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Bovine Factor X_{1a} (Activated Stuart Factor). Evidence of Homology with Mammalian Serine Proteases†

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ABSTRACT: Amino acid sequences of peptides containing the amino-terminal and active-site regions of the heavy chain of bovine factor X_{1a} (activated Stuart factor) have been determined. The amino-terminal sequence is *Ile-Val-Gly-Gly*-Arg-Asp-*Cys*-Ala-Glu-Gly-Glu-Cys-*Pro-Trp-Gln*-Ala-Leu-*Leu*-Val-Asn-Glu-Glu-Asn-Glu-Gly-*Phe-Cys-Gly-Gly*-Thr-Ile-Leu-*Asn*-Glu-Phe-Tyr-*Val*-. The sequence which includes the reactive serine of the active site (capitalized) is *Phe-Cys-Ala-Gly-Tyr*-Asp-Thr-Gln-Pro-Glu-*Asp*-Ala-*Cys-Gln-Gly-Asp-SER-Gly-Gly-Pro*-His-*Val*-Thr-Arg-. The amino acids which are in italics are identical with those found in the amino-terminal region and active-site region of bovine trypsin, indicating considerable homology between the two proteins. These sequences are also very similar to the corresponding sequences of other proteases including bovine thrombin, bovine chymotrypsin, and human plasmin. It is concluded that bovine factor X_{1a} is a typical serine protease which has evolved from an ancestor common to other plasma and pancreatic proteolytic enzymes.

Factor X (Stuart factor)[1] is a protein which participates in the middle phase of blood coagulation (Davie *et al.*, 1969). The purified protein can be separated into two chromatographic fractions (factors X_1 and X_2) having similar chemical and biological properties (Jackson and Hanahan, 1968; Fujikawa *et al.*, 1972a).

An enzyme in Russell's viper venom activates factors X_1 and X_2 by hydrolyzing a peptide bond in the amino-terminal region of the heavy chain of the precursor molecule (Fujikawa *et al.*, 1972b). The activated protein (factor X_{1a} or X_{2a}) is a proteolytic enzyme which converts prothrombin to thrombin. It also hydrolyzes various synthetic esters such as *p*-toluenesulfonyl-L-arginine methyl ester and benzoyl-L-arginine ethyl ester (Esnouf and Williams, 1962; Milstone, 1964; Jackson and Hanahan, 1968; Aronson and Ménaché, 1968; Adams and Elmore, 1971). Like certain proteolytic enzymes, activated factor X is inhibited by soybean trypsin inhibitor (Breckenridge and Ratnoff, 1964; Milstone, 1964; Lundblad and Davie, 1965; Jackson and Hanahan, 1968) and by diisopropyl phosphorofluoridate (DFP)[2] although relatively high concentrations of this organic phosphate are required (Jackson and Hanahan, 1968; Leveson and Esnouf, 1968; Fujikawa *et al.*, 1972b).

This communication describes experiments which show that factor X_{1a} contains amino acid sequences which are homologous with sequences found in other proteolytic enzymes, specifically in bovine trypsin (Walsh and Neurath, 1964), chymotrypsin A (Hartley, 1964), elastase (Hartley and Shotton, 1971), and thrombin (Magnusson, 1971). These homologous sequences are found in the amino-terminal segment of the heavy chain of factor X_{1a} and in the region of the serine residue which reacts with DFP.

Experimental Procedure

Materials

Bovine factor X_1 and the enzyme from Russell's viper venom which activates factor X were prepared as previously described (Fujikawa *et al.*, 1972a,b). DFP was obtained from Pierce Chemical Co. and [^{32}P]DFP from Amersham/Searle Co. Sephadex products were obtained from Pharmacia Fine Chemicals and the anion-exchange resin, AG-1X2 (200–400 mesh), from Bio-Rad Laboratories. Porcine trypsin, a product of Novo Industri A/S, was purified on an affinity column as described by Robinson *et al.* (1971). Reagents used for the sequenator (sequenal grade) were from Pierce Chemical Co.

