Collagen of fibrocartilage: A distinctive molecular phenotype in bovine meniscus

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Fibrocartilage of bovine knee meniscus was analyzed for major and minor collagen constituents. The main fraction (~98%) of pepsin-solubilized collagen consisted of type I with a small proportion (< 10%) of type III molecules. The minor fraction (1–2%) isolated by salt precipitation could be further resolved into type V collagen that consisted of α1(V) and α2(V) chains and a type II-like molecule with chains that had all the characteristics of the 3α variant of α1(II) found in hyaline cartilage. The articular surface zone of the meniscus appeared 2–3-fold enriched in these minor collagens compared with deeper tissue, though qualitatively the same distinctive collagen phenotype was evident throughout.

Bovine *Cartilage* *Collagen* *Fibrocartilage* *Meniscus*

1. INTRODUCTION

The collagen phenotype of hyaline cartilage differs completely from that of other connective tissues. Not only is the bulk type II collagen a distinct genetic species, but also the minor collagens (the 1α, 2α and 3α chains [1] and type M collagen [2]) appear specific for cartilage. The collagen phenotype of fibrocartilage has not been studied in depth, though basically it seems to resemble that of skin, bone or tendon, rather than hyaline cartilage, in having type I collagen as its main constituent [3].

This study set out to identify minor collagen species that might be present in the semilunar meniscus of the knee-joint. By histology this tissue is a typical fibrocartilage, though it resembles articular cartilage in surface appearance, mechanical function and in some details of its proteoglycan chemistry [4,5]. It was of interest to determine whether any of the characteristic collagen species of hyaline cartilage were present in fibrocartilage.

Abbreviations: CNBr, cyanogen bromide; TFA, trifluoroacetic acid; EDTA, ethylenediamine tetraacetic acid

2. MATERIALS AND METHODS

Medial and lateral menisci, femoral articular cartilage and cortical bone were dissected from steer knee (stifle) joints bought from a meat distributor. The menisci were liberally trimmed of both ends to avoid ligament and of their outer third of vascularized tissue. Whole menisci were diced (~1 mm) for collagen preparation in bulk by pepsin digestion. Cross-sections (~1 mm) were also cut from which the articulating surfaces (< 0.5 mm deep) and deep tissue were separately sampled to assess site-related variations in collagen composition.

2.1. *Extraction of collagen*

Tissue was stirred with 4 M guanidine–HCl, 0.05 M Tris (pH 7.5) for 24 h at 4°C to remove proteoglycans. Only a small amount of soluble type I collagen was detected by electrophoresis of material in this extract. Residues were washed with water, equilibrated and homogenized (Polytron) in 3% acetic acid and digested with pepsin (1 : 10 of dry tissue wt) for 24 h at 4°C [6]. Insoluble material was sedimented and the separated super-

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natant was adjusted to 0.7 M with solid NaCl [7]. Precipitated collagen was recovered by centrifugation, dialysed against 0.1 M acetic acid and freeze-dried. [NaCl] in the supernatant was increased serially to 1.2 M and 2.0 M, the precipitates being recovered at each step [1,2,7]. Similar fractions were prepared from cortical bone (ground in liquid N_2 and decalcified by 0.5 M EDTA), articular cartilage and fetal calf dermis.

The 1.2 M fraction from meniscus was further fractionated by dissolving the protein in 3% acetic acid at 4°C and serially adding NaCl to 0.7 M, 0.9 M, then 1.8 M, collecting the precipitates by centrifugation. Less than 3% of the material precipitated at 0.7 M, about 2/3 precipitated at 0.9 M and 1/3 at 1.8 M.

2.2. *Electrophoresis*

Samples were run in SDS–polyacrylamide slab gels as in [8], staining with Coomassie blue R250. For CNBr digestion and amino acid analysis of isolated collagen α-chains, 15-cm sample streaks were run on a 5% gel; individual α-bands were visualized by soaking the gel in 1 M KCl [9], cut out with a razor blade, washed in 50% methanol and, for amino acid analysis, hydrolyzed in 6 M HCl for 24 h at 108°C or, for peptide mapping, digested with CNBr in 70% formic acid [10].

