THE SEPARATION OF TWO SOFT-TISSUE COLLAGENS BY COVALENT CHROMATOGRAPHY

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1. Introduction

Several genetically distinct collagens are now known to occur in mammalian tissues, each differing in the primary structure of their α-chain subunits. The most ubiquitous and widely studied, referred to systematically as Type I, is composed of two different chains α_1 (I) and α_2 occurring in the molecule in the ratio of 2 : 1 [1]. This heterogeneity is not found in the other collagens so far described. Type II collagen α_1 $(II)_3$ occurs exclusively in cartilagenous structures [2], while Type IV α_1 $(IV)_3$ is associated with basement membrane [3]. Type III collagen [α_1 $(III)_3$] is found in a variety of tissues notably skin and blood vessel walls and always in association with Type I. Moreover their relative abundance within a tissue varies with age, Type III being more predominant in early life [7]. Divergences from normal ratios have been implicated in the human pathological conditions of osteogenesis imperfecta [8,9] and Ehlers-Danlos IV [10].

Purification of Type III collagen invariably involves separation from Type I and this has been approached previously in two ways. Some workers have employed variations on the technique first described by Chung and Miller [5] which exploits an apparent difference in solubility of the two collagens in neutral salt solutions. Others have used ion-exchange chromatography to achieve a separation [6]. This paper described a new method which takes advantage of the thiol groups present in Type III collagen.

2. Materials and methods

Sephadex G-25 and thiol-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Pepsin, EC 3.4.4.1. (2 × crystallized) was a product of Sigma Chemical Company, London; 2, 2′ dipyridyl disulphide was obtained from Aldrich Chemical Company, London. Other materials were the products of B.D.H.

2.1. *Preparation of collagen*

Skin collagen was derived from two sources. Human material was obtained from 20–30 week-old foetuses as a result of therapeutic abortions. Calf skin came from animals two months old. Subcutaneous fat was removed by dissection before the samples were homogenised and cleaned for 48 h at 4°C with several changes of 0.05 M Tris/HCl pH 7.4, 0.9% w/v NaCl. The insoluble material was suspended in 100 vols. of 0.5 M acetic acid containing pepsin (0.2 mg ml^{-1}) and digestion continued for 16 h at 4°C. The insoluble residue was resuspended in the same volume of pepsin solution and incubated for a further period of 16 h at 4°C. The small residue was removed by centrifugation and collagen precipitated from the combined supernatants by addition of 5 M NaCl to a final concentration of 2 M. The precipitate was redissolved in the original volume of 0.5 M acetic acid and salting out repeated twice more before dialysis against 0.1 M acetic acid and freeze-drying.

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2.2. *Reduction and denaturation*

40 mg aliquots of the freeze-dried material were suspended in 10 ml 0.1 M Tris/HCl pH 8.0, 1.0 M urea, 0.3 M NaCl, 1 mM EDTA, 5 mM dithiothreitol and incubated for 30 min at 60°C. The solutions were clarified by passage through millipore filters to a final pore size of 0.45 μm.

2.3. *Molecular sieve chromatography*

Dithiothreitol was removed from the collagen solution by passage through a column (38 X 2.6 cm.) of Sephadex G-25 which had been equilibrated with the same buffer but which lacked dithiothreitol.

The column effluent was monitored at 240 nm and the excluded peak pooled.

2.4. *Covalent chromatography*

5 g of thiol-activated Sepharose 4B were swollen in 0.1 M Tris/HCl pH 8.0, 1.0 M urea, 0.3 M NaCl, 1 mM EDTA and the excess buffer removed. A portion of the excluded peak from the G-25 column was added and the mixture was shaken gently for 2 h in a water bath maintained at 42°C. The slurry was then deaerated and packed into a jacketed column 1.6 cm in diameter which, too, was kept at 42°C. To avoid bubble formation in the column, all buffers used subsequently were deaerated. Buffer was pumped through the column at 120 ml·h^{-1} and the effluent monitored at 240 nm. When the absorbance had reached the baseline level non-specifically bound material was eluted with the same buffer made 1 M with respect to NaCl. When, once again, the absorbance had fallen to the baseline, the same Tris buffer, this time supplemented with dithiothreitol to 5 mM, was pumped onto the column at 30 ml·h^{-1} until the absorbance of the eluate began to rise. At this point the pump was stopped and the reduction allowed to continue for 40 min before the released material was pumped from the column.

