HUMAN AORTA COLLAGENS: EVIDENCE FOR THREE DISTINCT SPECIES*

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SUMMARY

Three different molecular species of collagen and a soluble form of elastin were obtained by digestion of human aortas with pepsin. Two of the three collagens contain 1/2 cystine, present in interchain disulfide crosslinkages, and appear to represent type IV collagen previously described in basement membranes and type III collagen, recently found in fetal skin. The third collagen species is type I, the molecule found in a wide variety of connective tissues including skin, bone, tendon and ligaments.

Pepsin has recently been successfully used on non-lathyritic tissues to isolate both type I and type II collagens because of selective degradation of crosslinked regions in the non-helical portions of the molecule (1, 2). It has also been used to extract the disulfide crosslinked type IV collagen from basement membranes (3), suggesting that investigation of both normal and pathological human tissues in which the collagens are quite insoluble and in which the lathyritic state cannot be induced might be approached by use of peptic digestion. As part of a study of the vascular scarring which occurs in arteriosclerosis we report here initial observations on the collagens and an elastin from normal human aortas.

MATERIALS AND METHODS

Aortas were obtained at autopsy from patients of widely varying ages (20-70),

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who died of non-cardiovascular diseases, and dissected free of adhering fat and separated into inner and outer portions by an arbitrary dissection plane through the media. The heterogeneous population of molecules described below is present in both parts of the preparation. Extraction of homogenized aortas with .16M phosphate buffer pH 7.6; 0.5M acetic acid; 2M Mg_2Cl or chloroform-methanol prior to pepsinization did not increase subsequent yields of collagen. The fresh tissues were homogenized at 4°C in a Sorvall Omni-Mixer in 0.5M acetic acid. Pepsin (2X crystallized Worthington Biochemical Corp.) was added at 20 mg per gm wet weight and the tissues digested for 3 days at 4°C with continuous stirring. The clarified extract was neutralized to inactivate peptic activity and the collagens precipitated overnight at room temperature by addition of sodium chloride to a final concentration of 20%. The precipitate was then collected and resolubilized in 0.16M phosphate buffer, pH 7.6, clarified by centrifugation and the collagens reprecipitated by addition of sodium chloride to 15%. This procedure was repeated 3 times. The final precipitate was collected in 0.5M acetic acid, dialyzed against 0.1% acetic acid and lyophilized.

Chromatography on CM-cellulose was performed as previously described (4) both with and without prior reduction of the denatured collagens with dithiothreitol (DTT). Molecular sieve chromatography was performed on an 8% Agarose gel equilibrated with 1M calcium chloride and .05M tris pH 7.5 (5). Amino acid analyses were done as previously described (6). Carbohydrates were analyzed after methanolysis on a Perkin Elmer Model 900 gas liquid chromatograph (7). Electrophoresis on SDS-polyacrylamide gels (10%), both with and without DTT reduction, was performed as previously described (8).

RESULTS AND DISCUSSION

The solubility of aorta collagens in neutral buffers or weak organic acids is less than 1.0% whereas 15 to 20% is soluble in native triple helical form after digestion

Table I

Amino Acid and Carbohydrate Composition of Aortic Elastin and Collagens*

	Solubilized Elastin	300,000 MW Collagens CMC Fig. 2 Peak 1	300,000 MW Collagens CMC Fig. 2 Peak 2	100,000 MW Collagens CMC Fig. 4 Peak 1	100,000 MW Collagens CMC Fig. 4 Peak 2	100,000 MW Collagens CMC Fig. 4 Peak 3
3-Hydroxyproline	0	4	2	1	3	1
4-Hydroxyproline	9	118	118	102	110	91
Aspartic Acid	8	50	47	43	45	42
Threonine	18	24	21	19	22	16
Serine	12	33	37	38	32	35
Glutamic Acid	26	85	76	82	80	79
Proline	121	86	95	106	103	93
Glycine	261	330	347	340	331	334
Alanine	270	46	77	96	69	89
1/2 Cystine	4	7	3	0	1	0
Valine	116	28	20	24	27	32
Methionine	1	2	8	7	11	8
Isoleucine	24	26	17	12	21	23
Leucine	46	42	27	26	36	40
Tyrosine	38	7	6	2	5	6
Phenylalanine	15	23	13	15	16	15
Hydroxylysine	0	39	15	13	24	18
Lysine	6	12	24	25	19	25
Histidine	1	7	7	5	6	9
Arginine	7	31	40	44	39	44
Isodesmosine	7					
Desmosine	10					
Galactose		4.0	1.1			
Glucose		3.7	1.0			
Mannose		0.2	0.1			

* Values for amino acids in residues/1000. Values for isodesmosine and desmosine in leucine equivalents. Carbohydrate values in gm hexose/100 gm protein.

with pepsin. Repepsinization of tissues which had yielded 15% in the first digestion, solubilized an additional 15%. Repeated digestions to establish the complete extent of solubility have not been done.

