CHARACTERISATION OF ANOTHER SHORT-CHAIN DISULPHIDE-BONDED COLLAGEN FROM CARTILAGE, VITREOUS AND INTERVERTEBRAL DISC

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1. Introduction

Cartilage is an avascular gel-like tissue having a collagenous meshwork made up mainly of a type of collagen (type II) present only in tissues of a similar nature such as vitreous and intervertebral disc [1–3]. Minor collagens 1α2α3α [4] and disulphide-bonded phosphate-soluble collagens [5–7] have been demonstrated in cartilage and intervertebral disc which are not present in other dissimilar tissues. At present, the configuration of the phosphate soluble collagens is not clearly established. Here we describe the isolation and characterisation of a second such collagen from cartillage, vitreous and intervertebral discs.

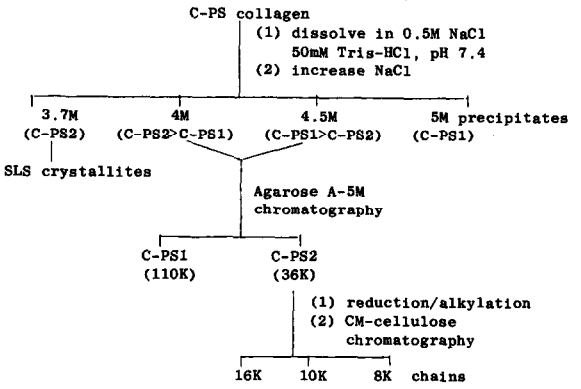

Fig.1. Isolation and characterisation of C-PS2 collagen from cartilage, intervertebral disc and vitreous. C-PS collagen was prepared as in [6].

2. Materials and methods

2.1. *Isolation of C-PS2 collagen*

Bovine nasal cartilage and human intervertebral discs were digested with pepsin and the total solubilised collagen fractionated to give type II, the 1α2α3α collagens and a phosphate-soluble fraction (C-PS) as in [6]. Bovine vitreous was centrifuged at 50 000 × *g* and the pellet treated as for cartilage and disc.

The C-PS collagen fraction was further purified by slow differential salt precipitation at pH 7.4 and the various precipitates obtained used as indicated (fig.1) to characterise C-PS2 collagen in the native and denatured states. Except where otherwise stated detailed characterisation was carried out on the cartilage collagen.

2.2. *Characterisation of C-PS2 collagen*

2.2.1. Native state

Segment long spacing (SLS) crystallites of the unreduced native C-PS2 collagen (3.7 M precipitate) were prepared as in [8]. 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) or negatively with 1% phosphotungstic acid (pH 7.4). The grids were examined in a Jeol electron microscope.

2.2.2. Denatured state

Agarose A-5m chromatography was carried out as in [9] and the separated C-PS2 component thus obtained was reduced, alkylated [10] and fractionated on carboxymethyl-cellulose [11].

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was as in [12]. Peptide bands were located with Coomassie blue and glycosylated peptides by the periodic acid Schiff (PAS) reaction [13]. Cyanogen bromide peptides of unreduced C-PS2 were prepared according to [14]. Molecular masses (M_r) of

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the various peptides were estimated by comparison with the cyanogen bromide peptides of type I collagen and are expressed in k where k = 10^3.

Samples were hydrolysed in constant boiling HCl under nitrogen for 24 h at 110°C and analysed for amino acids on a Jeol-6AH amino acid analyser.

