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Note

One-step chromatographic isolation of collagen cross-links

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One of the unusual features of the connective tissue proteins collagen and elastin is the extensive post-translational modification that occurs after synthesis of the polypeptide chains. These include hydroxylations of prolines and lysines, glycosylations of hydroxylysines, Schiff base condensations between aldoses and lysyl residues and cross-link formation based on lysine and hydroxylysine residues[1–4]. These cross-links link the polypeptide chains but only comprise a small proportion of the total composition of the protein[5–7]. This paucity makes their isolation and characterization somewhat difficult.

Cellulose media have been used for the chromatography of the pyridinium cross-links desmosine and isodesmosine, found in elastin[8–10]. In acid hydrolyzates of collagen that have been reduced with tritiated sodium borohydride there are the pyridinium cross-links, pyridinoline (Pyr) and 3-hydroxypyridinoline (Hypyr)[6,11] as well as the radioactively labelled monohydroxylysinonorleucine (MLNL), dihydroxylysinonorleucine (DLNL), lysinonorleucine (LNL) and histidinohydroxymerodesmosine (HHMD)[12]. In this paper we describe a method for the quantitative isolation of these cross-links by cellulose chromatography.

EXPERIMENTAL

Collagens were purified from rat tail tendon[13], sheep periodontal ligament[14], sheep blood vessels[15], bovine achilles tendon[16] and calf skin[17]. The preparations were reduced with tritiated sodium borohydride (The Radiochemical Centre, Amersham, U.K., 9.1 Ci/mmole) that had been diluted to 10 mCi/mmole according to the procedure of Light and Bailey[12]. LNL was purchased from Elastin Products (Pacific, MO, U.S.A.) and histidinohydroxymerodesmosine was kindly provided by Kathleen Smolenski. Cellulose powder was from Riedel de Haen and *n*-butanol and acetic acid were from May and Baker.

The collagen preparations were hydrolysed *in vacuo* for 24 h at 110°C in 6 *M* hydrochloric acid. Amino acid analysis was used to check the purity of the collagens.

For the isolation of the cross-links, hydrolyzates were mixed with glacial acetic acid, cellulose slurry and *n*-butanol, loaded on to a column of celulose (10 × 1 cm) and chromatographed firstly with at least 25 ml of *n*-butanol–acetic acid–water (4:1:1)

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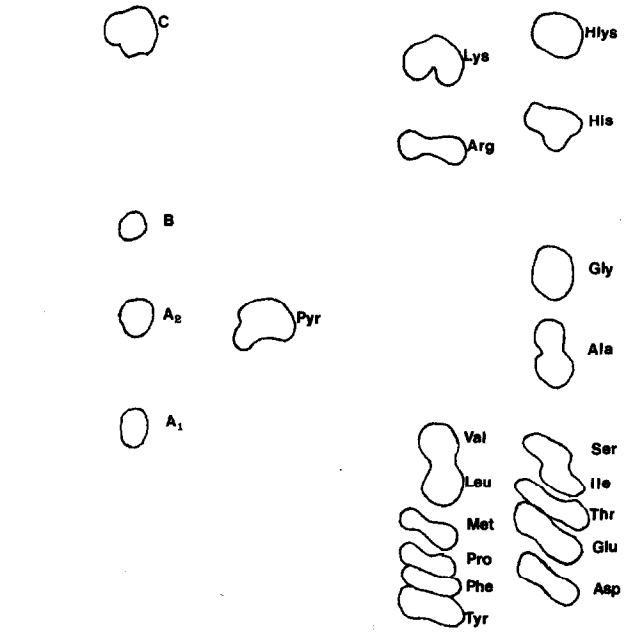
and then with 5 ml of water according to the method of Skinner[9]. The two eluates were dried before redissolving in 0.01 *M* hydrochloric acid for further analysis.

Amino acid analyses were performed on a Beckman 119CL amino acid analyzer. For radioactivity profiles, 1-min fractions were collected after elution through the photometer and 0.5-ml aliquots were taken for counting.

