Isolation and Characterization of Cyanogen Bromide Peptides from the Collagen of Bovine Articular Cartilage

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Significant amounts of native collagen can be extracted from bovine articular cartilage after removal of the acid mucopolysaccharides by controlled proteolysis. The fraction thus solubilized upon denaturation gives rise to three identical α chains. Cleavage of these chains with CNBr generated nine peptides, all of which contain glycine as one-third of their total amino acid residues. Two of the smaller peptides CB-1 and CB-2 contain partially hydroxylated proline. A similar CNBr digest of intact cartilage also gives a series of peptides identical with those obtained from the soluble cartilage collagen. The absence of cross-linking peptides, the fact that only few β components are seen in articular cartilage collagen and the similarity in peptide pattern between the two collagen fractions investigated, suggests that this collagen is stabilized by a different cross-linking mechanism, possibly involving an association with the tissue proteoglycans.

Articular cartilage is a tissue of primary significance because of its involvement in the pathogenesis of joint diseases. Its principal components, collagen and proteoglycans, have defied attempts to investigate them primarily because of difficulties in isolation. The disruptive and dissociative techniques or extraction with denaturing solvents (Sadjara & Hascall, 1969; Rosenberg *et al.*, 1970) yield very low amounts of collagen and in a partially degraded form.

Recently Strawich & Nimni (1971) made use of papain at 4°C to remove significant amounts of glycosaminoglycans from bovine articular cartilage. Subsequent extraction with 0.45 M-NaCl yielded 18–20% of the total collagen in solution. This collagen fraction had all the characteristics of native collagen, contained high amounts of hydroxylysine and glycosidically bound hexoses and on denaturation gave a single component, which migrated like an α_1 chain. Therefore mammalian cartilagenous tissues appeared to have the same unusual features as avian cartilage (Miller & Matukas, 1969; Miller, 1971). The collagen extracted from bovine articular cartilage contained only very few β components and only traces of aldehydic cross-linking precursors. This caused us to think that this collagen may be deficient in the inter- and intra-molecular covalent cross-links which are found in other tissues, and suggested that this collagen may be stabilized in a different way, probably by ionic or covalent interactions with proteoglycans of the ground substance. Since about 80% of the articular cartilage collagen remained insoluble after papain treatment, the possibility could not be ruled out that another type of collagen was also present which cannot be extracted by the above procedures.

We have attempted to isolate and characterize the CNBr peptides of the collagen extracted from bovine articular cartilage by the method of Strawich & Nimni (1971) and compare them with the CNBr peptides of intact cartilage.

Experimental

Extraction of collagen

Articular cartilage from bovine long bones was cleaned, chopped into small pieces (1–2 mm) and incubated with papain at 4°C for 48 h as described by Strawich & Nimni (1971). The insoluble residue was washed with 10 mM-iodoacetic acid to inactivate the enzyme followed by 0.15 M-NaCl, pH 7. To extract collagen this material was homogenized in 0.45 M-NaCl, pH 7, and kept shaking at 4°C for 48 h. The soluble collagen was further purified by successive precipitations with NaCl at acid and neutral pH as described previously (Strawich & Nimni, 1971) and centrifuged at 100 000g for 90 min. The extracted collagen was dialysed against water and freeze-dried.

Cleavage with CNBr

Finely sliced bovine articular cartilage (1–2 mm cuboid pieces) or the freeze-dried soluble collagen preparation were suspended in 70% (v/v) formic acid, N₂ was bubbled through the suspensions and 100–200-fold molar excess of CNBr, relative to the methionyl residues, was added. The reaction mixtures were incubated at 30°C for 4–6 h, diluted with cold water and freeze-dried to remove the excess of CNBr.

The peptide mixture was suspended either in 0.02M-sodium citrate buffer, pH 3.6, or 0.1M-acetic acid and centrifuged at 15000 rev./min for 30 min. The supernatant contained peptides derived from collagen and the residue, mostly proteoglycans.

