ISOLATION AND CHARACTERISATION OF AN UNUSUAL COLLAGEN FROM HYALINE CARTILAGE AND INTERVERTEBRAL DISC

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Received 10 November 1980

1. Introduction

The major collagen of hyaline cartilage is type II collagen, consisting of 3 identical α1(II) chains [1,2]. However, additional minor collagenous components have been observed (but not identified) in chick scleral cartilage [3] and chick embryonic limb cartilage [4], and the αB (or α1(V)) chain of type V collagen was reported in epiphyseal cartilage [5]. In [6], 3 collagen α-sized chains from human hyaline cartilage were isolated and partially characterised. Two of these chains designated 1α and 2α, were very similar to the αB (α1(V)) and αA (α2(V)) chains, respectively, of type V collagen but were shown to be genetically distinct by several physicochemical criteria. The third chain 3α, however, resembled the α1(II) chain of type II collagen and could be a post-translational modification of this chain.

We have studied the collagens of bovine nasal cartilage and human intervertebral disc and have identified the 1α, 2α and 3α chains in addition to type II collagen in both tissues. However, we have never observed the αI(V) chain which we believe originates only from contaminating perichondrium. We have also isolated an unusual collagen which is different from any other collagen reported in cartilage or disc. This report describes its isolation and characterisation.

2. Materials and methods

Bovine nasal cartilage was freed from adhering perichondrium and fragmented in a stainless steel bomb under liquid nitrogen. The powdered tissue was extracted with 2 M $MgCl_2$, 50 mM Tris–HCl (pH 7.4) at 4°C for 48 h to remove proteoglycans, washed with distilled water and incubated in 0.5 M acetic acid (pH 2.8) with pepsin (E:S, 1:50) for 48 h at 4°C. The pepsin digest was clarified by centrifugation at $1.8 \times 10^5 \times g$-min and the various collagenous components isolated as shown in fig.1. The cartilage phosphate precipitate (C-PP) contained the 1α, 2α, 3α and traces of the α1(II) chains and the phosphate supernatant (C-PS) contained the unusual collagen fraction investigated here.

The nucleus and annulus from human lumbar intervertebral discs were examined separately and the C-PP and C-PS fractions observed in both. With the exception of the electron microscopic and amino acid analyses which have not yet been undertaken, the results described apply to both cartilage and disc.

The C-PS fraction and an albumin control were incubated in 20 mM Tris–HCl, 2 mM $CaCl_2$ (pH 7.4) with clostridiopeptidase B (E:S, 1:100) for 3 h at 37°C. Phenylmethanesulphonyl fluoride and *N*-ethylmaleimide were included to inhibit any non-specific protease activity.

Carboxymethyl cellulose chromatography in the native and denatured states was performed as in [7] and [8], respectively. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (with and without prior reduction with dithiothreitol) was as in [9]. Peptide bands were located by Coomassie blue and glycosylated peptides by the Periodic acid Schiff (PAS) reaction in [10]. Molecular sieve chromatography on agarose A-5m was as in [11]. Reduction and alkylation of the α-sized component isolated on agarose A-5m was as in [12]. Samples were hydrolysed in constant boiling HCl under nitrogen for 24 h at 110°C and analysed on a Jeol-6AH amino acid analyser.

Segment long spacing (SLS) segments of the unre-

duced native C-PS collagen were prepared as in [13]. Samples were applied to thin carbon films and stained positively with both 1% phosphotungstic acid (pH 3.4) and 1% uranyl acetate (pH 4.2). The grids were examined in a Jeol electronmicroscope.

3. Results and discussion

The preparation and purification of the various collagens in hyaline cartilage or the intervertebral disc is shown in fig.1. Type II collagen was almost completely precipitated at 0.86 M NaCl (pH 2.8) and the remaining collagenous components subsequently precipitated at 2 M NaCl (pH 2.8). The 2 M precipitate was reprecipitated at 4–5 M NaCl (pH 7.4) and showed 3α-sized components which were PAS-positive (i.e., extensively glycosylated) migrating in similar positions to the 1α, 2α and 3α chains [6]. However the intensity of the Coomassie blue stain was more marked in the 1α position (fig.2A(1)). Subse-