2.3. *Chromatography*

Samples of pepsin-solubilized collagen were also fractionated by high pressure liquid chromatography (HPLC) on a Brownlee Aquapore RP 300 column (4.6 mm × 25 cm). Collagen samples (200 μg) were heat denatured in 1% trifluoroacetic acid (TFA) and eluted at 1 ml/min with a gradient of 20–23% B in 4 min then 23–32% B in 50 min (A = 0.1% TFA in water; B = 0.085% TFA in acetonitrile–propanol, 3:1, v/v). Fractions (2 ml) were collected, freeze-dried and analyzed by electrophoresis.

3. RESULTS

Fig.1 compares the results of electrophoretic analysis of the various fractions of pepsin-solubilized collagen. Type I collagen predominated in the 0.7 M NaCl precipitate which accounted for about 98% of the solubilized meniscus collagen. After reduction with dithiothreitol, some α1(III) was evident running just above α1(I) (fig.1a, 0.7 M lane) in the position of the α1(III) chain from embryonic bovine skin (not shown). Densitometry measured type III at no more than 10% of the total collagen in the 0.7 M NaCl fraction. The 1.2 M NaCl precipitate showed 3 prominent bands in the α-region of the gel (fig.1). The slower two had identical mobilities to the α1(V) and α2(V) chains that are abundant in 1.2 M NaCl fractions prepared from skin, bone (fig.1b) and many other connective tissues. The third, prominent band ran ahead of α2(V) with a mobility slightly slower than that of α1(I) or α1(II), and in the position of the