Chromatography of CNBr peptides on CM-cellulose

The peptide mixture was applied to a CM-cellulose column equilibrated with 0.02M-sodium citrate buffer, pH 3.6, and maintained at 40°C. The elution was carried out with a linear gradient of 0–0.14M-NaCl.

Fractions corresponding to each peak were pooled, freeze-dried and de-salted on a column (1 cm × 100 cm) of Bio-Gel P-2, equilibrated with 0.15M-acetic acid at room temperature.

Molecular sieve chromatography on Bio-Gel P-4

The CNBr digest was passed through a column (1.6 cm × 100 cm) of Bio-Gel P-4 (100–200 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) equilibrated with 0.1M-acetic acid. This procedure was used to separate low-molecular-weight peptides which eluted at the beginning of the CM-cellulose column run and which sometimes caused an overlapping pattern.

Amino acid analysis

The de-salted peptides were hydrolysed with 6M-HCl in sealed ampoules under N₂ at 108°C over-

night. Standard amino acid mixtures were hydrolysed at the same time as the samples and were included in each automatic run of the analyser, thus correcting for any losses due to degradation of amino acids. The hydrolysates were dried and analysed by using a Jeol amino acid analyser. The molecular weights of peptides were calculated from their amino acid content and compared with those obtained by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Determination of hydroxylysine-bound hexoses

The peptides were treated with 0.3M-sodium periodate at room temperature for 5 h as described by Aronson *et al.* (1967). Excess of periodate was removed by passing the solution through ion-exchange resin AG-1 X8 (Bio-Rad Laboratories) and by dialysis against water. The dialysed samples were hydrolysed with 6M-HCl as described above and analysed for hydroxylysine on the amino acid analyser. The hydroxylysine residues that resisted periodate oxidation were considered to be in the O-glycosylated form. Hexoses in these peptides were also determined directly on the individual peptides by the anthrone method (Roe, 1955).

Molecular-weight measurements by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

The method of Weber & Osborn (1969) was used for this purpose. Proteins of different molecular weights and CNBr peptides from α_1 or α_2 chains of

Fig. 1. Elution pattern of CNBr peptides obtained from whole cartilage

The peptide mixture was applied to a column (2.5 cm × 20 cm) of CM-cellulose equilibrated with 0.02M-sodium citrate buffer, pH 3.6, and maintained at 40°C. Elution was carried out with a linear gradient of 0–0.14M-NaCl in the same buffer (1 litre of each) (Deshmukh & Nimni, 1971). The arrow marks the start of elution. The flow rate was adjusted to 100 ml/h. Fractions (5 ml) were collected after monitoring the effluent through a flow cell at 234 nm. The numbered peaks are discussed in the text.

Fig. 2. Elution pattern of CNBr peptides of soluble cartilage collagen fraction

These peptides were solubilized after digestion of proteoglycans with papain and separated on CM-cellulose under the same conditions as described for Fig. 1.

Fig. 3. Separation of CNBr peptides from whole cartilage collagen by using Bio-Gel P-4

Elution was carried out with 0.1M-acetic acid from a column (1.6cm $\times$ 100cm) of Bio-Gel P-4. Fractions (3ml) were collected after the absorbance at 230nm was recorded. The numbered peaks are discussed in the text. The separation for peptides from the soluble collagen fraction is not shown but gave the same components.

rat skin acid-soluble collagen were used as reference standards (see Fig. 4). Molecular weights of individual peptides of untreated cartilage or the cartilage collagen were determined from a standard curve.