The identities of the unbound and displaced materials were examined by: (1) polyacrylamide gel electrophoresis; (2) carboxymethyl cellulose chromatography. (3) amino acid analysis.

2.5. *Polyacrylamide gel electrophoresis*

Subunit composition of the separated collagen fractions were examined by polyacrylamide gel electrophoresis. 3 mg samples of freeze-dried material were dissolved in 500 μl of 1% w/v sodium sulphate (SDS) and incubated at 60°C for 30 min. Some samples were reduced by adding 2-mercaptoethanol to 1% v/v before incubation. 25 μl aliquots were run in ten parallel tracks in an advanced slab apparatus using a method essentially the same as described by Sykes and Bailey [12] except that for the human samples a gel concentration of 5.2% w/v was used.

2.6. *Carboxymethyl cellulose chromatography*

The elution profiles of unbound and displaced materials were examined using a column (16 X 130 mm) of carboxymethyl cellulose maintained at 42°C. The resin was equilibrated in 0.02 M potassium acetate buffer and collagen eluted with a linear gradient of 0.02 to 0.10 M LiCl.

The column was standardised by acid-soluble rat tail tendon collagen and pepsin-treated calf skin collagens partially purified by differential salt precipitation [5]. After dialysis and freeze-drying, materials from the eluted peaks were identified by SDS-polyacrylamide gel electrophoresis as described above.

3. Results

3.1. *Molecular sieve and covalent chromatography*

The figures relate to a typical experiment using 40 mg of purified calf-skin collagen. The reduced and denatured chains (fig.1 peak I) were effectively separated from reducing agent (peak II). Half the eluted material (18 ml) was reacted immediately with

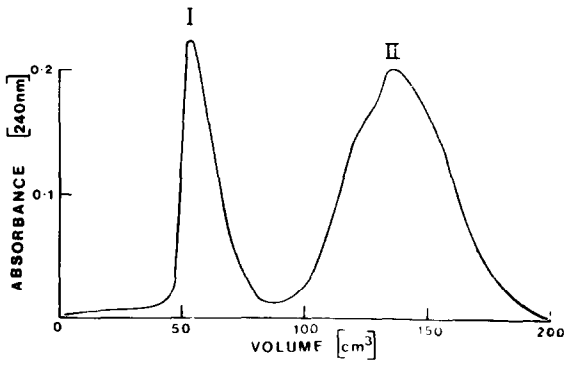

Fig.1. Elution profile of reduced and denatured pepsin-treated calf skin from Sephadex G-25.

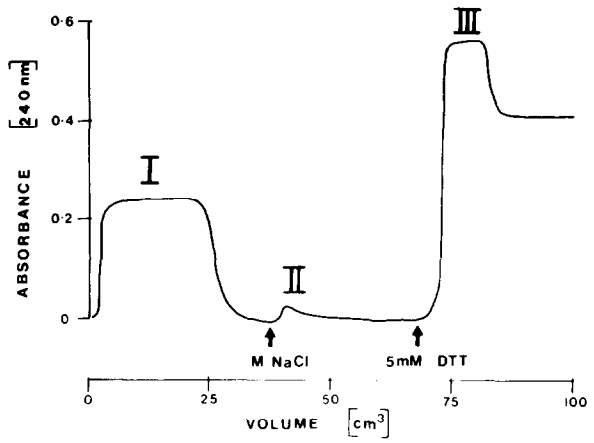

Fig.2. Stepwise elution pattern of reduced and denatured pepsin-treated calf skin collagen from thiol-Sepharose column.

the thiol-Sepharose. The bulk of the collagen (12.4 mg) did not bind to the column (fig.2 peak I) while a small amount (peak II, less than 1 mg) was eluted with 1 M NaCl. The addition of 5 mM dithiothreitol displaced a further 6.2 mg of material (peak III).