3. Results and discussion

A new phosphate-soluble collagen has been isolated from cartilage, intervertebral disc and vitreous. This collagen appears to be of the same family as that in [5–7]. Both are disulphide-bonded collagens and have been isolated by virtue of their extreme solubility in 0.02 M disodium hydrogen phosphate solutions at pH 9.2. The original of these collagens (C-PS1) had been characterised as a short-chain collagen consisting of 3 equal length chains of $M_r \sim 33$ k [6]. The new phosphate soluble collagen, C-PS2 can be separated from C-PS1 in the native state by slow differential salt precipitation (fig.1). The 3.7 M precipitate consisted purely of C-PS2 collagen and was used for SLS crystallite formation (see below). C-PS2 had an app. M_r 36 k in the case of cartilage or disc and like C-PS1 was PAS positive, that is highly glycosylated. A minor component of $M_r \sim 70$ k consistently copurified with C-PS2 in the native state and is probably (though not certainly) a dimeric form (fig.2A, lane 3).

When C-PS1 and C-PS2 from cartilage (or disc) were compared with those from vitreous (fig.2A, lanes 1,2) two major differences were observed:

(i) Two components migrating rather slower than C-PS1 were more pronounced in vitreous. These are believed to be higher M_r forms of C-PS1 and will be reported elsewhere (S. A., J. B. W., in preparation).

(ii) Vitreous C-PS2 consistently migrated with a slower mobility. However, since vitreous C-PS2 was more PAS positive than that of cartilage we believe the decreased mobility relates to the extent of glycosylation rather than to an increase in M_r value. This is further supported by amino acid analysis (see below).

C-PS2 was also separated from C-PS1 in the denatured state by agarose chromatography (fig.3) and the amino acid analyses of the two collagens compared (table 1A). C-PS2 had a higher imino acid, aspartic acid and cysteine and lower glutamic acid and glycine content than C-PS1, the lower glycine indicating the presence of a non-collagenous moiety or non-collagenous sequences in the triple helix. The amino acid composition of vitreous C-PS2 was similar to that of cartilage but the hydroxylysine content and consequently the number of potential sites for glycosylation was markedly increased. This supports the contention that vitreous C-PS2 is more glycosylated than that of cartilage or disc.

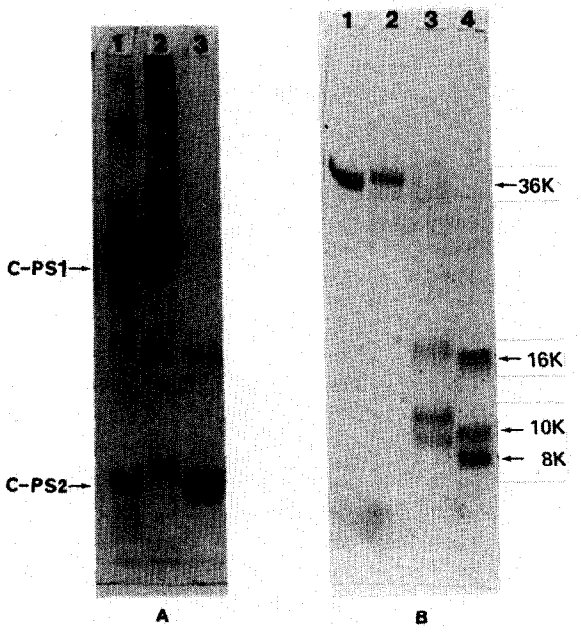

Fig.2. (A) SDS–polyacrylamide gel electrophoresis (5%) of the 4.5 M NaCl (pH 7.4) precipitate from cartilage or disc (lane 1) vitreous (lane 2) and the 3.7 M NaCl (pH 7.4) precipitate of cartilage (lane 3). (B) SDS–polyacrylamide gel electrophoresis (12%) of cartilage C-PS2 before (1) and after (4) reduction and vitreous C-PS2 before (2) and after (3) reduction.

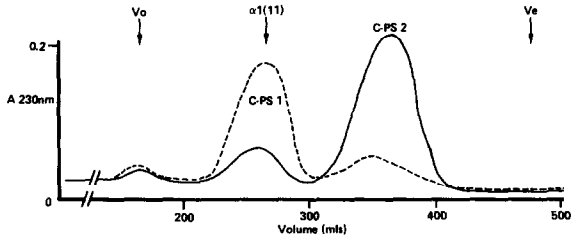

Fig.3. Agarose A-5m chromatography of the 4 M (——) and 4.5 M (– – –) NaCl (pH 7.4) precipitates of cartilage C-PS collagen. Fractions were eluted with 1 M $CaCl_2$, 50 mM Tris–HCl (pH 7.4). The elution position of α1(II) chains is shown as a reference.

