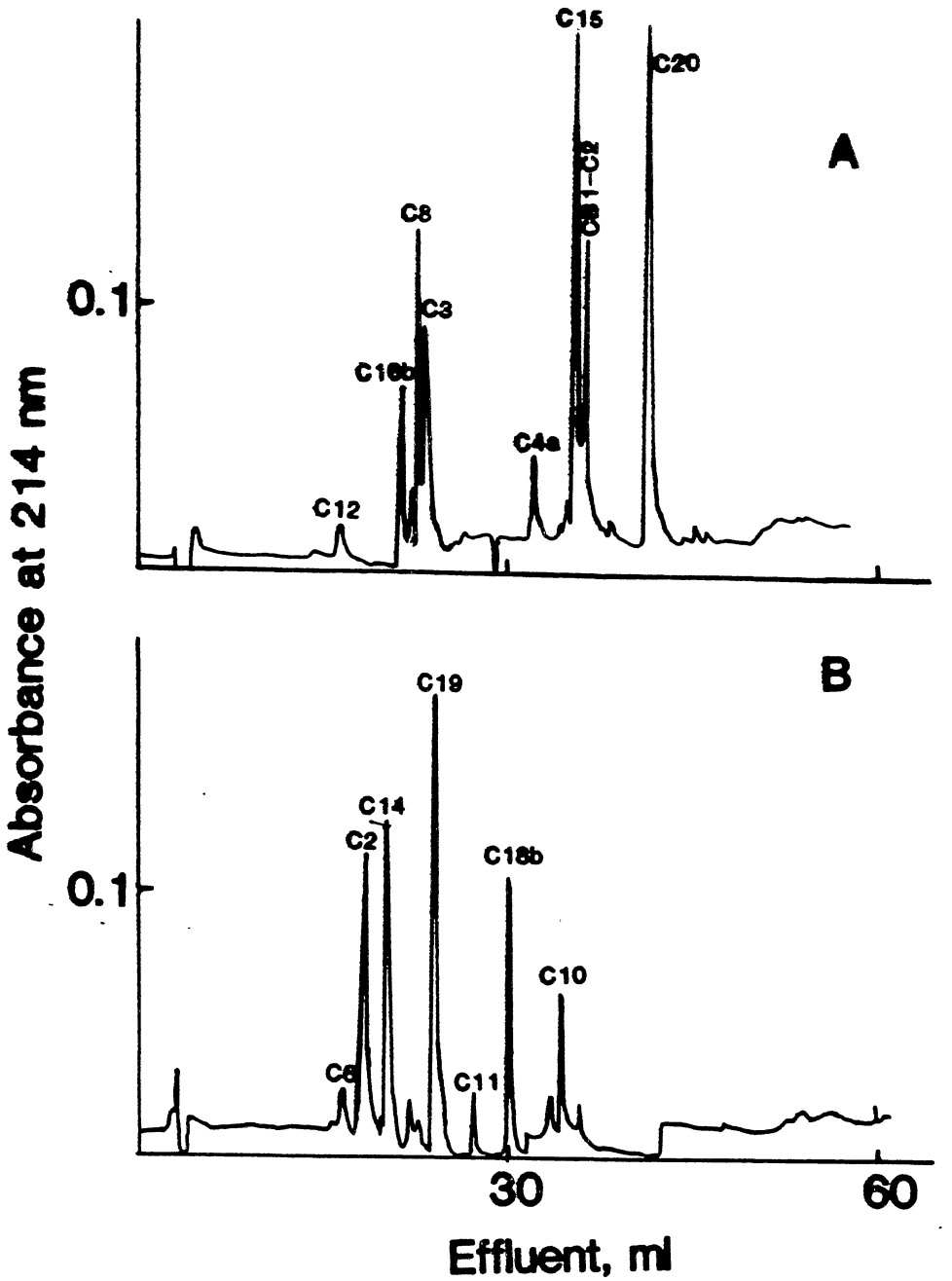

C15
C20
A
C8
C3
C16b
C4a
C12
0.1
Absorbance at 214 nm
B
C19
C14
C2
C16b
C10
C6
C11
0.1
30
60
Effluent, ml

Figure 26. Isolation of (A) Chymotryptic and (B) Thermolytic Peptides of Ovine CB4. Conditions were the same as in Figure 9.