Results

The elution patterns of CNBr peptides of soluble collagen and untreated cartilage after chromatography on the CM-cellulose column are shown in Figs. 1 and 2. The elution of the respective peptides in both cases is similar, although the separation of

peptides eluting in the latter part of the chromatogram is better in the case of soluble collagen. In some runs, where peptides 1-4 eluted as diffused peaks, better separation was achieved on phosphocellulose (Deshmukh & Nimni, 1971) or Bio-Gel P-4 columns. The separate identity of peptides 1, 2, and 3 was confirmed by running a portion of the CNBr-peptide mixture on a Bio-Gel P-4 column (Fig. 3). The large peptides eluted as one peak at the beginning of the run, whereas the small-molecular-weight peptides separated very well. The purity of all the peptides was checked by polyacrylamide-gel electrophoresis and repeated amino acid analysis of the peaks from different runs on CM-cellulose and Bio-Gel P-4 columns.

The amino acid compositions of all the peptides originating from soluble collagen or the untreated cartilage are given in Tables 1 and 2. Amino acid composition of peptides 1, 2, and 3 obtained by chromatography on the Bio-Gel P-4 column was more reproducible and the peptides were purer than those obtained from CM-cellulose. The identity of the peptides when obtained by both procedures was evident by amino acid analysis as well as by their behaviour during rechromatography of the initial peptides from CM-cellulose on Bio-Gel P-4. The relative proportions of peptides 1, 2, and 3 obtained by these two procedures, as well as from one run to another, were not always stoichiometric; the reason for this variability is not clear. As is evident from the results, the total of the individual amino acids in all the peptides is close to the amino acid composition of α_1 (II) chains as reported by Strawich & Nimni (1971). Glycine comprises one-third of the total amino acids in each of the peptides, suggesting that all these peptides originated from the helical region. In the case of peptides 1 and 2, proline residues were partially hydroxylated and the rate of hydroxylation

Table 1. *Amino acid composition of CNBr peptides of a soluble collagen fraction obtained from bovine articular cartilage after removal of the proteoglycans with papain*

The values in parentheses represent the means of three separate runs. The nearest integers are shown and were used to estimate the peptide size. Peptides 1, 2, and 3 were obtained from Bio-Gel P-4 chromatography, whereas the rest were collected during CM-cellulose chromatography.

Amino acid	Amino acid composition											Total	α_1 (II)*
	Peptide ...	1	2	3	4	5	6	7	8	9			
Hydroxyllysine	—	—	—	1 (0.9)	4 (4.1)	1 (1.0)	4 (4.1)	5 (4.9)	5 (4.8)	4 (4.2)		24	28
Lysine	—	—	—	—	3 (2.7)	2 (2.1)	3 (3.2)	2 (1.9)	4 (4.0)	2 (2.1)		16	15
Histidine	—	—	—	—	1 (0.9)	—	1 (1.0)	1 (0.8)	2 (2.1)	1 (0.9)		6	6
Arginine	—	—	—	—	—	—	6 (5.8)	8 (7.9)	14 (13.7)	8 (7.5)		49	46
Hydroxyproline	—	—	—	2 (1.8)	6 (5.7)	5 (4.9)	14 (13.7)	16 (16.2)	16 (16.2)	13 (13.2)		92	95
Aspartic acid	0-1 (0.6)	1 (1.0)	0-1§ (0.6)	3 (3.1)	20 (20.4)	10 (10.3)	8 (8.3)	6 (6.0)	8 (7.6)	6 (5.8)		46	49
Threonine	1 (0.9)	—	2 (1.8)	1 (1.0)	8 (7.8)	6 (5.8)	2 (2.0)	3 (2.9)	4 (3.5)	2 (2.1)		17	21
Serine	—	—	—	—	4 (3.9)	2 (1.9)	3 (3.1)	4 (3.8)	4 (4.1)	2 (1.9)		21	27
Glutamic acid	1 (0.9)	—	—	1 (1.0)	4 (3.9)	2 (2.0)	12 (12.4)	12 (12.4)	13 (12.7)	8 (8.0)		76	80
Proline	3 (2.9)	—	2 (2.1)	3 (3.2)	16 (15.9)	7 (6.7)	18 (17.8)	18 (17.5)	20 (19.5)	13 (13.2)		116	114
Glycine	4 (4.1)	—	2 (2.1)	5 (4.9)	22 (22.2)	12 (12.2)	50 (49.5)	54 (54.4)	63 (63.4)	43 (42.5)		332	329
Alanine	8 (8.2)	—	5 (4.8)	11 (10.7)	63 (62.5)	35 (35.3)	19 (19.4)	16 (15.7)	20 (20.0)	15 (14.7)		112	103
Valine	3 (3.1)	—	2 (2.1)	5 (5.2)	20 (20.1)	12 (11.7)	4 (3.8)	4 (4.2)	5 (4.8)	2 (1.9)		21	20
Isoleucine	—	—	1 (1.0)	1 (1.0)	2 (1.9)	2 (1.9)	2 (2.1)	2 (2.1)	2 (2.2)	1 (1.1)		10	12
Leucine	—	—	—	—	2 (1.9)	1 (0.8)	2 (2.1)	4 (3.7)	6 (6.3)	3 (3.2)		27	26
Tyrosine	1 (0.9)	—	—	—	4 (3.8)	5 (4.6)	4 (3.9)	—	1 (0.9)	—		2	2
Phenylalanine	—	—	—	—	1 (1.1)	—	—	—	3 (2.7)	1 (1.0)		12	15
Homoserine†	—	—	—	—	2 (2.0)	2 (1.6)	2 (2.1)	2 (1.8)	1	1		8	8-10†
Total	1	1	1	34	183	105	153	157	191	125		987	