Table 1
Amino acid composition of (A) C-PS1 and C-PS2 collagens of cartilage and vitreous and (B) the 3 constituent chains of cartilage C-PS2

	(A) Cartilage			(A) Vitreous C-PS2	(B)					
	C-PS1 (110 k)	C-PS2 (36 k)			16 k		10 k		8 k	
		(a)	(b)		(a)	(b)	(a)	(b)	(a)	(b)
OHPro	70	115	43	91	105	18	119	13	143	12
Asp	47	60	22	61	59	10	36	4	32	3
Thr	17	15	6	23	17	3	18	2	19	2
Ser	36	31	12	34	38	6	32	3	16	1
Glu	107	73	27	80	76	13	76	8	71	6
Pro	90	106	39	89	102	17	122	13	82	7
Gly	328	282	104	286	315	53	322	34	309	26
Ala	58	75	28	62	78	13	70	8	74	6
½Cys	1	13	5	15	12	2	5	1	4	1
Val	37	25	9	31	20	3	21	2	30	2
Met	7	11	4	6	–	–	7	1	24	2
Ileu	26	24	9	28	26	4	14	1	34	3
Leu	47	48	18	59	48	8	47	5	36	3
Tyr	4	6	2	6	8	1	4	1	8	1
Phe	6	9	3	16	2	1	11	1	7	1
OHLys	32	14	5	28	12	2	21	2	17	1
Lys	31	21	8	23	18	3	17	2	26	2
His	11	14	5	15	11	2	4	1	20	2
Arg	46	57	21	47	55	9	55	6	46	4
Average residue weight	95.8	97.0		96.5	94.5		94.3		96.3	

Amino acid residues are expressed as: (a) residues/1000 total amino acids; (b) residues/peptide using the calculated average residue mass