High-voltage paper electrophoresis was performed at either pH 6.4 or pH 2.1 in a Savant electrophoresis enclosure on either Whatman No. 1 paper for analytical separations or on Whatman 3MM paper for preparative separations. Analytical electropherograms were stained with ninhydrin–cadmium acetate reagent[18]. For radio-



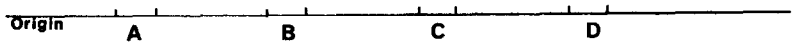


Fig. 1. Electrophoresis of water eluate of cellulose chromatography of bovine achilles tendon collagen hydrolyzate (lane A), pyridinoline (lane B), amino acid markers (lanes C and D). Electrophoresis was at pH 1.9 for 40 min. The paper was stained with ninhydrin–cadmium acetate reagent[18]. See Table II for identification of components in Lane A.

activity detection, 1-cm squares were cut from the paper and placed in scintillation counting vials. Compounds of interest were eluted from paper strips in an elution chamber using 2% acetic acid. Measurement of radioactivity was performed by adding 4.5 ml of ACSII (The Radiochemical Centre) to the sample in the vial and counting in a Philips PW4700 beta counter.

RESULTS

When 40 mg of an acid hydrolyzate of collagen is chromatographed on a cellulose mini-column as described under Experimental, Pyr is eluted in the water phase. Its identity was confirmed by high-voltage electrophoresis at pH 1.9 (Fig. 1), an ultraviolet absorption peak at 295 nm, a fluorescence emission at 395 nm after excitation at 295 nm and amino acid analysis. Pyr was not detected by any of these methods in the butanol–acetic acid eluate. Pyr was isolated by this method from hydrolyzates of collagen from sheep blood vessels, rat tail tendon, periodontal ligament and bovine achilles tendon but was not detected in calf skin collagen.

When 40 mg of an acid hydrolyzate of sodium borohydride-treated collagen was chromatographed, the higher specific radioacitivity (cpm of tritium per nmol of amino acid) was in the water eluate (Table I).

When this water phase was electrophoresed at pH 6.5 (Fig. 2A) and at pH 1.9 (Fig. 2B), several ninhydrin-positive components are detected. Details of their radioactivity and electrophoretic behaviour and ultraviolet properties are listed in Table II. As expected, Pyr was detected and confirmed in these hydrolyzates. It was not radioactive and had the same properties as mentioned above for the unreduced hydrolyzates.

TABLE I

PARTITIONING OF TRITIUM AND AMINO ACIDS DURING CELLULOSE CHROMATOGRAPHY OF HYDROLYZATES OF NaB^3H_4-TREATED COLLAGENS

Tissue	*Radioactivity (cpm/100 µl)*	*Amino acids [nanomoles (leucine equiv.)/100 µl]*	*Specific radioactivity (cpm/nmol)*
Rat tail tendon			
Mobile phase	62780	349.0	179.9
Water phase	7030	5.0	1406.0
Sheep periodontal ligament			
Mobile phase	25750	269.0	95.7
Water phase	74040	75.0	987.2
Sheep blood vessel			
Mobile phase	9090	659.0	13.8
Water phase	3300	92.0	35.9
Bovine achilles tendon			
Mobile phase	8420	335.9	25.1
Water phase	7755	28.6	271.2