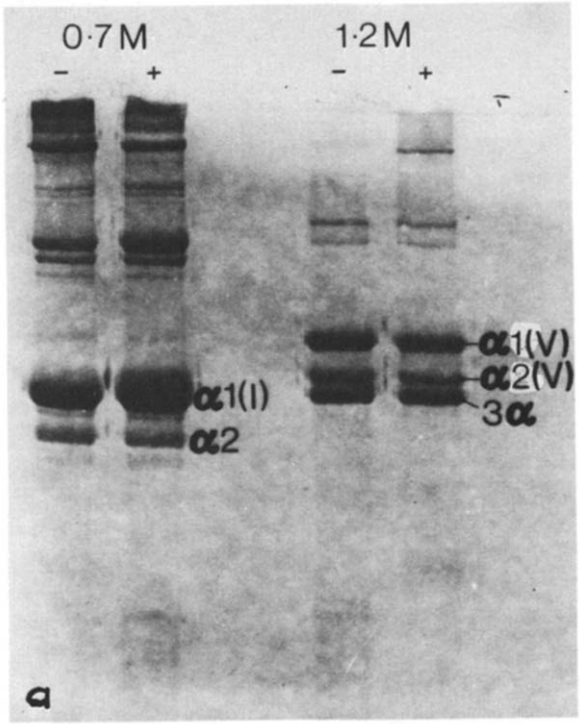
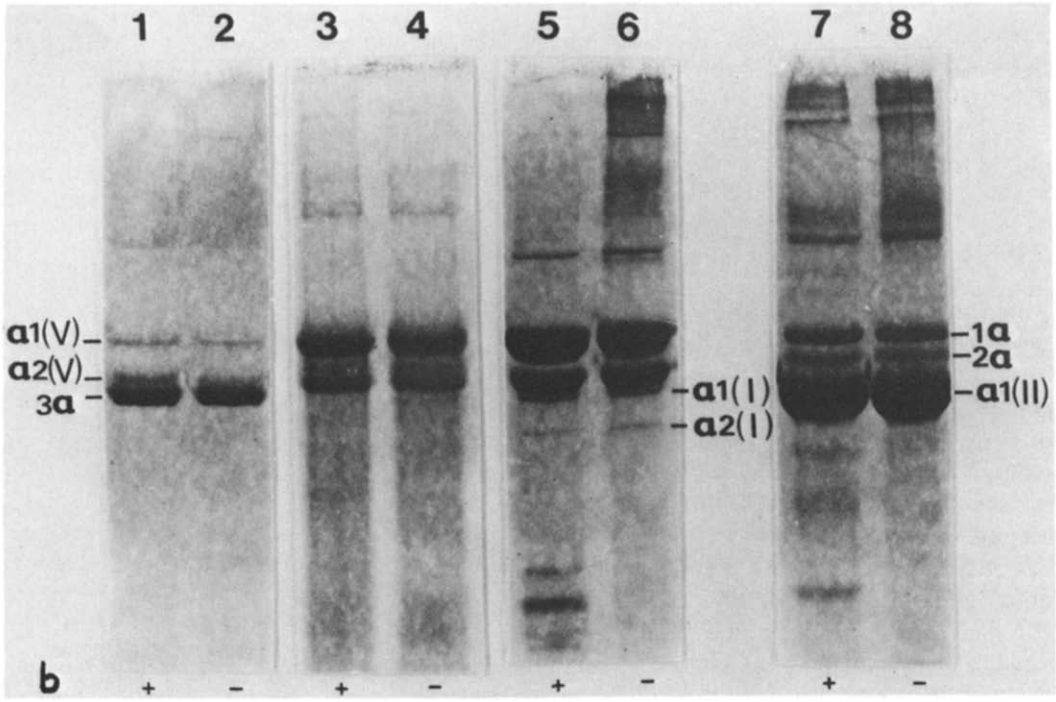
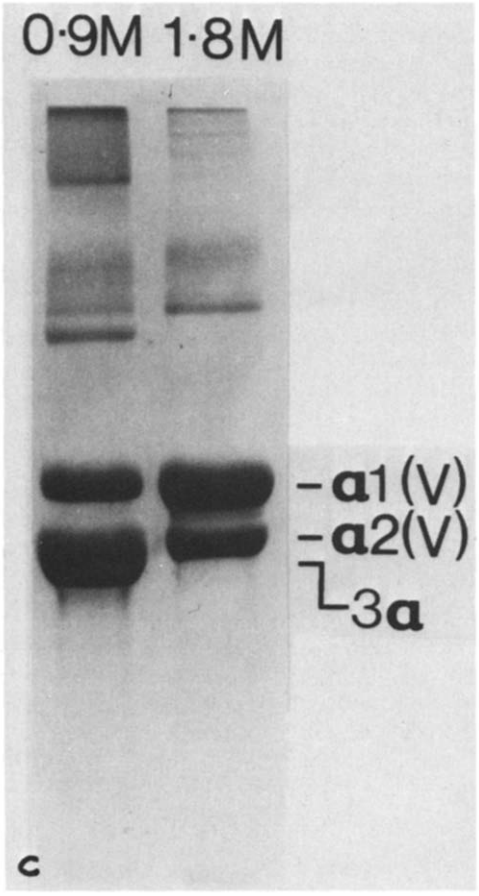


Fig.1. Electrophoresis in SDS–5% polyacrylamide of collagen fractions from bovine meniscus compared with minor collagen preparations from other tissues. (a) Pepsin-solubilized meniscus collagen fractions precipitating at 0.7 M and 1.2 M NaCl, pH 2.6 with (+) and without (−) reduction by dithiothreitol. (b) The 1.2 M NaCl fractions prepared from bovine meniscus [1,2], bone [3,4], skin [5,6] and articular cartilage [7,8]. Samples in lanes 1, 3, 5 and 7 were reduced with dithiothreitol, samples in lanes 2, 4, 6 and 8 were unreduced. (c) Sub-fractions separated from the 1.2 M NaCl preparation by reprecipitation from dilute acetic acid using 0.9 M NaCl and 1.8 M NaCl.