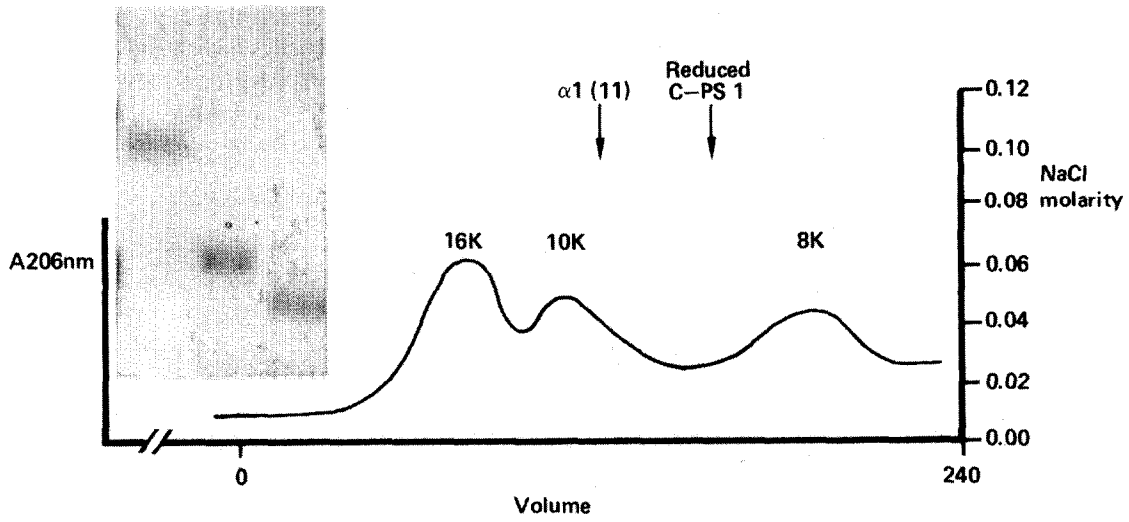

Fig.4. CM-cellulose chromatography of the reduced and alkylated cartilage C-PS2 component isolated by agarose chromatography. The column was equilibrated with 1 M urea, 0.02 M sodium acetate (pH 4.8) at 42°C and developed with a superimposed linear NaCl gradient 0–0.2 M over 400 ml. Inset shows the purity of the 3 separated components.

Fig.2B shows the effect of reduction on C-PS2 from cartilage (lanes 1,4) and vitreous (lanes 2,3). Three components were observed after reduction but each vitreous component migrated more slowly (and was therefore probably more glycosylated) than the respective cartilage component. This result also suggested that glycosylation was not restricted to one part of the C-PS2 molecule. Thus the whole collagen in vitreous appears to be more glycosylated probably reflecting differences in the activity of the lysine hydroxylase or glycosyl transferases between the 2 tissues. The apparent M_r-values of the 3 cartilage C-PS2 components were 16 k, 10 k and 8 k. These could be separated, after complete reduction and alkylation of C-PS2, by CM-cellulose chromatography (fig.4). The 16 k and 10 k components eluted early in the chromatogram whereas the 8 k component was more basic and eluted later. The amino acid analyses of the 3 separated components are shown in table 1B. The glycine content increased in all 3 components indicating the removal of some non-collagenous sequences during reduction and alkylation. Each component was significantly different from the others but their combined analyses (in terms of residues per peptide) agreed closely with that of the orginal 36 k component. The smallest (8 k) component had a very high hydroxyproline and relatively low proline content reminiscent of basement membrane (type IV) collagen.

Analyses of the cyanogen bromide peptides of C-PS2 showed that no new peptides arose after reduction (fig.5) although an increase in the lower M_r peptides (5–16 k) occurred at the expense of the peptides of M_r 20–30 k. This suggests that the larger peptides are dimers or trimers of the smaller ones linked by disulphide bonds, the major 24 k peptide probably being a dimer of the 16 k and 8 k peptides. It is probable that the 16 k cyanogen bromide peptide is identical to the 16 k component observed on reduction alone (fig.2B) since this component lacks methionine (table 1B) and appears to be unaffected by cyanogen bromide (unpublished). However, it is not yet certain which of the remaining cyanogen bromide peptides are derived from the 8 k and 10 k component chains.

The molecular configuration of C-PS2 collagen was determined from the SLS crystallites of the native 3.7 M precipitate (fig.6). The segments which formed dimeric or polymeric crystallites were short (±41 nm) ~1/7th the normal length of a collagen monomer. This indicated that the 3 component chains of 16 k, 10 k and 8 k were linked side-by-side by interchain disulphide bonds in a short triple helical segment of M_r 36 k. The 16 k component is probably the linking chain of the molecule since it contains twice as much cysteine as the 10 k and 8 k components (table 1B).