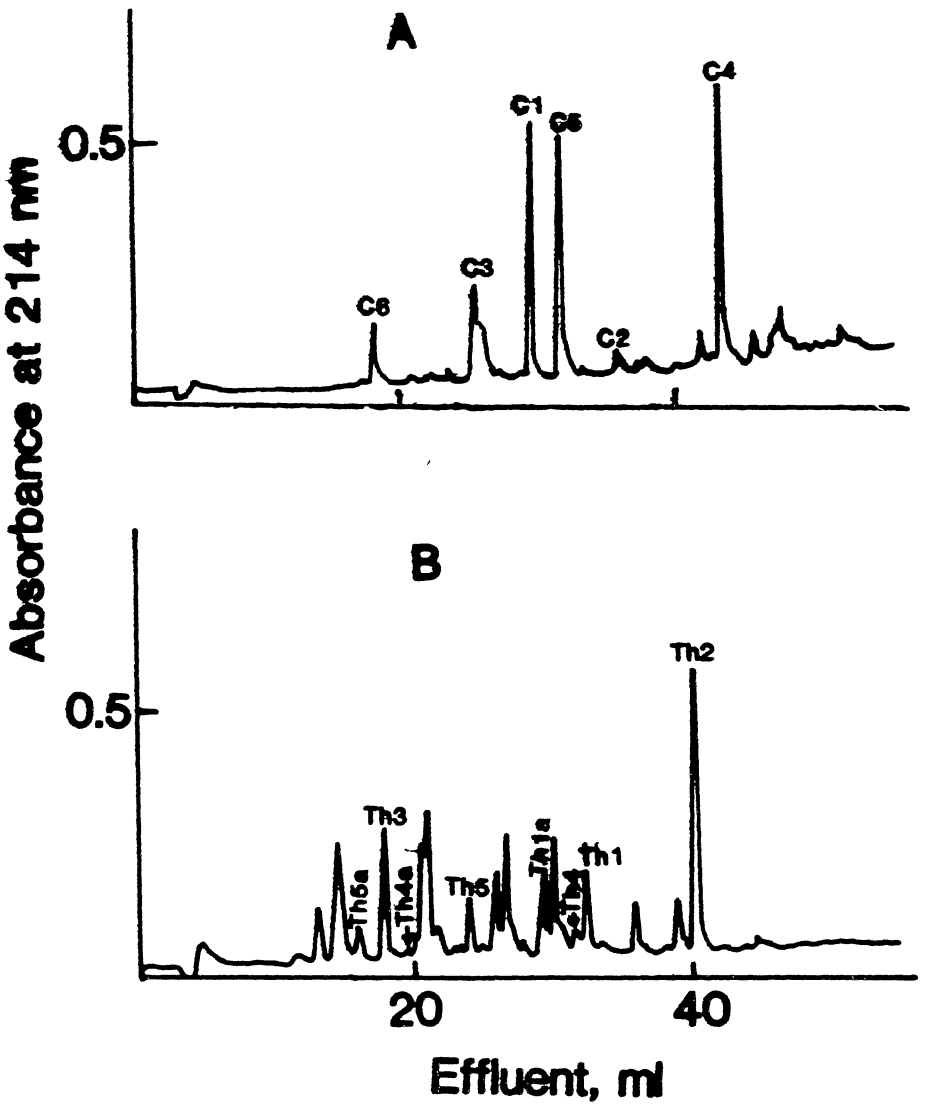

A
B
C1
C5
C4
C3
C6
C2
Th2
Th3
Th1a
Th1
Th6a
Th4a
Th5
Th1b
0.5
0.5
Absorbance at 214 nm
20
40
Effluent, ml

Figure 27. Isolation of Chymotryptic Peptides (o) CB4-C1, and (o) CB4-C2 of Ovine DNase. Performic acid oxidized ovine CB4 was digested with chymotrypsin. Peptide isolation conditions were the same as in Figure 9.