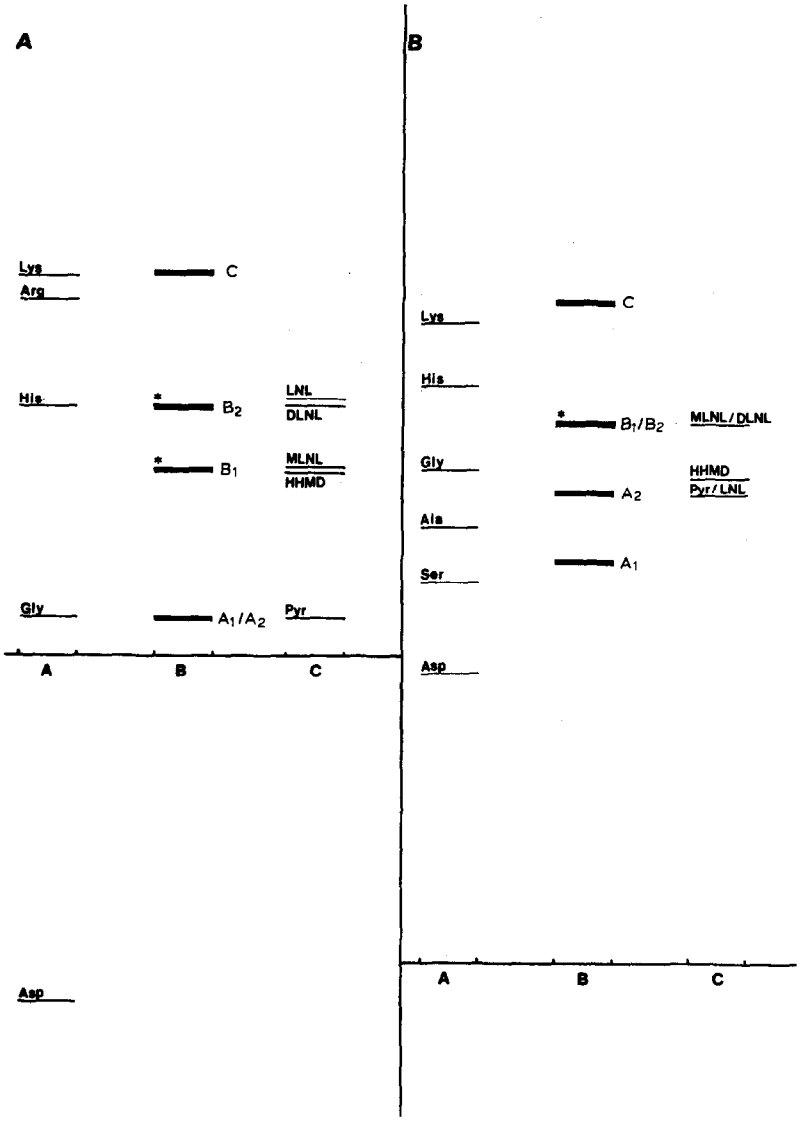


Fig. 2. Electrophoresis of water eluate of cellulose chromatography of ^{3}H-labelled bovine achilles tendon collagen hydrolyzate (lane B), amino acid markers (lane A) and cross-link markers (lane C). See Table II for identification of components in lane B. The paper was stained with ninhydrin–cadmium acetate reagent. An asterisk (*) indicates the position of elution of the tritium label. (A) Electrophoresis was at pH 6.4 for 40 min. (B) Electrophoresis was at pH 1.9 for 40 min.

There were two radioactive species detected, both of which were basic at pH 6.5 with B2 being more basic than B1. However, at pH 1.9, both B1 and B2 had similar mobilities. When these species were electrophoresed preparatively, eluted and chromatographed on the amino acid analyzer, they were identified as MLNL (B1) and DLNL (B2).

TABLE II

CHARACTERIZATION OF WATER ELUATE FROM CELLULOSE CHROMATOGRAPHY OF HYDROLYZATES OF NaB^3H_4-TREATED COLLAGEN

See Fig. 2 for electrophoresis.

	*Mobility at pH 6.5**	*Mobility at pH 1.9***	*Radioactivity*	*UV Absorbance*	*Fluorescence*	*Identity*
A1	0	1.05	–	–	–	Serine
A2	0	1.25	–	+ (295 nm)	+	Pyr
B1	−0.39	1.40	+	–	–	MLNL
B2	−0.55	1.40	+	–	–	DLNL
C	−0.90	1.73	–	–	–	Hydroxylysine

* Mobile relative to aspartic acid. A negative value indicates migration is in the opposite direction to aspartic acid.