Methods

Activation of Factor X_1. The conversion of factor X_1 to the active form (factor X_{1a}) by Russell's viper venom and its subsequent inactivation with DFP or [^{32}P]DFP were carried out as previously described (Fujikawa *et al.*, 1972b).

Reduction, pyridylethylation, and separation of the heavy and light chains of factors X_1, X_{1a}, and DIP factor X_{1a} were also carried out as previously described (Fujikawa *et al.*, 1972b).

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[1] The nomenclature for various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] Abbreviations used are: DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl.

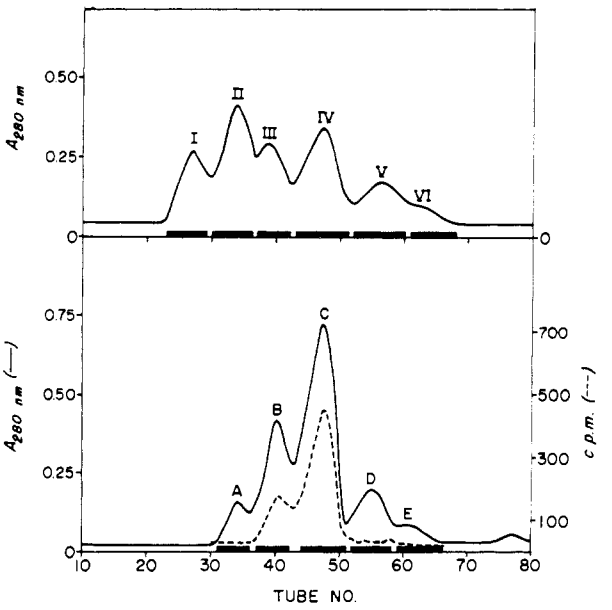

FIGURE 1: Gel filtration of the cyanogen bromide digest of the *S*-pyridylethyl heavy chain of bovine factor X_1 (top). The lyophilized digest (125 mg) was dissolved in approximately 5 ml of 25% aqueous formic acid and applied to a 2.5 × 110 cm column of Sephadex G-50 equilibrated and eluted with 9% formic acid. The flow rate was approximately 35 ml/hr; fractions were collected every 15 min. Fractions were combined as indicated by black bars and peptides were recovered by lyophilization. Gel filtration of the cyanogen bromide digest of the *S*-pyridylethyl heavy chain of [^{32}P]DIP factor X_{1a} (bottom). The digest (60 mg) in 5 ml of 9% formic acid was applied to a 2.5 × 110 cm column of Sephadex G-50 and eluted as above. The column was monitored by absorption at 280 nm (——) and by radioactivity present in 100-μl aliquots (-----).

Cleavage with Cyanogen Bromide. The *S*-pyridylethyl heavy chain of factor X_1 was dissolved in 70% formic acid (20 mg of protein/ml) and solid cyanogen bromide was added to a final concentration of approximately 40 mg/ml. After reaction for 20 hr in the dark at room temperature, the solution was diluted fivefold with water and lyophilized.

For the isolation of the cyanogen bromide peptides from the activated enzyme, 40 mg of the *S*-pyridylethyl heavy chain of factor X_{1a} and 20 mg of the *S*-pyridylethyl heavy chain of [^{32}P]DIP factor X_{1a} (750 × 10^3 cpm) were dissolved in 6 ml of 80% formic acid. Approximately 60 mg of cyanogen bromide was then added and the reaction was allowed to continue overnight in the dark at room temperature. The mixture was then diluted with nine volumes of water and lyophilized.

Tryptic Digestion. *S*-Pyridylethylated fragments of factor X_1 were dissolved in 1% NH_4HCO_3 (pH 8) to form solutions containing 10 mg of peptide/ml. After incubation for 16 hr with porcine trypsin (0.2 mg/ml) at 37°, the digests were lyophilized.