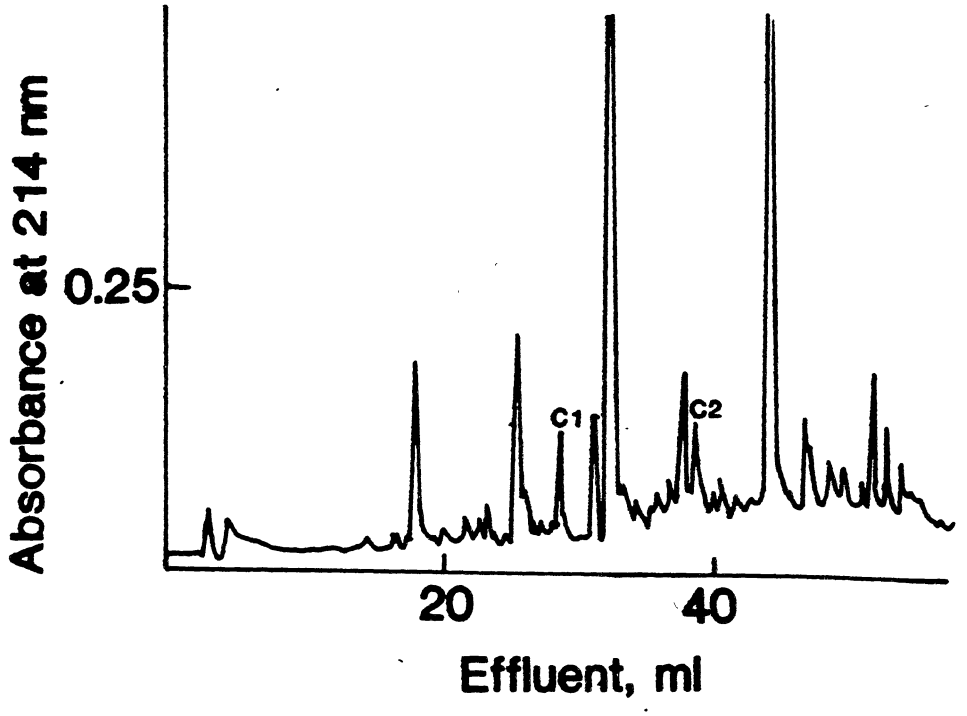

Absorbance at 214 nm
0.25
C1
C2
20
40
Effluent, ml

Figure 28. Isolation of Thermolytic Peptides of Ovine CB5 by HPLC. For HPLC, conditions, refer to Figure 9.

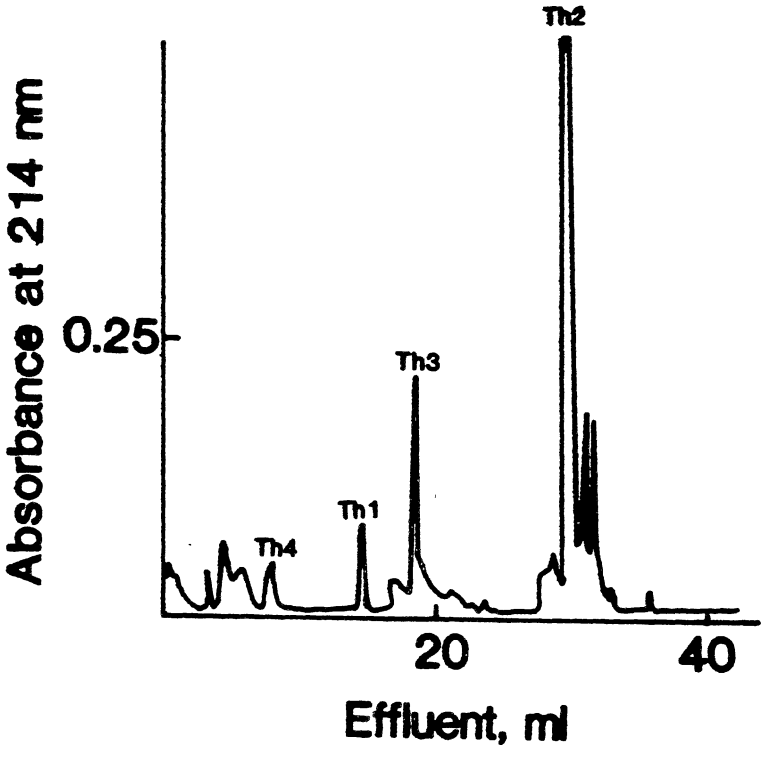

Th2
Absorbance at 214 nm
0.25
Th3
Th1
Th4
20
40
Effluent, ml

Figure 29. Complete Amino Acid Sequence of Ovine Pancreatic DNase. The sequence is based on the partial sequence analysis by automated sequenator of large peptides, manual sequencing of a few peptides, amino acid composition of small peptides obtained by proteolytic digestion of CNBr-fragments of the enzyme, and the known sequence of bovine DNase. Residues identified by Edman degradation are indicated by heavy lines.