C-PS2 is almost certainly the same as the CF2 col-

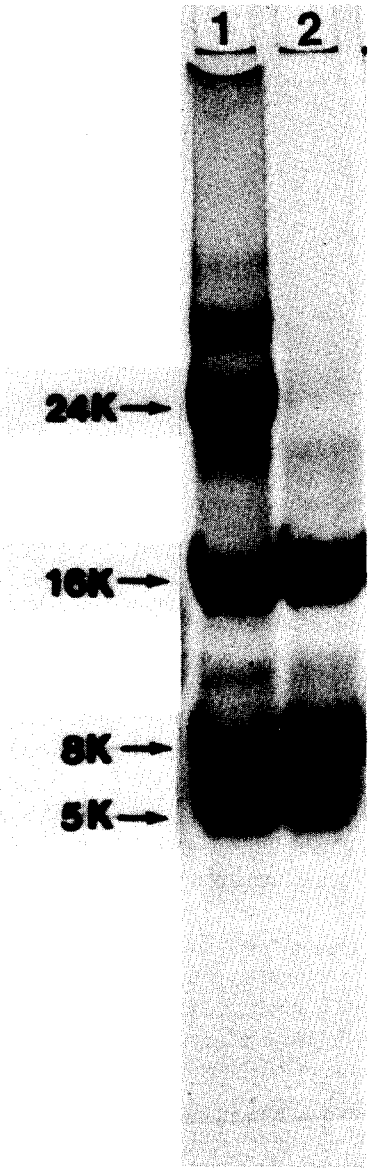

Fig.5. SDS–polyacrylamide gel electrophoresis (12%) of the cyanogen bromide peptides of C-PS2 collagen (1) before and (2) after reduction with dithiothreitol.

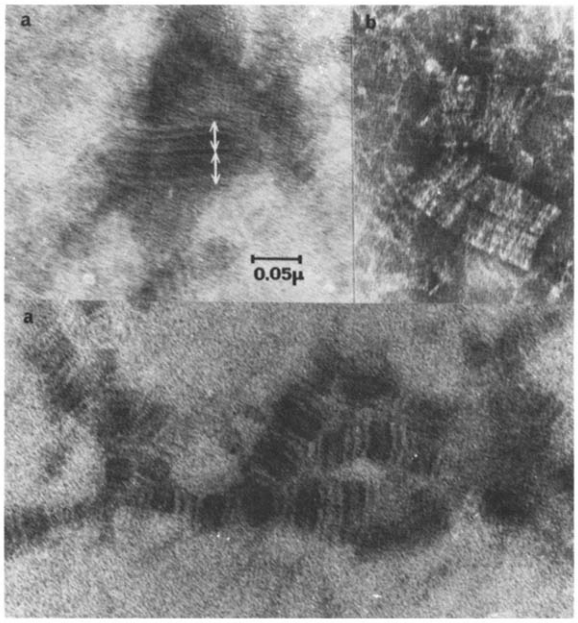

Fig.6. Segment long spacing (SLS) crystallites of cartilage C-PS2 collagen stained: (a) positively with 1% phosphotungstic acid (pH 3.4) and 1% uranyl acetate (pH 4.2) and (b) negatively with 1% phosphotungstic acid (pH 7.4). The arrows indicate the length of the molecules and where arrows join indicates point of dimerisation.

lagen (35 k) isolated from articular cartilage [15] and reported to consist of 3 identical chains each with app. M_r 12 k (estimated by gel electrophoresis on a 5% gel). The function of C-PS collagens is unknown. They account for ~5% of the collagen in bovine nasal cartilage, the ratio of C-PS1 to C-PS2 being ~2:1. However, as both C-PS1 and C-PS2 are independent triple helical forms this ratio will not be significant in terms of their structural association. It is more likely that they represent helical portions of one larger complete molecule. Support for this concept is derived from preliminary studies using immunofluorescent localisation which indicate that both C-PS1 and C-PS2 collagens are located in the pericellular/perilacunar area of the chondrocytes suggesting a similar distribution within the tissue.

These new disulphide bonded collagens in some ways resemble type IV (basement membrane) collagen although no basement membrane is present in cartilage, intervertebral disc or vitreous. Type IV collagen is the only other known collagen that is soluble in phosphate buffers [16], has several pepsin susceptible regions in the triple helix [17] and has a high ratio of hydroxyproline to proline as noted particularly for the 8 k chain of C-PS2 collagen. We have suggested that C-PS1 collagen could be related to the $1\alpha 2\alpha 3\alpha$ collagens [6] since both are highly soluble in salt solutions at acid and neutral pH and have a similar amino acid composition. Until further information is available both suggestions must remain speculative.

Acknowledgements

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