* From Strawich & Nimni (1971).

† Represents methionine residues.

‡ Includes homoserine lactone.

§ Variable amounts of hydroxyproline (average 0.6 residues/peptide).

Table 2. *Amino acid composition of collagen CNBr peptides obtained from intact cartilage*

The values in parentheses represent the means of three separate runs. The nearest integers are shown and were used to estimate the peptide size. Peptides 1, 2, and 3 were obtained from Bio-Gel P-4 chromatography, whereas the rest were collected during CM-cellulose chromatography.

Amino acid	Peptide ...	Amino acid composition										Total	α_1 (II)*
		1	2	3	4	5	6	7	8	9			
Hydroxylysine	—	—	—	1 (0.8)	4 (3.8)	1 (1.0)	4 (4.0)	5 (5.1)	5 (4.8)	5 (4.9)		25	28
Lysine	—	—	—	—	3 (3.1)	1 (1.1)	3 (2.8)	2 (2.0)	4 (3.7)	2 (2.0)		15	15
Histidine	—	—	—	—	1 (1.1)	1 (0.9)	1 (0.9)	—	2 (2.0)	1 (1.0)		6	6
Arginine	—	—	—	2 (1.9)	6 (5.7)	5 (4.8)	7 (6.8)	7 (6.7)	14 (13.9)	7 (6.7)		48	46
Hydroxyproline	Trace	Trace	Trace	2 (2.0)	20 (19.5)	9 (8.7)	14 (13.7)	16 (15.5)	14 (14.2)	14 (14.2)		89	95
Aspartic acid	1 (0.9)	2 (1.8)	2 (2.1)	8 (7.7)	4 (3.6)	4 (3.6)	7 (6.5)	6 (6.1)	8 (8.1)	6 (6.1)		44	49
Threonine	—	—	—	4 (3.8)	2 (2.1)	3 (2.9)	3 (2.9)	3 (3.2)	4 (3.9)	2 (2.0)		18	21
Serine	1 (0.8)	1 (0.9)	1 (1.0)	4 (4.1)	2 (2.0)	3 (3.1)	3 (3.1)	4 (4.2)	3 (2.9)	2 (1.9)		21	27
Glutamic acid	4 (3.8)	3 (3.1)	2 (1.9)	15 (15.2)	7 (6.7)	9 (8.8)	12 (12.3)	13 (12.6)	20 (20.4)	8 (7.8)		73	80
Proline	5 (4.7)	3 (2.8)	5 (4.8)	20 (20.3)	13 (12.9)	19 (18.7)	17 (16.6)	17 (16.6)	20 (20.4)	14 (13.9)		116	114
Glycine	7 (6.9)	5 (4.7)	11 (10.7)	65 (64.5)	37 (36.6)	48 (48.4)	54 (53.7)	65 (65.2)	65 (65.2)	43 (42.7)		335	329
Alanine	3 (3.1)	1 (0.9)	4 (3.7)	18 (18.2)	16 (16.3)	20 (20.2)	16 (15.9)	16 (15.9)	20 (19.5)	15 (14.5)		113	103
Valine	—	1 (1.0)	1 (0.8)	2 (1.9)	2 (1.8)	4 (3.6)	3 (2.8)	3 (2.8)	2 (1.8)	3 (3.0)		18	20
Isoleucine	—	—	—	2 (2.1)	1 (0.9)	2 (1.8)	2 (1.8)	2 (2.1)	3 (3.1)	1 (0.9)		11	12
Leucine	1 (1.0)	1 (1.1)	—	4 (4.0)	5 (5.1)	4 (3.9)	4 (4.0)	4 (4.0)	6 (6.0)	4 (3.8)		29	26
Tyrosine	—	—	—	1 (1.0)	—	—	—	—	1 (0.9)	—		2	2
Phenylalanine	—	—	—	2 (1.9)	2 (1.7)	2 (2.1)	2 (2.1)	2 (1.9)	3 (3.1)	2 (1.8)		13	15
Homoserine†	1	1	1	1	1	1	1	—	1	1		8	8-10†
Total	23	18	32	180	109	151	153	153	188	130		984	