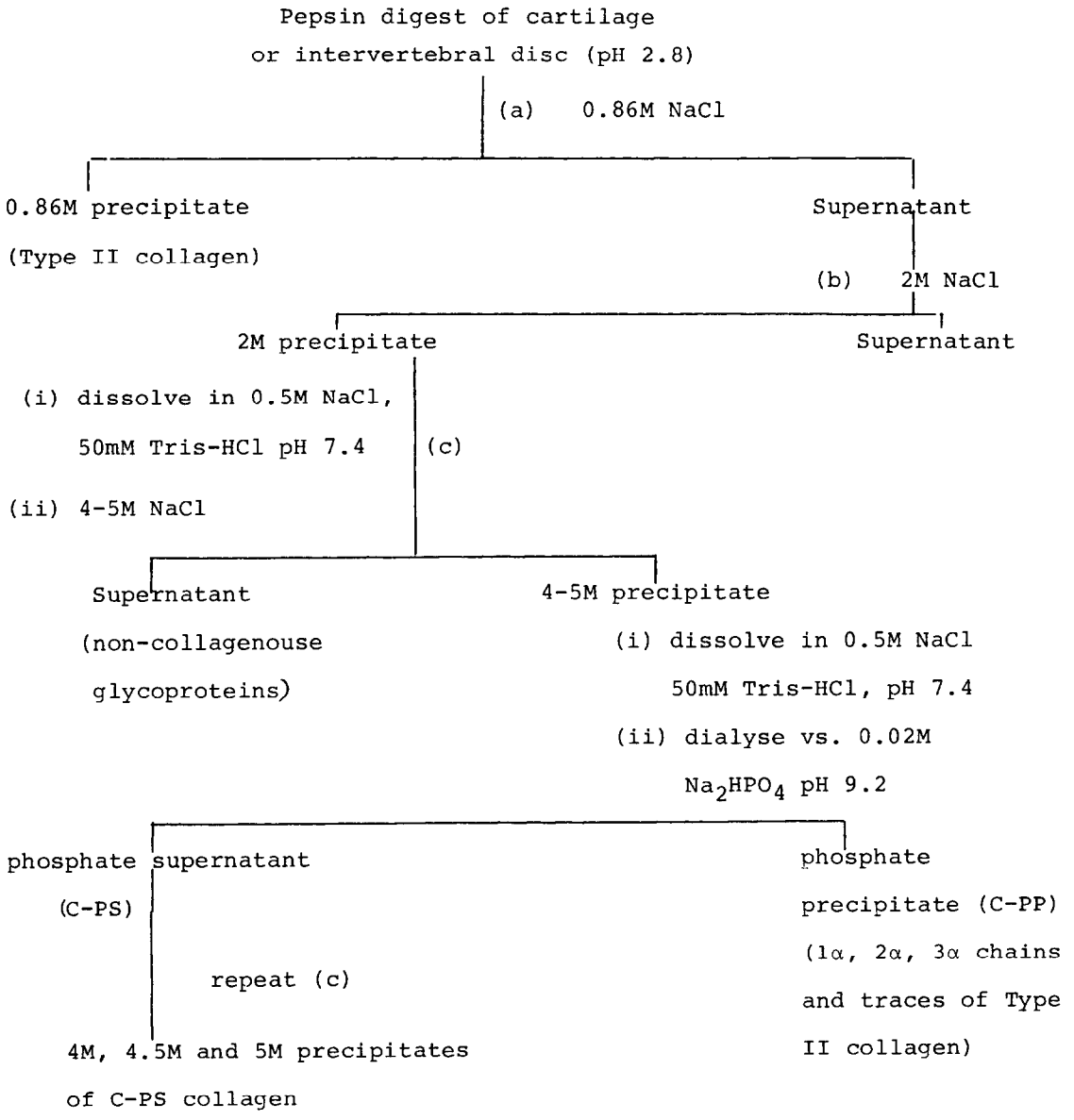

Fig.1. Preparation and purification of native cartilage or intervertebral disc phosphate soluble (C-PS) collagen.

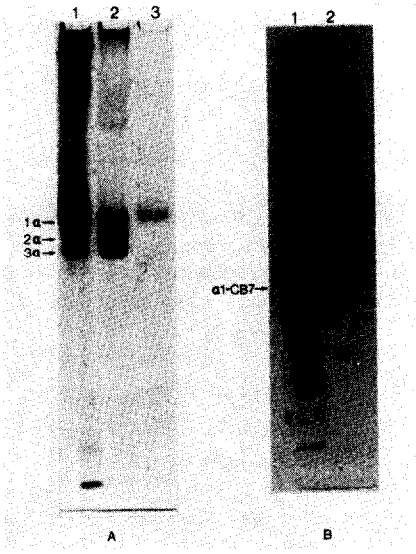

Fig.2. (A) SDS–polyacrylamide gel electrophoresis (5%) of various collagen fractions isolated during the preparation and purification of C-PS collagen: (1) 4–5 M NaCl (pH 7.4); (2) phosphate precipitate (C-PP); (3) phosphate supernatant (C-PS). (B) SDS–polyacrylamide gel electrophoresis (10%) of the reduced C-PS collagen component: (1) cyanogen bromide peptides of type I calf skin collagen; (2) reduced C-PS component. Staining was with Coomassie blue, 0.2% in 20% trichloroacetic acid.

quent dialysis against 0.02 M Na_2HPO_4, resolved the 4–5 M NaCl precipitate into 2 fractions, a phosphate precipitate (C-PP) and a phosphate supernatant (C-PS). Gel electrophoresis of the C-PP fraction revealed 3 non-reducible bands of similar intensity which were identified as 1α, 2α and 3α (fig.2A(2)). The C-PS fraction showed a major PAS-positive component in the 1α region (fig.2A(3)) and was completely digested with clostridiopeptidase B under conditions which failed to digest an albumin control, indicating that it was a collagen.