3α chain from articular cartilage [1]. This chain was not present in the 1.2 M fractions from skin and bone (fig.1b). The ratio of α1(V) to α2(V) in different preparations of meniscus collagen was constant at about 2:1. The ratio of the type V chains to 3α showed some variability between different bulk preparations, though 3α usually predominated. Further analysis showed that the native molecules of type V collagen, of composition [α1(V)]$_2\alpha$2(V), could be resolved from the native 3α variant of type II collagen by sequential precipitation from dilute acetic acid at 0.9 M and 1.8 M NaCl (fig.1c). Some type V chains are still evident in the 0.9 M fraction but the 1.8 M fraction shows only α1(V) and α2(V) bands at a 2:1 ratio.

Electrophoretic analysis of CNBr-digests of the whole 1.2 M NaCl fraction from meniscus, and of the 3α-like chain isolated from the gel, showed a peptide map basically typical of α1(II) (fig.2). The amino acid composition of the excised band was also typical of α1(II), but with a 25% higher content of hydroxylysine compared with α1(II) from articular cartilage (table 1). The presence of the type II-like molecules in the 1.2 M fraction and not in the 0.7 M fraction, and the slower elec-

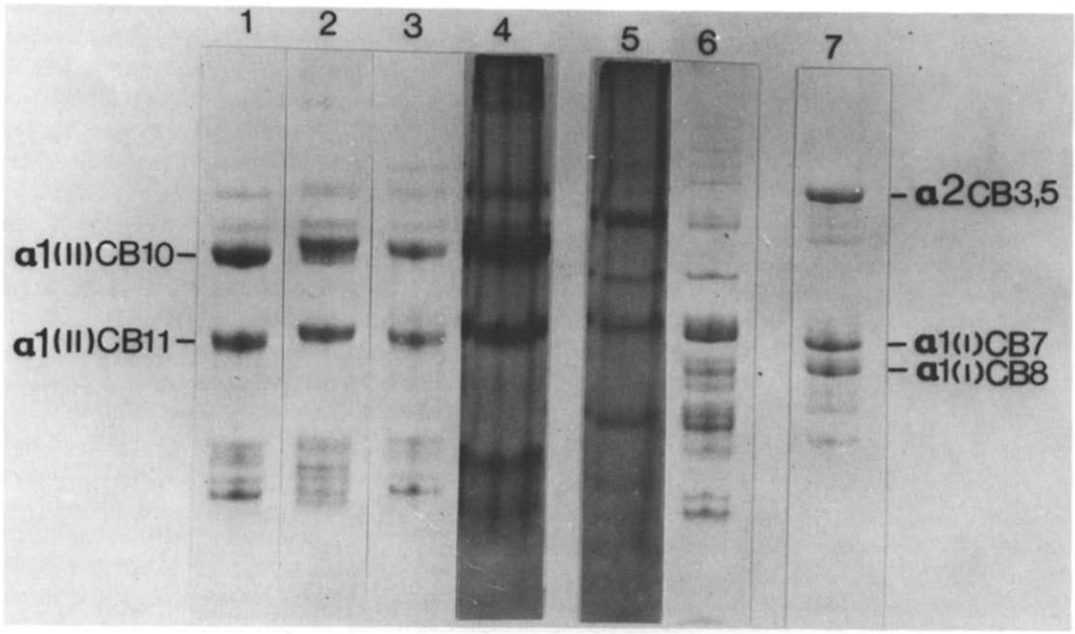


Fig.2. SDS–10% polyacrylamide electrophoresis of CNBr-digests of collagen preparations from bovine meniscus: (1) type II collagen from bovine articular cartilage; (2) 3α from bovine articular cartilage; (3) 1.2 M NaCl fraction from bovine meniscus; (4) meniscus 3α chain isolated by electrophoresis of the 1.2 M NaCl fraction; (5) meniscus α1(V) chain isolated by electrophoresis of the 1.2 M NaCl fraction; (6) type V collagen from bovine skin (1.2 M NaCl fraction); (7) type I collagen from bovine meniscus (0.7 M NaCl fraction).