* From Strawich & Nimni (1971).

† Represents methionine residues.

‡ Includes homoserine lactone.

seemed greater in the soluble collagen fraction. The stoichiometric recovery of peptides was quite adequate except as mentioned for peptides 1, 2, and 3, which because of unpredictable relative variations became hard to ascertain.

Table 3 describes the total content of hydroxylysine, *O*-glycosidically bound hydroxylysine (stable after oxidation by sodium periodate) and the total hexose content of the peptides from soluble collagen and whole cartilage. From the number of periodate-stable hydroxylysine residues and hexose residues, it was possible to estimate the amounts of disaccharide units. Peptides 1, 2, and 3 were devoid of hexose residues.

The molecular weights of individual peptides calculated from the amino acid analyses and by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis are given in Table 4. Fig. 4 represents the standard curve of molecular weights with different proteins and CNBr peptides of α_1 and α_2 chains of rat skin acid-soluble collagen as reference standards.

Discussion

Collagen in articular cartilage proved to be difficult to work with because of its insolubility. To solubilize significant amounts of this material, we were forced to enzymically cleave the cartilage proteoglycans (Strawich & Nimni, 1971). The lack of any intramolecular cross-links in the collagen solubilized after treating the articular cartilage with papain at

Fig. 4. Standard curve comparing the molecular weights of different proteins and CNBr peptides of α_1 and α_2 chains of rat skin acid-soluble collagen with their electrophoretic mobilities on sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (10% acrylamide)

Standards (mol.wt.) are: A, ovalbumin (45 000); B, peptide α_2 CB₃ (30 600); C, chymotrypsinogen (25 000); D, peptides α_1 CB₇ and α_1 CB₈ (24 000); E, lysozyme (14 400); F, peptide α_1 CB₃ (13 600); G, ribonuclease A (13 700).

Table 3. Peptides derived from acid-hydrolysed collagen analysed for their hydroxylysine content before and after treatment with sodium metaperiodate

The hydroxylysine residues which resisted this treatment are considered to be involved in glycosidic linkage. Hexoses were determined in the untreated peptides. The values in parentheses correspond to the numbers of disaccharide units/peptide.