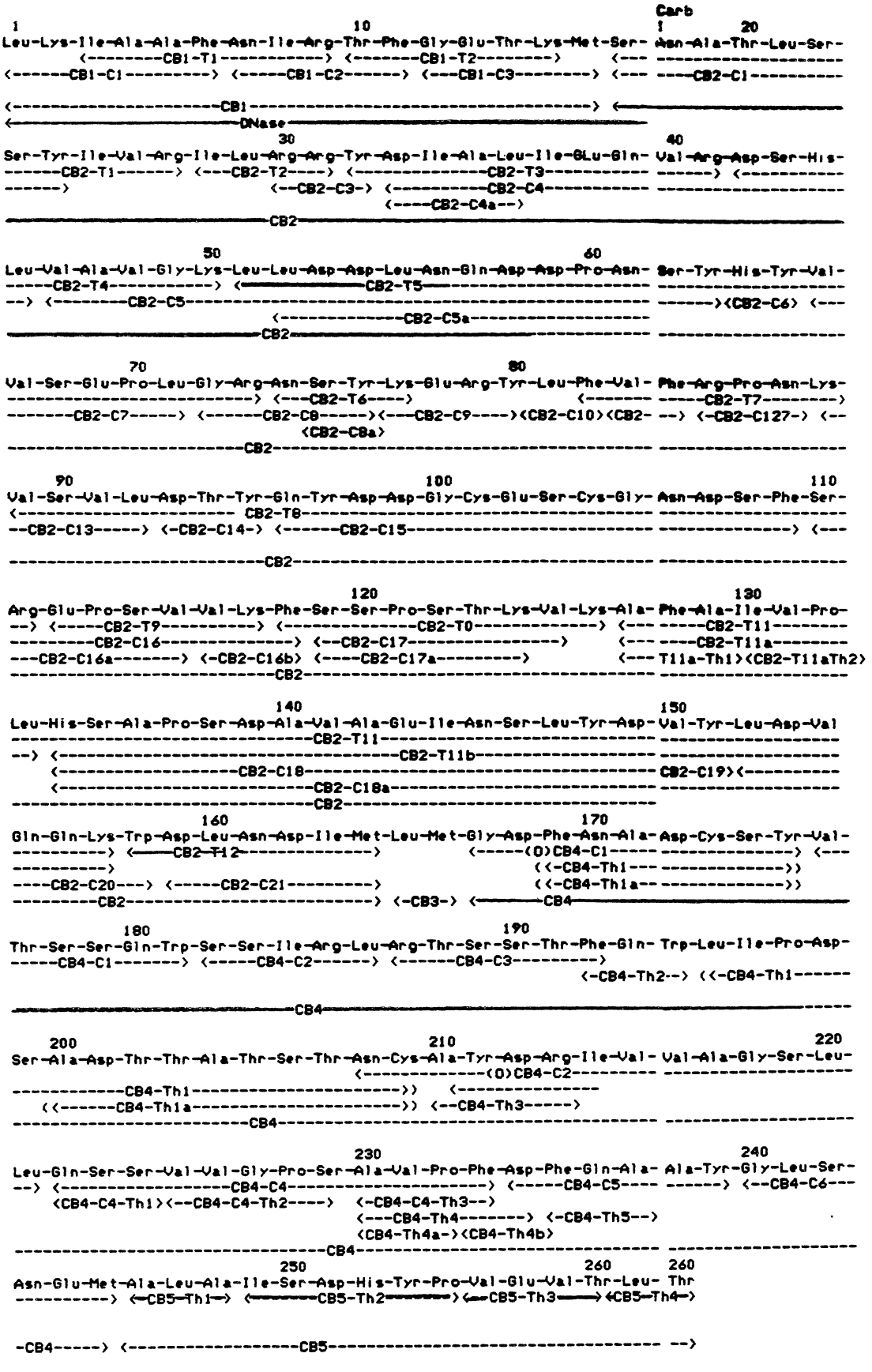

Carb
1 10 1 20
Leu-Lys-Ile-Ala-Ala-Phe-Asn-Ile-Arg-Thr-Phe-Gly-Glu-Thr-Lys-Met-Ser- Asn-Ala-Thr-Leu-Ser-
CB1-T1 CB1-T2 CB1-C1 CB1-C2 CB1-C3 CB2-C1
CB1
DNase
30 40
Ser-Tyr-Ile-Val-Arg-Ile-Leu-Arg-Arg-Tyr-Asp-Ile-Ala-Leu-Ile-Glu-Gln- Val-Arg-Asp-Ser-His-
CB2-T1 CB2-T2 CB2-T3 CB2-C3 CB2-C4 CB2-C4a
CB2
50 60
Leu-Val-Ala-Val-Gly-Lys-Leu-Leu-Asp-Asp-Leu-Asn-Gln-Asp-Asp-Pro-Asn- Ser-Tyr-His-Tyr-Val-
CB2-T4 CB2-T5 CB2-C5 CB2-C5a CB2-C6
CB2
70 80
Val-Ser-Glu-Pro-Leu-Gly-Arg-Asn-Ser-Tyr-Lys-Glu-Arg-Tyr-Leu-Phe-Val- Phe-Arg-Pro-Asn-Lys-
CB2-T6 CB2-C7 CB2-C8 CB2-C8a CB2-C9 CB2-C10 CB2-C12 CB2-T7
CB2
90 100 110
Val-Ser-Val-Leu-Asp-Thr-Tyr-Gln-Tyr-Asp-Asp-Gly-Cys-Glu-Ser-Cys-Gly- Asn-Asp-Ser-Phe-Ser-
CB2-T8 CB2-C13 CB2-C14 CB2-C15 CB2-C16
CB2
120 130
Arg-Glu-Pro-Ser-Val-Val-Lys-Phe-Ser-Ser-Pro-Ser-Thr-Lys-Val-Lys-Ala- Phe-Ala-Ile-Val-Pro-
CB2-T9 CB2-T10 CB2-T11 CB2-C16 CB2-C17 CB2-T11a CB2-C16a CB2-C16b CB2-C17a T11a-Th1 CB2-T11aTh2
CB2
140 150
Leu-His-Ser-Ala-Pro-Ser-Asp-Ala-Val-Ala-Glu-Ile-Asn-Ser-Leu-Tyr-Asp- Val-Tyr-Leu-Asp-Val
CB2-T11 CB2-T11b CB2-C18 CB2-C19 CB2-C18a
CB2
160 170
Gln-Gln-Lys-Trp-Asp-Leu-Asn-Asp-Ile-Met-Leu-Met-Gly-Asp-Phe-Asn-Ala- Asp-Cys-Ser-Tyr-Val-