Sequenator Analyses. Sequenator analyses of the proteins and protein fragments were carried out by the method of Edman and Begg (1967) as modified by Hermodson *et al.* (1972).

Detection of Phosphoserine. Radioactive peptides were hydrolyzed at 110° with 2 N HCl in sealed tubes for 10 hr. The hydrolysate was subjected to electrophoresis at pH 2.1 (2000 V, 1 hr) in pyridine–formic acid–water. Radioautographs of the electropherograms were compared to standards of *O*-phosphoserine and *O*-phosphothreonine.

Results

Fragmentation with Cyanogen Bromide. After treatment of the heavy chain of factor X_1 with cyanogen bromide, the products were subjected to gel filtration on Sephadex G-50. Six fractions were separated and numbered consecutively I through VI (Figure 1, top). Similar fractionation of the products obtained from the heavy chain of [^{32}P]DIP factor X_{1a} (750 × 10^3 cpm, 2 μmol) yielded five fractions labeled A–E (Figure 1, bottom). The elution profiles of the cyanogen bromide fragments of the precursor differ from those of the activated protein since during activation of factor X_1 an amino-terminal peptide with a molecular weight of approximately 11,000 (Fujikawa *et al.*, 1972b) is split from the heavy chain. It is also evident from these experiments that most of the ^{32}P radioactivity (520 × 10^3 cpm, approximately 70%) present in the heavy chain of factor X_{1a} appeared in one fraction (C) which eluted from the column in the same position as fraction IV of the precursor. Sequenator analyses of 2 mg of sample from fractions IV and C revealed that fraction IV was a pure peptide containing phenylalanine as the amino-terminal residue, followed by cystine and alanine. Sequenator analyses of fraction C indicated that it was a mixture of two peptides.

Subfractionation of Fraction C. The components of fraction C were further separated by chromatography on SE-Sephadex in 8 M urea, as shown in Figure 2. Three separate peak fractions were obtained, but only one of these (C-3) was radioactive and therefore it represented the fragment containing the active site. Amino acid analysis of fraction C-1 yielded only traces of amino acids; hence this fraction was not further characterized. Sequenator analyses of fraction C-2 and C-3 revealed that each contained a pure peptide. The amino-terminal sequence of C-2 was Ile-Val-Gly- and of C-3, Phe-Cys-Ala-. Fraction C-3 (derived from the activated enzyme had the same amino-terminal sequence and amino acid composition as fraction IV (derived from the precursor) and therefore represented the same region of the heavy chain.

Amino-Terminal Sequences of Fraction C-2 and of the Heavy Chain of Factor X_{1a}. Extended sequenator analyses of fraction

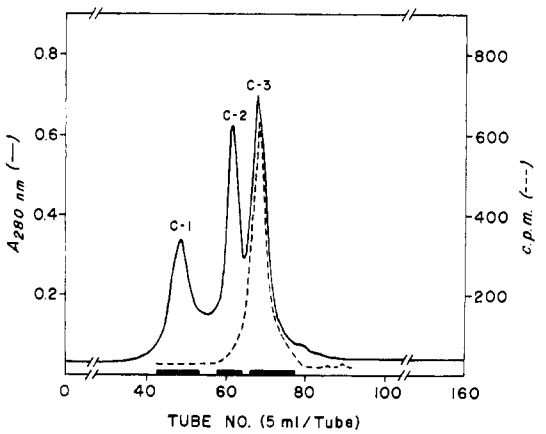

FIGURE 2: Subfractionation on SE-Sephadex of fraction C derived from factor X_{1a} (see Figure 1). The sample (approximately 20 mg, containing 400 × 10^3 cpm) was dissolved in 5 ml of 0.05 M sodium formate containing 8 M urea (pH 2.7) and applied to a 2.0 × 40 cm column of SE-Sephadex C-25 equilibrated at 50° with the same buffer. The column was eluted at 50° with a flow rate of 50 ml/hr. A linear gradient was established from 400 ml of the equilibrating buffer to 400 ml of 1.0 M sodium formate–8 M urea (pH 4.4). The column was monitored by absorption at 280 nm and for radioactivity. Fractions were pooled as indicated by black bars. Peptides were recovered by desalting on a 2.5 × 40 cm column of Sephadex G-25 equilibrated with 9% formic acid and subsequently lyophilized.