3.2. *SDS - polyacrylamide gel electrophoresis*

The patterns of unbound and displaced collagens as well as the starting material are shown (fig.3). Material displaced by 1 M NaCl (not shown) gave essentially the same pattern as the unbound collagen. The designation of chain type is based on comparison with samples of many different collagens and chains separated from them by CMC-chromatography. In both calf and human examples the material which did not bind to thiol-Sepharose contained substantially only Type I collagen while the bound material appeared to contain only Type III sub-units. Under conditions of electrophoresis, calf α_1(III) migrates at a significantly lower rate than α_1(I). Human α_1(III) is more difficult to resolve from α_1(I), hence the lower gel concentration required to effect separation. In samples which had not been previously reduced and denatured, the addition of mercaptoethanol to the incubation medium and consequent reduction of disulphide bonds altered the electrophoretic pattern. Disulphide bonded trimers $\alpha_1(III)_3$ in the starting material migrated in the γ-chain region but moved, on reduction, to the position excepted for monomers. A minor band appeared on reduction of the starting material in the position of β-chain dimers, particularly easy to see in the human example. This band was also visible in the material displaced by dithiothreitol from the thiol-Sepharose column and is probably $\beta_1(III)_2$ i.e. $\beta_1(III)_1(III)$. The interchain crosslink in this dimer is not a disulphide bridge as it persists even after prolonged reduction. (5% mercaptoethanol, 4 h at 60°C).