** Mobility relative to serine.

In separate experiments, ^{3}H-labelled HHMD and unreduced LNL were added to 40 mg of non-reduced tendon collagen hydrolyzates. Most of the HHMD was insoluble in butanol–acetic acid but over 80% LNL was eluted in the mobile phase (Table III).

When hydrolyzed radioactive collagens were chromatographed on the cellulose column and both the organic eluate and the water eluate were analyzed on the amino acid analyzer for the distribution of radioactivity, peaks appeared in the water phase at the positions expected for the mjaor cross-links (Fig. 3). As expected there was very little radioactivity in the mobile organic phase. The majority of the radioactivity in this solvent was in the vicinity of the hexitol–lysyl and hexitol–hydroxylysyl residues. But as there were labelled species in the same position in the water phase, the partitioning of these derivatives did not seem to be quantitative.

DISCUSSION

In elastin, there are unique interchain cross-links of which the principal ones are the pyridinium-based desmosine and isodesmosine. These structures can be separated from the other constituents in hydrolyzates by cellulose chromatography[8–10]. Most amino acids are soluble in butanol–acetic acid–water mixtures but these compounds are not[19]. For collagen, most of the characterized cross-links are acid-labile, aliphatic compounds that precursors of the more complex non-reducible mature

TABLE III

PARTITIONING OF RADIOACTIVITY IN CELLULOSE CHROMATOGRAPHY OF COLLAGEN SUPPLEMENTED WITH ^{3}H-LABELLED HHMD OR LNL

	Collagen + HHMD (%)	*Collagen + LNL (%)*
Mobile phase	23.7	82.7
Water phase	76.3	17.3

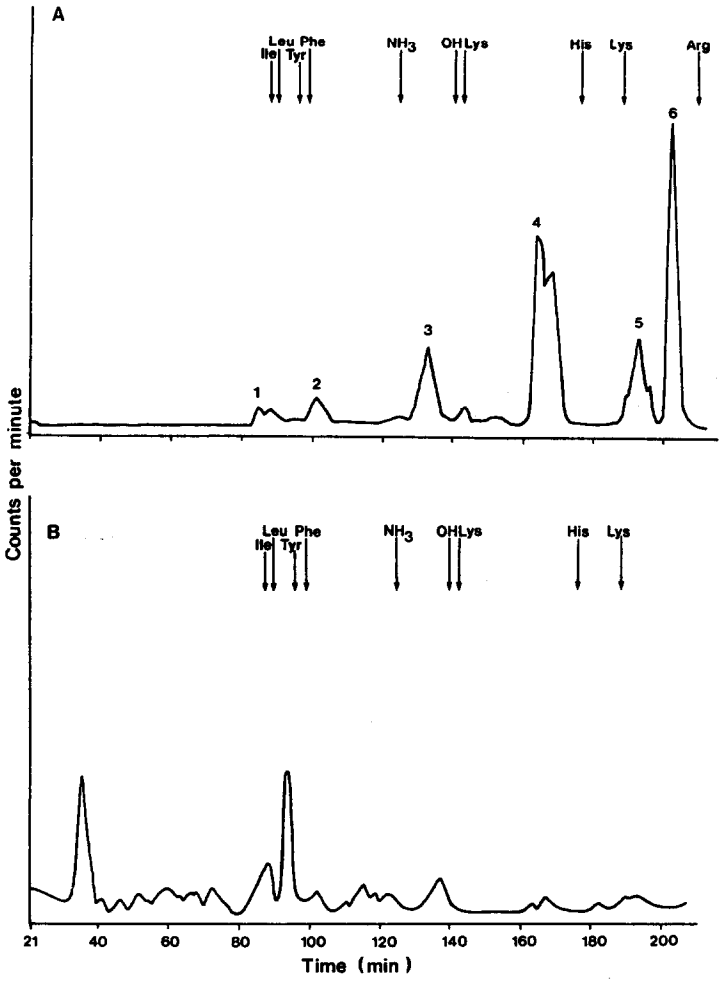


Fig. 3. Ion-exchange chromatography on amino acid analyzer of water phase (A) and mobile phase (B) of ^{3}H-labelled acid hydrolyzate of bovine achilles tendon collagen. Peaks: 1 = hexitol–hydroxylysines, 2 = hexitol–lysines, 3 = MLNL, 4 = DLNL, 5 = LNL, 6 = HHMD.