TABLE I: Amino-Terminal Sequences of Fragment C-2 of the Heavy Chain of Factor X_{1a} and of Several Other Proteolytic Enzymes.

Enzyme	Amino-Terminal Sequence[a]
	5 10
Bovine factor X_{1a} (fragment C-2)	*Ile-Val-Gly-Gly*-Arg-*Asp-Cys*-Ala-Glu-*Gly*-Glu-Cys-
Bovine trypsin[b]	*Ile-Val-Gly-Gly*-Tyr-Thr-*Cys*-Gly-Ala-Asn-Thr-Val-
Bovine thrombin[c]	*Ile-Val*-Glu-*Gly*-Gln-*Asp*-Ala-Glu-Val-*Gly*-Leu-Ser-
Bovine chymotrypsin A[d]	*Ile-Val*-Asn-*Gly*-Glu-Glu-Ala-Val-Pro-*Gly*-Ser-Trp-
Human plasmin[e]	Val-*Val-Gly-Gly*-Gln-Val-Ala-His-Pro-His-Ser-Trp-
	15 20 25
Bovine factor X_{1a}	-*Pro-Trp-Gln*-Ala-Leu-*Leu*-Val-Asn-Glu-Glu-Asn-Glu-Gly-
Bovine trypsin	-*Pro*-Tyr-*Gln*-Val-Ser-*Leu*-Asn-Ser-Gly-Tyr-His- *[f] *[f]
Bovine thrombin	-*Pro-Trp-Gln*-Val-Met-*Leu*-Phe-Arg-Lys-Ser-Pro-Gln-Glu-Leu-[g]
Bovine chymotrypsin A	-*Pro-Trp-Gln*-Val-Ser-*Leu*-Gln-Asp-Lys-Thr-Gly-Phe-His-
Human plasmin	-*Pro-Trp-Gln*-Val-Val-*Leu*-Leu-Arg-
	30 35
Bovine factor X_{1a}	-*Phe-Cys-Gly-Gly*-Thr-Ile-Leu-*Asn-Glu*-Phe-Tyr-*Val*-
Bovine trypsin	-*Phe-Cys-Gly-Gly*-Ser-Leu-Ile-*Asn*-Ser-Gln-Trp-*Val*-
Bovine thrombin	-Leu-*Cys-Gly*-Ala-Ser-Leu-Ile-Ser-Asp-Arg-Trp-*Val*-
Bovine chymotrypsin A	-*Phe-Cys-Gly-Gly*-Ser-Leu-Ile-*Asn-Glu*-Asn-Trp-*Val*-

[a] Amino acids in italics are homologous with factor X_{1a}. [b] Walsh and Neurath (1964). [c] The B chain of thrombin (Magnusson, 1971). [d] The B chain of α-chymotrypsin (Hartley, 1964). [e] The B chain of plasmin (Robbins *et al.* (1972). [f] Deletions in bovine trypsin correspond to residues 24 and 25 of factor X_{1a}. [g] Leucine represents an apparent insertion in bovine thrombin between residues 25 and 26 of factor X_{1a}.

TABLE II: Amino Acid Sequences of the Sites Which React with DFP in the Heavy Chain of Bovine Factor X_{1a} and in Other Serine Proteases.