CB2-T12 (O)CB4-C1 CB4-Th1 CB2-C20 CB2-C21 CB4-Th1a
CB2 CB3 CB4
180 190
Thr-Ser-Ser-Gln-Trp-Ser-Ser-Ile-Arg-Leu-Arg-Thr-Ser-Ser-Thr-Phe-Gln- Trp-Leu-Ile-Pro-Asp-
CB4-C1 CB4-C2 CB4-C3 CB4-Th2 CB4-Th1
CB4
200 210 220
Ser-Ala-Asp-Thr-Thr-Ala-Thr-Ser-Thr-Asn-Cys-Ala-Tyr-Asp-Arg-Ile-Val- Val-Ala-Gly-Ser-Leu-
(O)CB4-C2 CB4-Th1 CB4-Th1a CB4-Th3
CB4
230 240
Leu-Gln-Ser-Ser-Val-Val-Gly-Pro-Ser-Ala-Val-Pro-Phe-Asp-Phe-Gln-Ala- Ala-Tyr-Gly-Leu-Ser-
CB4-C4 CB4-C4-Th1 CB4-C4-Th2 CB4-C4-Th3 CB4-Th4 CB4-C5 CB4-Th5 CB4-Th4a CB4-Th4b CB4-C6
CB4
250 260 260
Asn-Glu-Met-Ala-Leu-Ala-Ile-Ser-Asp-His-Tyr-Pro-Val-Glu-Val-Thr-Leu- Thr
CB5-Th1 CB5-Th2 CB5-Th3 CB5-Th4
CB4 CB5

Figure 30. Isolation of the Two Forms of Ovine DNase by DEAE Cellulose Chromatography. Ovine DNase after ammonium sulfate fractionation, CM cellulose, Blue Sepharose, and soybean trypsin inhibitor agarose chromatographies, was chromatographed on a 2x10 cm DEAE cellulose column equilibrated in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0. DNase activity appeared in two peaks on elution with a gradient 100 ml each of 2 and 10 mM $CaCl_2$ in the starting buffer. Pool 1 and Pool II were separately dialyzed and lyophilized.