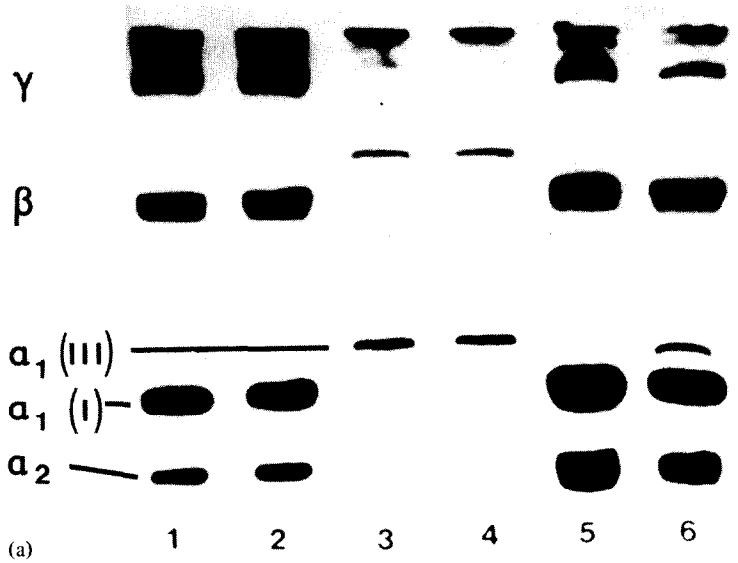

Fig.3

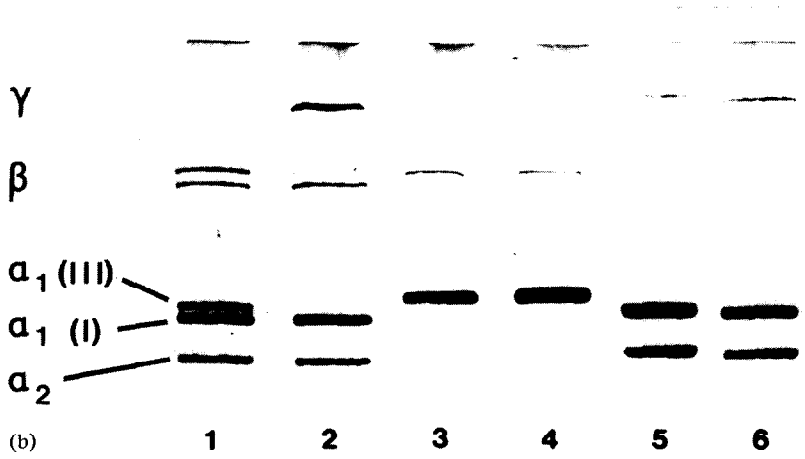

Fig.3. SDS-polyacrylamide electrophoresis. (a) Pepsin-treated calf skin collagens separated by covalent chromatography. Tracks 2, 4 and 6 were incubated in the presence of 1% v/v 2-mercaptoethanol. Tracks 1 and 2; unbound material. 3 and 4; materials displaced by dithiothreitol. 5 and 6; starting material before reduction and denaturation. (b) Pepsin-treated human skin collagens separated by covalent chromatography. Tracks 1, 3 and 4 were incubated in the presence 1% v/v 2-mercaptoethanol. Tracks 1 and 2; starting material before reduction and denaturation. 3 and 4 material displaced by dithiothreitol. 5 and 6 unbound material.

3.3. *Carboxymethyl cellulose chromatography*

The elution profiles are shown in fig.4. Fig.4(a) shows the elution of pepsin-treated calf skin collagens partially purified by differential salt precipitation compared to rat tail tendon collagen. The material precipitating at 1.5 M NaCl gave a major peak close to the region of rat β_{12}. This was assumed to be Type III collagen by comparison with other published data [5,6] and its electrophoretic behaviour (not shown).

The collagen precipitating at 2.5 M NaCl gave the pattern expected for Type I collagen. This was confirmed by electrophoresis (not shown). Fig.4(b) shows that the material which was bound to the thiol-Sepharose column eluted as a single peak in the position of Type III while the unbound collagen gave a profile conforming to that of Type I.

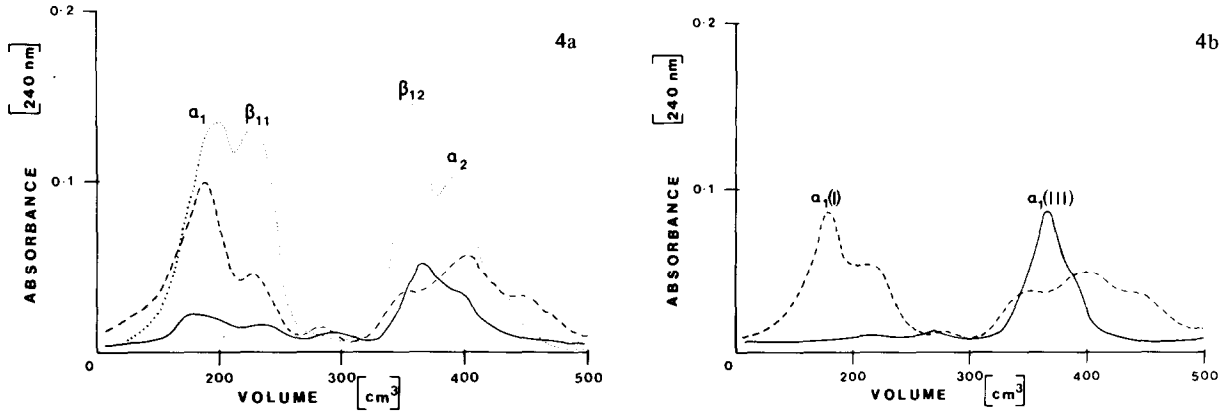

Fig.4. Elution profiles of denatured collagen chains from carboxymethyl cellulose. (a) Acid extracted rat tail tendon collagen (dotted line). Pepsin treated calf skin collagens partially purified by differential salt precipitation. Precipitates were collected at 1.5 M NaCl (solid line) and 2.5 M NaCl (dashed line). Chain notation refers to the rat tail sample. (b) Pepsin treated calf skin collagens separated by covalent chromatography. Unbound fraction (dotted line) and material displaced by dithiothreitol (solid line).

3.4. *Amino acid analysis*

The table shows the amino acid compositions of the unbound and displaced collagens from the experiment using calf collagen as the starting material. These are consistent with the identification of the unbound collagen as Type I and the bound material as Type III [5].

4. Discussion

The separation described above relies on the covalent binding of free thiol groups in reduced, denatured Type III collagen to thiol groups immobilised on Sepharose. Covalent chromatography was first developed to separate active papain, which contains a free thiol group, from the inactive enzyme where this group is blocked, most commonly by cysteine [11].