Enzyme	Amino Acid Sequence[a]
	170 175
Bovine factor X_{1a}	*Phe-Cys-Ala-Gly-Tyr*-Asp-Thr-Gln-Pro-Glu-
Bovine trypsin[b]	*Phe-Cys-Ala-Gly-Tyr*-Leu-Glu-Gly-Gly-Lys-
Bovine thrombin[c]	*Phe-Cys-Ala-Gly-Tyr*-Lys-Pro-Gly-Glu-Gly-Lys-[d]Arg-[d]Gly-[d]
Bovine chymotrypsin A[e]	Ile-*Cys-Ala- Gly*-*[f]- Ala-Ser-Gly-Val-*[g]
Porcine elastase[h]	Val-*Cys-Ala-Gly*-*[f]- Gly-Asn-Gly-Val-Arg-
	180 185 190
Bovine factor X_{1a}	-*Asp-Ala-Cys-Gln- Gly-Asp-SER-Gly-Gly-Pro*-His-*Val*-Thr-Arg-
Bovine trypsin	-*Asp*-Ser-*Cys-Gln- Gly-Asp-SER-Gly-Gly-Pro*-Val-*Val*-Cys-Ser-
Bovine thrombin	-*Asp-Ala-Cys*-Glu-*Gly-Asp-SER-Gly-Gly-Pro*-Phe-*Val*-Met-Lys-
Bovine chymotrypsin A	-Ser-Ser-*Cys*-Met- *Gly-Asp-SER-Gly-Gly-Pro*-Leu-*Val*-Cys-Lys-
Porcine elastase	-Ser-Gly-*Cys-Gln- Gly-Asp-SER-Gly-Gly-Pro*-Leu-His-Cys-Leu-

[a] Amino acids in italics are homologous with factor X_{1a}. The numbers refer to the amino acid sequence of bovine trypsinogen (Walsh and Neurath, 1964). [b] Walsh and Neurath (1964). [c] The B chain of thrombin (Magnusson, 1971). [d] Lysine, arginine, and glycine are apparent insertions in bovine thrombin between residues 176 and 177 of bovine trypsin. [e] The B chain of α-chymotrypsin (Hartley, 1964). [f] Deletions in bovine chymotrypsin and porcine elastase correspond to residue 171 of bovine trypsin. [g] Deletion in bovine chymotrypsin corresponds to residue 176 of bovine trypsin. [h] Hartley and Shotton (1971).

C-2 and of the heavy chain of factor X_{1a} revealed identical amino-terminal sequences. The analysis was definitive for 37 cycles of fraction C-2 and for 19 cycles of the heavy chain. The 37-residue sequence is shown in Table I and compared with the amino-terminal sequences of several serine proteases.

Sequence of the Active-Site Fragment. In view of the identity of fractions C-3 and IV, they were combined in a ratio of 1:7 and subjected to sequenator analysis. The sequence of the first 24 residues is shown in Table II together with homologous sequences of several serine proteases.

Fractions C-3 (1 mg, 50×10^3 cpm) and IV (12 mg) were combined and digested with trypsin. The products of tryptic digestion (2 μmol) were separated by gel filtration on Sephadex G-25 and 80% of the radioactivity (40×10^3 cpm) was recovered in the fraction associated with the largest peptide (Figure 3). The amino acid composition of this peptide (T-1) was identical with that calculated from the 24 amino-terminal residues of fraction C-3 (Table III). The peptide was obtained in 91% yield (1.83 μmol, specific activity 22×10^3 cpm/μmol).