Table 1
Partial amino acid compositions of 3α and α1(V) collagen chains from adult bovine meniscus

	Residues/1000 residues				
	Meniscus		Articular cartilage[a]		Placenta[b]
	3α	α1(V)	3α	α1(II)	α1(V)
3-Hyp	1	3	2	1–2	3
4-Hyp	104	96	104	104	109
Thr	25	23	21	21	19
Ser	26	39	26	26	26
Gly	326	328	332	333	322
Ala	118	63	100	100	46
Ile	11	21	9	10	19
Leu	28	39	25	25	39
Tyr	3	6	2	2	2
Phe	14	12	13	13	12
Hyl	20	32	21	17	35
His	3	6	3	3	8
Arg	50	38	52	50	50

[a] Adult bovine articular cartilage
[b] Human placenta; data from [13]

trophoretic mobilities of both the intact chain and its major CNBr-peptides are properties in common with the 3α collagen fraction of hyaline cartilage [1]. Even overloading a gel with a CNBr digest of the 0.7 M NaCl fraction failed to reveal α1(II)-peptides, indicating that essentially all the type II-like collagen in meniscus had behaved as 3α by precipitating at 1.2 M NaCl. With hyaline cartilage under these conditions, 96% of the type II collagen precipitates at 0.7 M NaCl [2,7].

The identity of the α1(V) chain was confirmed by its CNBr-peptide profile (fig.2) and its amino acid composition (table 1) determined on the electrophoretic band excised from the gel. The electrophoretic CNBr-peptide profile of the type V collagen, purified from the 1.2 M NaCl fraction by salt fractionation at 1.8 M NaCl, was essentially identical to that of type V collagen prepared from skin or bone and quite dissimilar to peptide profiles of 1α and 2α chains isolated from hyaline cartilage (not shown). Reverse-phase HPLC also resolved the 3α chain from α1(V) and α2(V) (fig.3), and the latter two chains eluted identically