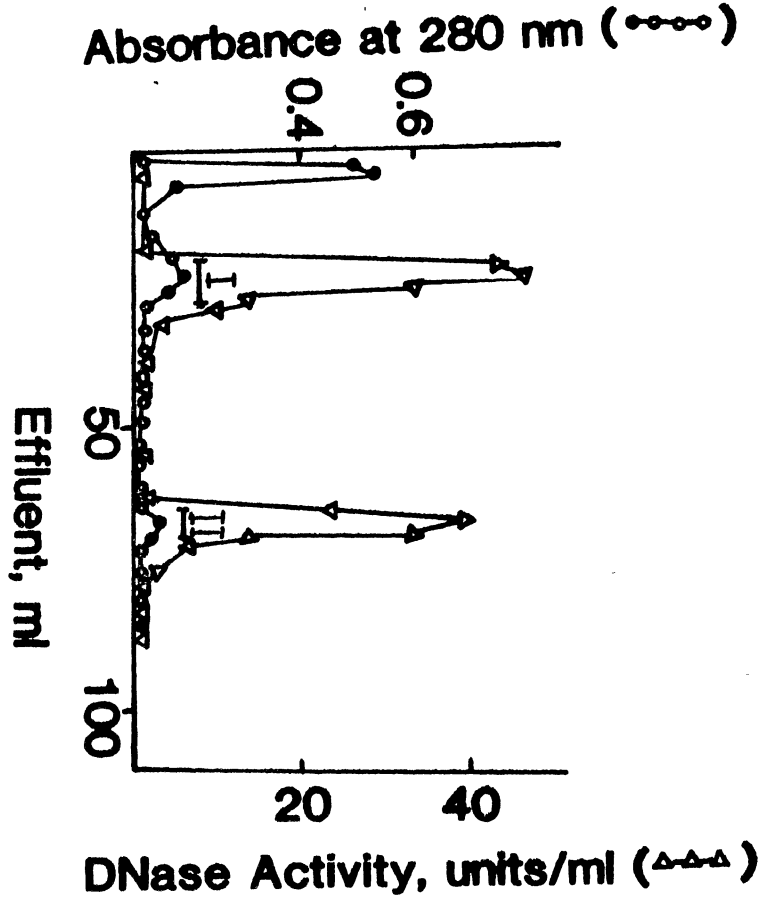

Absorbance at 280 nm (•-•-•)
0.4
0.6
Effluent, ml
50
100
20
40
DNase Activity, units/ml (Δ-Δ-Δ)

Figure 31. Isoelectric Focusing of Ovine DNase. Ovine DNases from Figure 26 were focused under conditions as described in Figure 5(A). (A) Pool I, (B) Ovine DNase, (C) Pool II.


A
B
C

Figure 32. Isolation of Acid Cleaved Peptides of Bovine DNase. About 5 mg of bovine DNase was cleaved at Asp-Pro bond with 88% formic acid. Peptide P-1 (residues 1 through 60) was isolated by HPLC (A). Column was Supelco LC-3DP (20x4.6 mm), flow rate 1 ml/min, temperature 40°C. Peptide P-1 was subjected to mild acid hydrolysis and the resulting peptide P-2 (residues 1 through 33) was isolated by HPLC (B). Conditions were the same as in Fifuge 9.