Differential salt precipitation of the C-PS fraction at pH 7.4 confirmed its high solubility property and 3 precipitates were obtained at 4, 4.5 and 5 M NaCl, each showing a similar electrophoretic pattern to the original C-PS fraction.

CM-cellulose chromatography in the native state of the 4, 4.5 or 5 M C-PS precipitate resulted in a peak eluting between 0.11 and 0.17 M NaCl. This is different from type IV collagen which elutes at 0.04 M NaCl and to types I, II, III and V collagens which elute at 0.09–0.1 M NaCl [7], but is similar to the

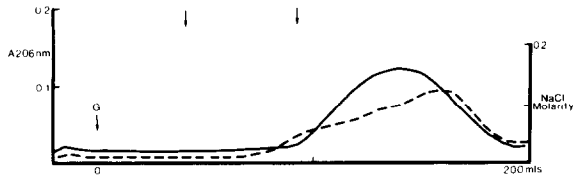

Fig.3. 3 CM-cellulose chromatography of native C-PS and C-PP collagens. The column was equilibrated with 2 M urea, 0.04 M sodium acetate (pH 4.8) and developed with a superimposed linear NaCl gradient of 0–0.2 M at 12°C. (——) Native C-PS collagen; (– – –) native C-PP collagen. Arrows denote positions of type IV collagen (0.04 M) and types I, II, III and V collagens (0.09–0.1 M).

C-PP fraction containing the 1α, 2α and 3α chains (fig.3).

Chromatography of the C-PS collagen in the denatured state on agarose A-5m (not shown) resulted in a major peak eluting in the α1(11) position, suggesting a lower M_r value than was indicated by gel electrophoresis. The amino acid analysis (table 1) indicates that ~1/3rd of the residues is glycine (confirming its collagenous nature), alanine is low and hydroxylysine and the hydrophobic residues are relatively high. The

Table 1
Amino acid composition of the C-PS collagen component isolated on agarose A-5m

OHPro	70.2
Asp	46.8
Thr	17.2
Ser	35.7
Glu	107.2
Pro	90.1
Gly	327.9
Ala	57.9
Cys	0.7
Val	37.0
Met	7.3
Ileu	25.8
Leu	46.8
Tyr	3.6
Phe	6.1
OHLys	32.0
Lys	30.8
His	11.1
Arg	45.6

% Hydroxylation Pro = 44%
% Hydroxylation Lys = 50.8%
Average residue weight = 95.8

Amino acids are expressed as residues/1000 total amino acids

total imino acids is low (160 compared to 200–220 for other collagens) and the proportion of basic amino acids high. The anomalous behaviour on SDS gels may be due to either the extent of glycosylation or to the higher average residue weight (95.8 compared to 91.2 for the α-chains of types I, II and III collagens). Both features have been implicated to explain similar observations for other glycosylated chains [6,14].

It appeared that the C-PS component was a genetically distinct α-chain. However, although the amount of cysteine detected was low (table 1), the C-PS component was reduced by dithiothreitol to subunits of ~33 000 M_r (estimated by comparison with the cyanogen bromide peptides of type I collagen (fig.2B)).

CM-cellulose chromatography in the denatured state of the reduced and alkylated subunits resulted in one basic peak eluting at ~0.077 M NaCl, indicating a single 33 000 M_r species (fig.4).

Two models for the native C-PS molecule are possible:

Either (1) there are 3α-sized chains each made up of 3 equal (33 000 M_r) subunits linked by intrachain disulphide bonds;

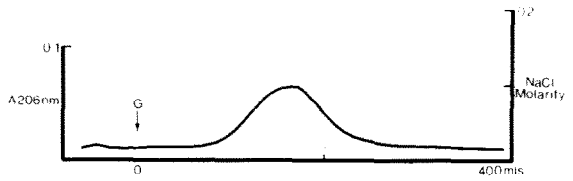

Fig.4. CM-cellulose chromatography of the reduced and alkylated C-PS component. The column was equilibrated with 1 M urea, 0.02 M sodium acetate (pH 4.8) at 4.2°C and developed with a superimposed linear NaCl gradient of 0–0.2 M.

Or (2) 3 identical chains of 33 000 M_r linked by interchain disulphide bonds to form a short triple-helical pseudo α-sized component.