The tryptic peptide T-1 contained *O*-phosphoserine as

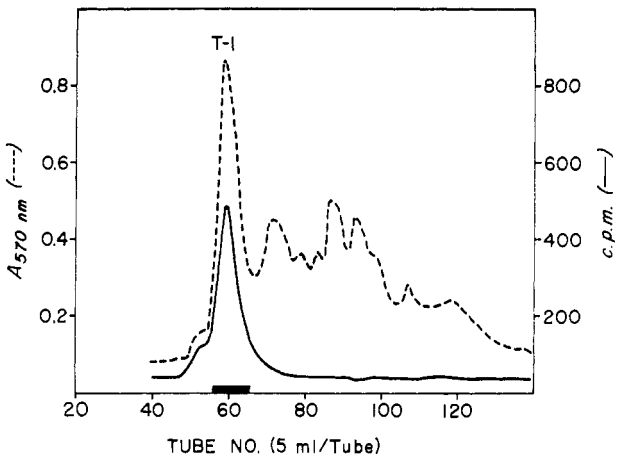

FIGURE 3: Isolation of the radioactive tryptic peptide T-1 from a mixture of fractions C-3 and IV. The tryptic digest (13 mg) was applied to a 2.5 × 100 cm column of Sephadex G-25 equilibrated with 9% formic acid and eluted with 9% formic acid at a flow rate of 50 ml/hr at room temperature. The effluent was monitored for radioactivity and for ninhydrin color at 570 nm after alkaline hydrolysis.

judged by partial acid hydrolysis and radioautographic comparison to standards. Thus the single serine residue in the peptide must be the site of attachment of the [^{32}P]DIP group.

Discussion

The present investigation demonstrates that two segments of the heavy chain of factor X_{1a} are homologous to corresponding regions of bovine trypsin and other mammalian serine proteases. These segments are (1) the amino-terminal region of factor X_{1a} and (2) the amino acid sequence surrounding the site which reacts with DFP.

Comparison of the amino-terminal sequences of bovine factor X_{1a}, trypsin, thrombin, and chymotrypsin A (Table I) demonstrates 9 identical residues among the first 37 (including gaps) and many conservative substitutions among the remaining residues. It is evident that in this region these four serine proteases are homologous. Comparison to human plasmin is limited to the first 20 residues (Table I) but here, too, homology with fragment C-2 of factor X_{1a} is evident.

A higher degree of homology exists in the sequence surrounding the reactive serine (Table II). The short-range sequence Gly-Asp-SER-Gly-Gly-Pro- is identical in bovine factor X_{1a}, trypsin, thrombin, chymotrypsin A, and porcine elastase. In addition, sequence homology extends over a 24-residue segment.

The heavy chain of factor X_{1a} also shares with mammalian serine proteases a characteristic composition of approximately 223–250 amino acid residues. The activation process is also similar and involves in each precursor protein the cleavage of a peptide bond in the amino-terminal portion relative to the active serine. Factor X_{1a} may also contain the "charge-relay system" Asp–His–Ser found in mammalian serine proteases and shares features common to the three-dimensional structures of chymotrypsin, trypsin, and elastase (Blow *et al.*, 1969; Freer *et al.*, 1970; Shotton and Watson, 1970).

The structural and functional significance of the light chain of factor X_{1a} is at present unknown. If this portion of the molecule serves as the recognition site for its specific protein substrate, it may also contain sequences that are homologous to corresponding sequences in other DFP-sensitive blood-clotting enzymes.

TABLE III: Amino Acid Composition[a] of Radioactive Peptide T-1 Isolated from a Tryptic Digest of a Cyanogen Bromide Fragment of [^{32}P]DIP Factor X_{1a}.

Amino Acid	Peptide T-1[b] Found	Peptide T-1[b] Nearest Integer	Active-Site Peptide[c] (First 24 Residues)
Lysine	0.13		
Histidine	0.66	1	1
Arginine	1.12	1	1
PE-cysteine[d]	1.51	2	2
Aspartic acid	3.04	3	3
Threonine	1.95	2	2
Serine	0.89	1	1
Glutamic acid	3.15	3	3
Proline	1.91	2	2
Glycine	4.17	4	4
Alanine	2.21	2	2
Valine	1.18	1	1
Methionine	0		
Isoleucine	0		
Leucine	0		
Tyrosine	0.82	1	1
Phenylalanine	*1.00*	1	1
Total	23.34	24	24
Yield (%)	91		

[a] Values from 24-hr hydrolysates, expressed relative to phenylalanine (taken as 1.00). Tryptophan analyses by the Ehrlich test were negative. [b] A similar peptide was isolated from a tryptic digest of the heavy chain of labeled factor X_{1a}. [c] Taken from Table II. [d] Pyridylethylcysteine.