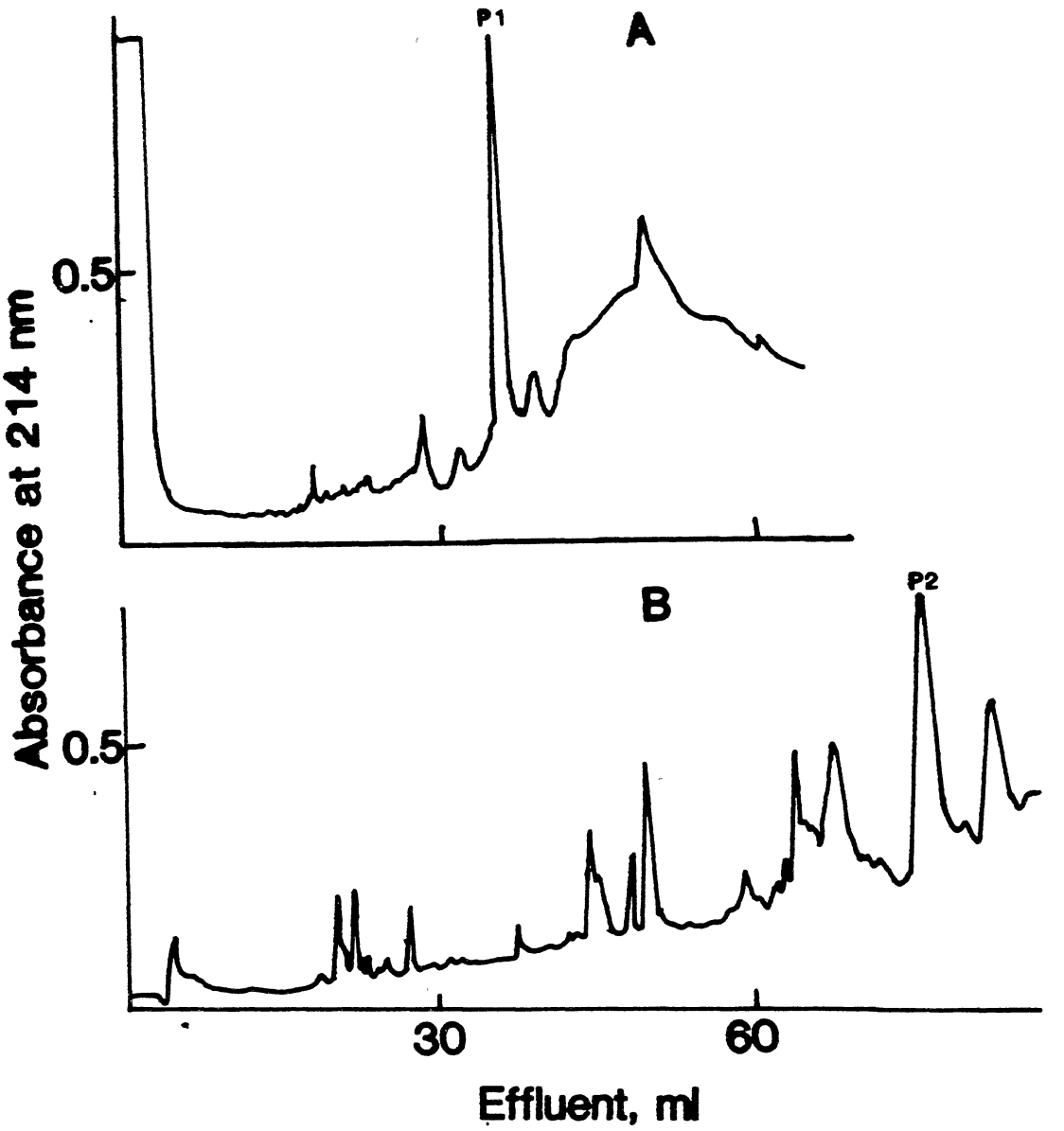
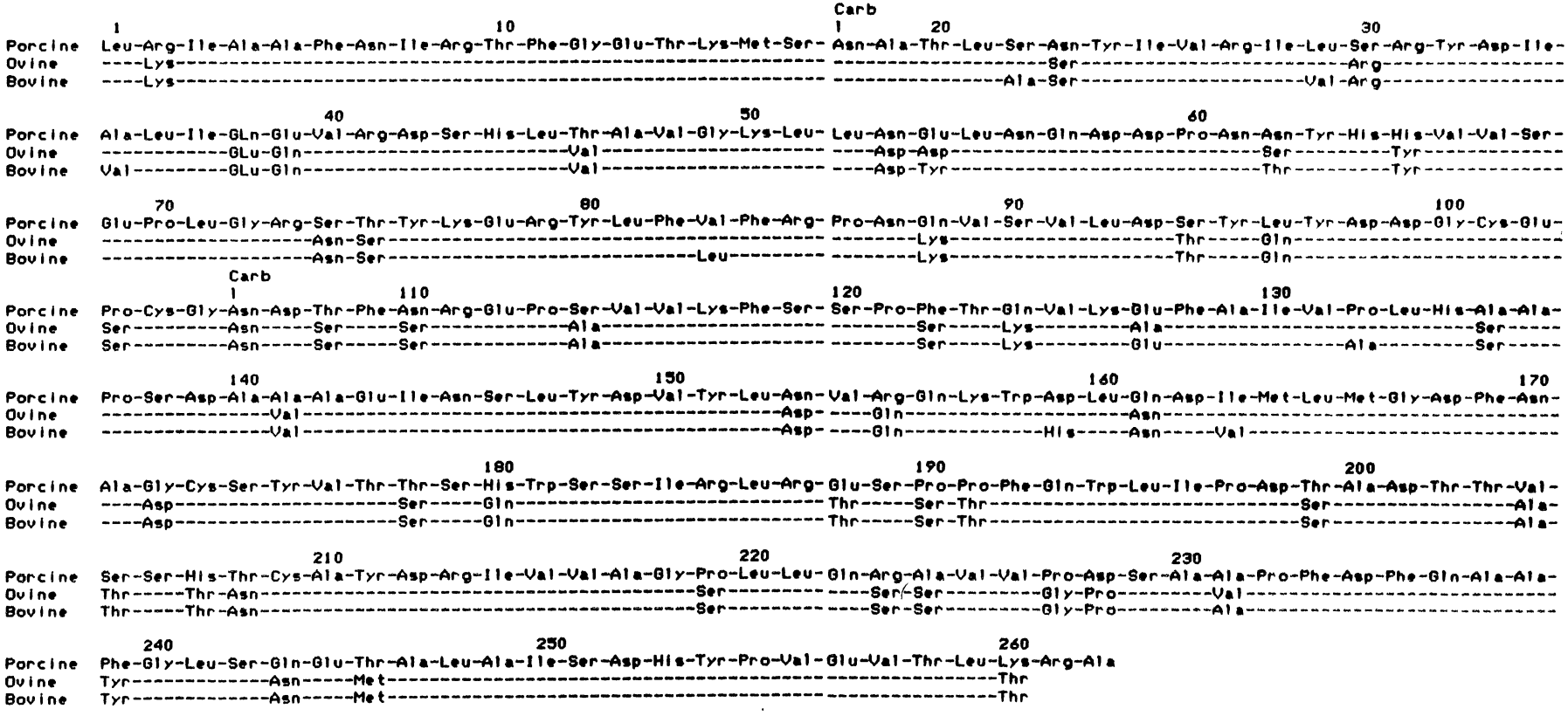

P1
A
0.5
Absorbance at 214 nm
B
P2
0.5
30
60
Effluent, ml

Figure 33. Comparison of Primary Structures of Porcine, Ovine and Bovine DNase.