cross-links[12]. Recently a stable, non-reducible pyridinium compound, pyridinoline, has been identified in a number of collagen hydrolyzates[6,11].

If the insolubility of the desmosines in butanol–acetic acid mixtures is due to the pyridinium nucleus, Pyr might be expected to behave similarly when collagen hydrolyzates are chromatographed. This paper shows that this is indeed so and that the subsequent recovery in the water phase is quantitative. However there are several other components present in this water phase. As well as hydroxylysine and serine, the major cross-links of immature collagen (MLNL, DLNL and HHMD) are present in this eluate. The exception is LNL which is not present in all collagen samples.

The occurrence of the amino acids serine and hydroxylysine in the water eluate is interesting. These, as well as the reducible cross-links, are aliphatic compounds

and are structurally distinct from the pyridinium cross-links. This suggests that there may be two mechanisms of chromatography occurring. One is based on the insolubility of pyridinium compounds in butanol–acetic acid and the second is for hydroxylated aliphatic amino compounds. The presence of most of the LNL in the mobile phase is consistent with this interpretation. This will need further evaluation so as to understand the mechanisms that are of importance in this system.

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REFERENCES

1 K. I. Kivirikko and R. Myllyla, *Methods Enzymol.*, 82 (1982) 245.
2 D. J. Prockop and L. Tuderman, *Methods Enzymol.*, 82 (1982) 305.
3 J. A. Foster, *Methods Enzymol.*, 82 (1982) 559.
4 J. H. Fessler, K. J. Doege, K. G. Duncan and L. I. Fessler, *J. Cell. Biochem.*, 28 (1985) 31.
5 R. J. Boucek, N. L. Noble and Z. Gunja-Smith, *Biochem. J.*, 177 (1979) 853.
6 D. R. Eyre, T. J. Koob and K. P. van Ness, *Anal. Biochem.*, 137 (1984) 380.
7 M. A. Paz, D. A. Keith and P. M. Gallop, *Methods Enzymol.*, 82 (1982) 571.
8 B. C. Starcher and M. J. Galione, *Prep. Biochem.*, 5 (1975) 455.
9 S. J. M. Skinner, *J. Chromatogr.*, 229 (1982) 200.
10 B. C. Starcher, *Anal. Biochem.*, 79 (1977) 11.
11 M. Barber, R. S. Bordoli, G. J. Elliott, D. Fujimoto and J. E. Scott, *Biochem. Biophys. Res. Commun.*, 109 (1982) 1041.
12 N. D. Light and A. J. Bailey, *Methods Enzymol.*, 82 (1982) 360.
13 A. J. Bailey, C. M. Peach and L. J. Fowler, *Biochem. J.*, 117 (1970) 819.
14 J. Sodek and H. F. Limeback, *J. Biol. Chem.*, 254 (1979) 10496.
15 P. F. Davis and Z. M. Mackle, *Anal. Biochem.*, 115 (1981) 11.
16 J. Einbinder and M. Schubert, *J. Biol. Chem.*, 188 (1951) 335.
17 P. M. Gallop and S. Seifter, *Methods Enzymol.*, 6 (1963) 635.
18 J. G. Heathcote and C. Haworth, *J. Chromatogr.*, 43 (1969) 84.
19 S. M. Partridge, D. F. Elsden and J. Thomas, *Nature (London)*, 197 (1963) 1297.