The process of blood coagulation involves several proteolytic activation reactions which are catalyzed by vitamin K dependent plasma proteins such as thrombin, factor IX_a, and factor X_a (Davie *et al.*, 1969). It will therefore be of interest to determine whether factor IX and perhaps factor VII are homologous with factor X and prothrombin. It has been reported that thrombin is homologous with chymotrypsin (Magnusson, 1971) and the present work extends this homology to factor X_{1a}. It seems quite possible, therefore, that the coagulation and digestion systems have evolved from a common ancestral serine protease.

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Structural Studies on Cartilage Collagen Employing Limited Cleavage and Solubilization with Pepsin†

Edward J. Miller

ABSTRACT: Insoluble cartilage collagen was prepared as the residue from the sternal cartilages of 10-week-old chickens by exhaustive extraction with 1.0 M NaCl at neutral pH and 0.5 M acetic acid. The extraction procedures were totally ineffective in solubilizing cartilage collagen but were useful as a means of removing proteoglycan components of the tissue. Amino acid analyses of the insoluble cartilage collagen residue revealed an amino acid composition closely resembling that of purified α1(II) chains. Characterization of the cyanogen bromide (CNBr) cleavage products derived from insoluble cartilage collagen indicated that they are, for the most part, qualitatively and quantitatively identical with those previously observed in CNBr digests of α1(II) prepared from soluble cartilage collagen. However, two additional CNBr peptides (designated peptides 14 and 15) comprising a total sequence of 21 amino acids derived from a nonhelical region of the cartilage collagen molecule were identified in the present study. Incubation of insoluble cartilage collagen in 0.5 M acetic acid containing pepsin (ratio of collagen: enzyme = 10:1, w/w) at 4° for 18 hr solubilized 60–70% of the collagen. Characterization of the pepsin-solubilized cartilage collagen with respect to chain composition, molecular weight of the component α chains and CNBr cleavage products of the chains indicated that the collagen was solubilized as monomeric molecules of the chain composition, $\{\alpha 1(II)\}_3$, and that the proteolytic activity of pepsin on the native cartilage collagen molecule is confined to relatively short sequences represented by the CNBr peptides, 1, 4, 14, 15, and the COOH-terminal portion of peptide 7. These results indicating that the cited sequences do not participate in collagen helix formation and that they are localized at the extremities of the α1(II) chains comprising the cartilage collagen molecule have been used, in conjunction with additional data on the location of peptides 1 and 4, to establish that the order of the CNBr peptides in the carboxy-terminal region of the α1(II) chain is: 7-14-15. These results further indicate that failure to detect peptides 14 and 15 in the CNBr cleavage products of α1(II) prepared from soluble cartilage collagen resulted from nonspecific proteolytic activity during extraction and purification of the collagen. It is proposed that the mechanism whereby the proteolytic activity of pepsin alters the solubility properties of cartilage collagen involves, at least in part, the degradation of the sequence represented by peptide 4, thus effectively eliminating a site of intermolecular cross-linking known to occur in this sequence.

In recent years, conclusive evidence has been presented indicating that cells of higher organisms possess several structural genes for collagen synthesis. Furthermore, current information indicates that expression of the genes for collagen synthesis is highly selective in certain cell types giving rise to some degree of specificity with respect to the type of collagen molecule found in various tissues. Cartilage collagen, for example, is comprised predominantly of molecules containing three identical α chains, designated α1(II) chains to distinguish them from the α1(I) and α2 chains common to the collagen in several other tissues such as bone, skin, and tendon (Miller and Matukas, 1969). Additional studies have shown that the chain composition of the collagen molecules in a variety of cartilaginous structures may be characterized as $\{\alpha 1(II)\}_3$. These include chick sternal (Trelstad *et al.*, 1970; Miller, 1971a), chick growth plate (Toole *et al.*, 1972), human

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