Carb
1 10 1 20 30
Porcine Leu-Arg-Ile-Ala-Ala-Phe-Asn-Ile-Arg-Thr-Phe-Gly-Glu-Thr-Lys-Met-Ser- Asn-Ala-Thr-Leu-Ser-Asn-Tyr-Ile-Val-Arg-Ile-Leu-Ser-Arg-Tyr-Asp-Ile-
Ovine ----Lys---------- ----------Ser----------Arg----------
Bovine ----Lys---------- ----------Ala-Ser----------Val-Arg----------
40 50 60
Porcine Ala-Leu-Ile-Gln-Glu-Val-Arg-Asp-Ser-His-Leu-Thr-Ala-Val-Gly-Lys-Leu- Leu-Asn-Glu-Leu-Asn-Gln-Asp-Asp-Pro-Asn-Asn-Tyr-His-His-Val-Val-Ser-
Ovine ----------Glu-Gln----------Val---------- ----Asp-Asp----------Ser---------Tyr----------
Bovine Val---------Glu-Gln----------Val---------- ----Asp-Tyr----------Thr---------Tyr----------
70 80 90 100
Porcine Glu-Pro-Leu-Gly-Arg-Ser-Thr-Tyr-Lys-Glu-Arg-Tyr-Leu-Phe-Val-Phe-Arg- Pro-Asn-Gln-Val-Ser-Val-Leu-Asp-Ser-Tyr-Leu-Tyr-Asp-Asp-Gly-Cys-Glu-
Ovine ----------Asn-Ser---------- --------Lys----------Thr-----Gln----------
Bovine ----------Asn-Ser----------Leu--------- --------Lys----------Thr-----Gln----------
Carb
1 110 120 130
Porcine Pro-Cys-Gly-Asn-Asp-Thr-Phe-Asn-Arg-Glu-Pro-Ser-Val-Val-Lys-Phe-Ser- Ser-Pro-Phe-Thr-Gln-Val-Lys-Glu-Phe-Ala-Ile-Val-Pro-Leu-His-Ala-Ala-
Ovine Ser---------Asn-----Ser-----Ser----------Ala---------- --------Ser-----Lys---------Ala----------Ser-----
Bovine Ser---------Asn-----Ser-----Ser----------Ala---------- --------Ser-----Lys---------Glu----------Ala---------Ser-----
140 150 160 170
Porcine Pro-Ser-Asp-Ala-Ala-Ala-Glu-Ile-Asn-Ser-Leu-Tyr-Asp-Val-Tyr-Leu-Asn- Val-Arg-Gln-Lys-Trp-Asp-Leu-Gln-Asp-Ile-Met-Leu-Met-Gly-Asp-Phe-Asn-
Ovine ----------Val----------Asp- ----Gln----------Asn----------
Bovine ----------Val----------Asp- ----Gln----------His-----Asn-----Val----------
180 190 200
Porcine Ala-Gly-Cys-Ser-Tyr-Val-Thr-Thr-Ser-His-Trp-Ser-Ser-Ile-Arg-Leu-Arg- Glu-Ser-Pro-Pro-Phe-Gln-Trp-Leu-Ile-Pro-Asp-Thr-Ala-Asp-Thr-Thr-Val-
Ovine ----Asp----------Ser-----Gln---------- Thr-----Ser-Thr----------Ser----------Ala-
Bovine ----Asp----------Ser-----Gln---------- Thr-----Ser-Thr----------Ser----------Ala-
210 220 230
Porcine Ser-Ser-His-Thr-Cys-Ala-Tyr-Asp-Arg-Ile-Val-Val-Ala-Gly-Pro-Leu-Leu- Gln-Arg-Ala-Val-Val-Pro-Asp-Ser-Ala-Ala-Pro-Phe-Asp-Phe-Gln-Ala-Ala-
Ovine Thr-----Thr-Asn----------Ser---------- ----Ser-Ser----------Gly-Pro---------Val----------
Bovine Thr-----Thr-Asn----------Ser---------- ----Ser-Ser----------Gly-Pro---------Ala----------
240 250 260
Porcine Phe-Gly-Leu-Ser-Gln-Glu-Thr-Ala-Leu-Ala-Ile-Ser-Asp-His-Tyr-Pro-Val-Glu-Val-Thr-Leu-Lys-Arg-Ala
Ovine Tyr----------Asn-----Met---------- ----------Thr
Bovine Tyr----------Asn-----Met---------- ----------Thr

VITA 2

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Doctor of Philosophy

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