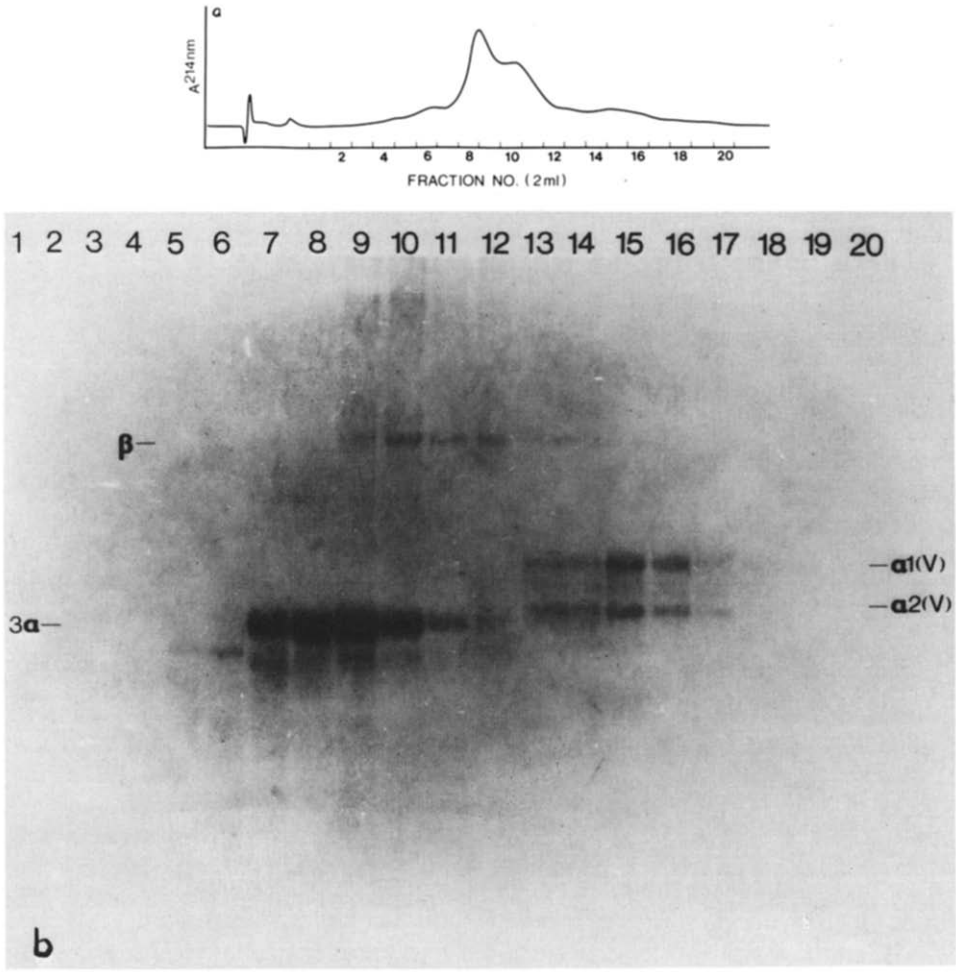


Fig.3. Reverse-phase high-performance liquid chromatography of the minor collagen fraction (1.2 M NaCl) from bovine meniscus: (A) elution profile; (B) SDS–5% polyacrylamide electrophoresis of collected fractions.

to α1(V) and α2(V) from bovine skin or bone and distinct from 1α and 2α chains of hyaline cartilage (not shown).

The 2 M NaCl fraction of the initial pepsin digest contained only traces of collagen, which apparently was mostly an overspill of the collagen species seen in the 1.2 M NaCl fraction (not shown). Bands characteristic of type M collagen, which is prominent in this fraction from young hyaline cartilage [2], were not evident.

The articulating surface zone of meniscus yielded more collagen in the 1.2 M fraction than did the meniscus interior, about 2.5% compared with 1% of the total pepsin-solubilized collagen. In the surface zone the ratio of type V to 3α chains also appeared higher than in deep tissue. Nevertheless, the qualitative composition of collagens in surface and deep regions of meniscus was similar, with type I, III, V and 3α species all present.

4. DISCUSSION

The results confirm that type I is the predominant collagen of meniscus fibrocartilage [3]. The small proportion of type III collagen must be a true matrix constituent and not derived from blood vessels since vascularized regions were avoided. The more soluble, minor fraction of meniscus col-

lagen, containing type V and a type II-like molecules, seems a unique combination that may be a distinctive feature of fibrocartilage. Type V is the ubiquitous minor collagen of bone, skin and other connective tissues in which type I collagen prevails [7]. The 3α chain was originally identified together with 1α and 2α chains in hyaline cartilage, where the bulk of the collagen is type II. The 1α, 2α and 3α chains have also been found in annulus fibrosus [11], which is a unique cartilage that incorporates both types I and II collagens in its fibrous structure [12].

The 3α variant of type II collagen seems to be the one collagen constituent that the different kinds of cartilage (annulus fibrosus, meniscus and hyaline cartilage) have in common, with meniscus distinguished by its lack of conventional type II collagen. Whether 3α proves to be a genetically distinct variant of type II collagen or is an example of post-translational microheterogeneity, it may have a special function in cartilaginous tissues, for instance in mediating interactions between the collagen network and proteoglycans. Meniscus, annulus fibrosus and hyaline cartilage all include a cartilage class of proteoglycans that are rich in chondroitin sulphate [4], and that aggregate specifically with hyaluronic acid [5]. An important step in understanding the significance of the various minor collagen species will be to define their ultrastructural distribution in the matrix, including their spatial relationships with types I and II collagen fibrils, with proteoglycans, and with the chondrocytes.

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