CNBr peptides	Hydroxylysine and hexose content (mol/mol of peptide)					
	Whole cartilage collagen			Soluble collagen fraction		
	Hydroxylysine			Hydroxylysine		
	Total	Periodate stable	Hexose	Total	Periodate stable	Hexose
1	—	—	—	—	—	—
2	—	—	—	—	—	—
3	1	—	—	1	—	—
4	4	2	2	4	2	2
5	1	1	2 (1)	1	1	2 (1)
6	4	3	4 (1)	4	4	6 (2)
7	5	4	6 (2)	5	3	4 (1)
8	5	2	3 (1)	5	2	4 (2)
9	5	5	7 (2)	4	4	7 (3)

Table 4. *Molecular-weight determinations of CNBr peptides of whole cartilage collagen and soluble collagen fraction*

Owing to the small sizes of peptides 1–3, their molecular weights could not be determined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Nevertheless, their molecular weights estimated by amino acid analysis are in agreement with their elution profile from Bio-Gel P-2.

Peptide	Molecular weight			
	Whole cartilage collagen		Soluble collagen fraction	
	Amino acid analysis	Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis	Amino acid analysis	Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis
1	2443		2387	
2	1988		1710	
3	3429		3663	
4	19497	19500	19902	19500
5	11684	12000	11451	11000
6	16487	16000	16714	15800
7	16426	15700	17212	16000
8	20931	19000	20982	19800
9	14257	14000	13787	13000
Total	107142		107808	

4°C raised the possibility that terminal non-helical regions of the molecule were being cleaved. This suspicion remained even though papain under similar experimental conditions was not able to remove the non-helical portions of insoluble rat skin collagen fibres (Strawich & Nimni, 1971). The CNBr-peptide patterns obtained from the soluble collagen fraction extracted from cartilage pretreated with papain and from intact cartilage do not show any qualitative differences. There are some minor quantitative differences, as can be seen by a decrease in the amounts of peptides 3 and 7 in whole cartilage. The smaller peptides 1 and 2 showed partial hydroxylation of their proline residues, thus causing the average hydroxyproline/peptide to be less than 1 residue. Another unusual feature relates to the glycine content. All peptides studied showed one-third of the residues as glycine, suggesting that they may all be in the triple-helical form. The absence of non-helical peptides lacking hydroxyproline, which is typical of collagens of the $(\alpha_1)_2\alpha_2$ type, may be a reflexion of its unusual function and location. In this connexion it is of significance that collagen isolated from basement membrane (Kefalides, 1972), which resembles cartilage collagen in that it also comprises three identical α chains, also gives peptides that contain one-third glycine and variable amounts of hydroxyproline distributed along the whole length of the molecule.

The molecular weights of the smaller peptides were estimated by their amino acid composition. The

larger peptides showed a good agreement between estimates based on amino acid analysis and on their mobility on sodium dodecyl sulphate electrophoresis. The total molecular weight based on the summation of the individual peptide components is 107000. Hexoses (24–25 residues/1000) are absent from the smaller peptides CB-1, CB-2 and CB-3 but are present in glycosidic linkage to hydroxylysine in all other peptides. The soluble collagen fraction, which represents 20% of the total cartilage collagen, and the residual collagen show essentially similar amounts and distribution of bound hexoses.

The present studies thus fail to reveal any differences between the collagen fraction that can be extracted from articular cartilage following papain treatment and the total collagen present in the tissue. Although at this stage these experiments fail to shed any light on the subject of cross-linking, since the peptide patterns from both types of collagen are essentially identical, losses of collagen peptides after CNBr treatment suggest that some peptides may be covalently associated with acidic polysaccharides.

The solubility of cartilage collagen after papain treatment, the absence of intramolecular cross-links or other types of conventional cross-linked intermediates, together with the identity of the two collagen fractions under consideration, suggest that the cross-linking in cartilage may be essentially different from that of other connective tissues and may involve a unique type of association of this collagen with the proteoglycans.

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