The technique used here employs as a support material the mixed disulphide formed between 2.2′-dipyridyl disulphide and glutatione coupled to CNBr-activated sepharose fig.5 reaction (a). This is capable of reacting with thiol groups in the solute through a second mixed disulphide reaction (b). The addition of a reducing agent cleaves this disulphide, displacing the bound material from the stationary phase reaction (c).

The technique has been used to separate thiol-containing from non-thiol-containing proteins for example in the purification of mercaptalbumin from commercial preparations of bovine serum albumin

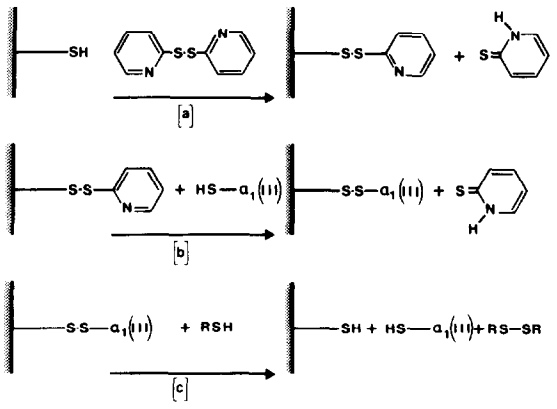

Fig.5. Reaction schemes for covalent chromatography of collagen. For details, see text.

Table 1
Amino acid compositions of unbound and displaced collagens

Amino acid	Unbound collagen	Displaced collagen
	Residues per thousand total residues	
OH-Pro	99	114
Asp	39	50
Thr	18	17
Ser	35	59
Glu	69	76
Pro	126	113
Gly	328	334
Ala	109	88
Val	21	16
Cys	0	2
Met	9	8
Ile	11	14
Leu	22	21
Tyr	1	2
Phe	11	11
OH-Lys	7	6
Lys	29	27
His	5	7
Arg	57	44

Figures for cysteine and methionine are the sum of amino-acids and their oxidation products

[13] but has not so far been used to separate proteins in which thiol groups are combined in intramolecular disulphide bridges. Although, theoretically, one disulphide bond between two of three identical chains in close register may leave the third thiol group free it seemed prudent when preparing collagen for covalent chromatography to reduce these bridges in order to expose the maximum of free thiol groups. Naturally the reducing agent must subsequently be removed to allow these groups to combine with the activated Sepharose. Finally, conditions must be such as to minimise the recombination of the thiol groups after the reducing agent has been removed. That this recombination did not occur to any great extent is demonstrated in fig.3. The banding patterns of those samples which had been chromatographed were, in contrast to the starting material, unaffected by the addition of mercaptoethanol to the incubation mixture. Had re-oxidation occurred to a significant degree at any stage, recombined dimeric and trimeric components would have migrated as α-chains in reduced samples.

Unfortunately, the factors which govern the migration of collagen on SDS gels are not yet fully understood. The unexpected separation of α_1(I) and α_2 chains was first described by Sykes and Bailey [12] and also by Furthmeyr and Timpl [14]. This anomalous behaviour has been variously attributed to differences between the chains of molecular weight, of conformation in SDS and of native charge [15]. The last, at least, is unlikely to be true since the more negatively charged molecule (α_1) is, in fact, the slower moving. In spite of its apparent irrationality, the method is widely used and remains an effective one for the analytical separation of collagen subunits. A further unexpected observation is that calf, α_1(III) migrates more slowly than α_1(I) fig.3(a) and these two components may be easily resolved using the original methods of Sykes and Bailey. Fig.3(b) demonstrates that, unhappily, the resolution between α_1(I) and α_1(III) from foetal human skin is not so marked and some modifications of the method were required to achieve even a modest separation.

However, combined with the evidence of carboxymethyl cellulose chromatography and amino acid analysis, it seems clear that an effective one-step separation of Type I and Type III collagens has been achieved by covalent chromatography. This new device may allow immobilization and separation of cysteine-containing collagens and peptides derived from them.

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