Support for the second model was provided by electron microscopy. SLS crystallites of the native unreduced 5 M C-PS fraction are shown in fig.5. The atypical segments form long tapes which interweave with each other. The general appearance of the segments is that they have fewer bands and are of much shorter length, ~1/3rd length of a collagen monomer. Their ribbon-like appearance suggests the tendency of the C-PS collagen to aggregate. The band pattern

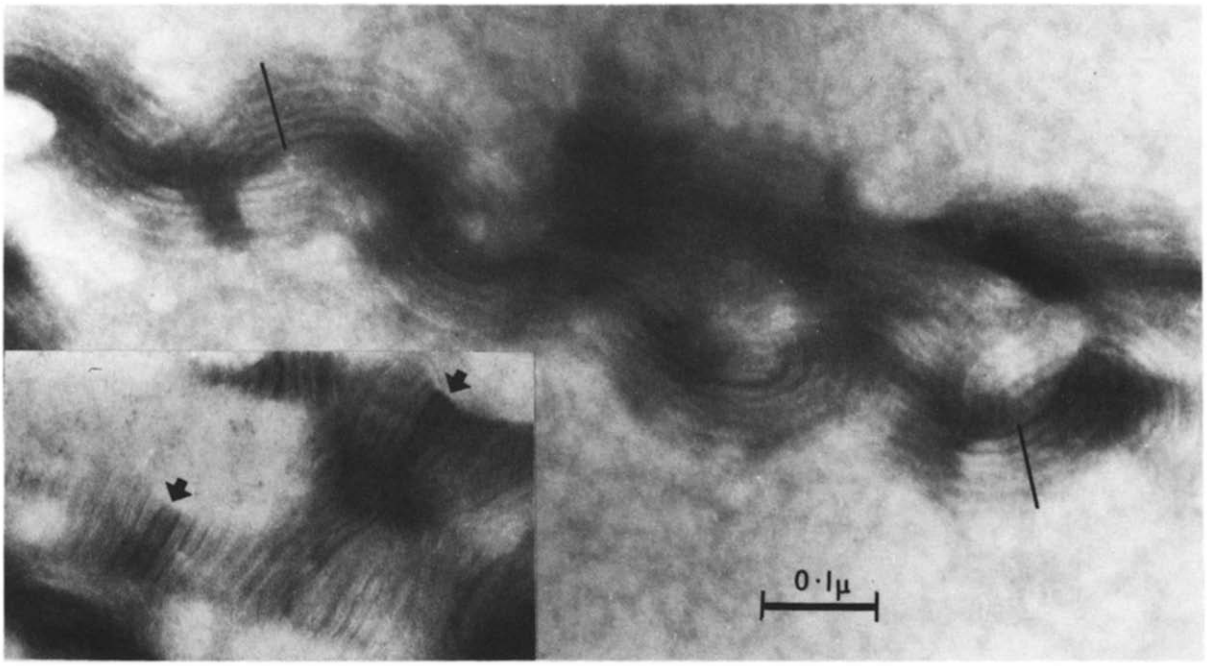

Fig.5. Segment long spacing (SLS) crystallites of C-PS collagen. Lines indicate approximate length of the molecule. Inset shows dimerisation of these short segments. Arrows indicate point of dimerisation.

shows rather evenly-spaced lines which tend to be perceptibly darker at one end perhaps indicating that the basic residues are clustered at this end.

The origin and function of the C-PS collagen is unknown. However, since it is isolated after pepsin digestion it (or its possible aggregates) may be part of a much larger structure in the cartilage and disc matrix.

There are certain striking similarities between the C-PS collagen and the disulphide bonded aggregates recently isolated from human placenta [15]. Both are extremely soluble and precipitate only at high salt concentrations, reduce to low M_r subunits and have a low hydroxyproline and high basic amino acid content. The subunits from placenta however are slightly larger than those from cartilage (40 000 M_r *vs* 33 000 M_r), contain <1/3rd glycine and large amounts of cysteine and tyrosine indicating non-collagenous regions and contain both acidic and basic chains. We have isolated similar aggregates from foetal skin and find these also intimately associated with non-collagenous glycoproteins [16]. The C-PS collagen could therefore represent a less aggregated and 'purer' analogue of the unusual collagen observed in placenta and skin. Alternately, it could be a distinct form specific to cartilaginous tissues only.

If C-PS collagen does represent a highly-soluble unique collagen common to such diverse tissues as skin, placenta, disc and cartilage, in which the major collagenous constituents differ considerably, it would suggest that it is probably not related to the major collagen types. Rather it may be related to the more soluble collagens: type V in skin and placenta and the 1α, 2α collagens in cartilage and disc.

Acknowledgements

We are grateful to Dr Adrian Shuttleworth for the amino acid analyses and to Mr John Murphy and Mr Simon Elstow for their excellent technical assistance. This work was supported by grants from the North West Regional Health Authority and the Medical Research Council.

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