The proteins solubilized by pepsin include both collagens and an elastin-like

molecule. The elastin-like protein is relatively soluble compared to the collagens and remained in solution in the neutral phosphate buffer at 15% NaCl and thus was separated from the collagens by the sequential salt precipitations. A typical amino acid composition of this elastin is reported in Table I and demonstrates low levels of acidic and basic residues, high levels of neutral amino acids especially glycine, alanine and valine and the presence of the crosslink compounds isodesmosine and desmosine.

Molecular sieve chromatography of the collagens on 8% Agarose revealed collagenous material eluting at the void volume ($V_E/V_{THO} = .30$) followed by a slightly retarded component ($V_E/V_{THO} = .37$) characteristic of a crosslinked collagen of molecular weight 300,000 (γ). A smaller amount of material eluted in the 200,000 (β) and 100,000 (α) molecular weight ranges. If elastin was not purified away by prior repeated salt precipitation a large broad peak eluted following the 100,000 MW α chain (Fig. 1).

Chromatography of the 300,000 molecular weight collagen on CM-cellulose revealed two incompletely separated peaks of material (Fig. 2). Amino acid analyses of the

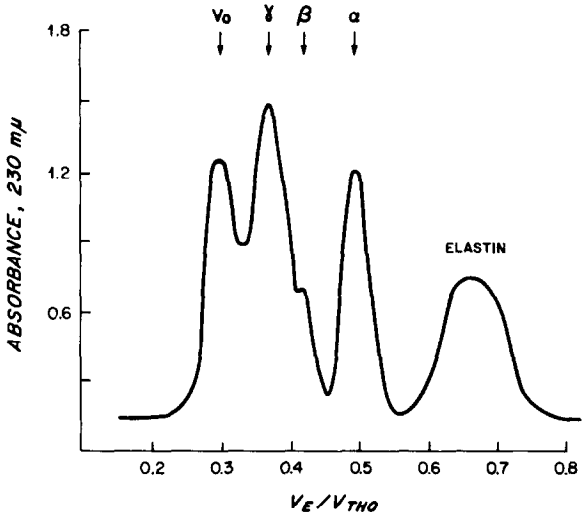

Figure 1: Chromatogram on 8% Agarose (Bio-Gel A 1.5M) of pepsin solubilized human aorta collagens. Elution positions of molecules with approximate molecular weights 300,000 (γ), 200,000 (β) and 100,000 (α) are indicated.

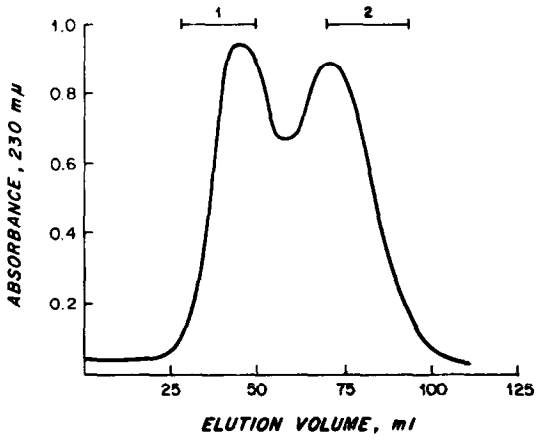

Figure 2: Chromatogram on CM-cellulose of γ fraction from Figure 1. Fraction 1 is principally type IV collagen, fraction 2, type III (Table I).

leading edge of the first peak and the trailing edge of the second peak are listed in Table I and show distinct differences. The first peak contained large amounts of 4 hydroxyproline, isoleucine, leucine and hydroxylysine and small amounts of alanine and arginine thus closely resembling basement membrane collagen (3). Although the second peak also contained unusually large amounts of 4 hydroxyproline and 1/2 cystine, it had less isoleucine, leucine, phenylalanine and hydroxylysine and more arginine and alanine than peak 1. The tentative identity of this material with type III collagen is discussed below. These two collagens also differed significantly in their carbohydrate content (Table I).

Electrophoresis of the 300,000 MW material on SDS-polyacrylamide gels revealed a major band at 1.0 mm and a minor band at 3.0 mm consistent with molecular weights of approximately 300,000 and 200,000 (Fig. 3). Following reduction with DTT the major band migrated to 5.0 mm, consistent with a molecular weight near 100,000, with minor bands at both 1.0 and 3.0 mm (Fig. 3). These data suggest the presence of disulfide interchain crosslinkages, which might be inter and/or intramolecular in nature

The very high molecular weight material excluded from the Agarose column was

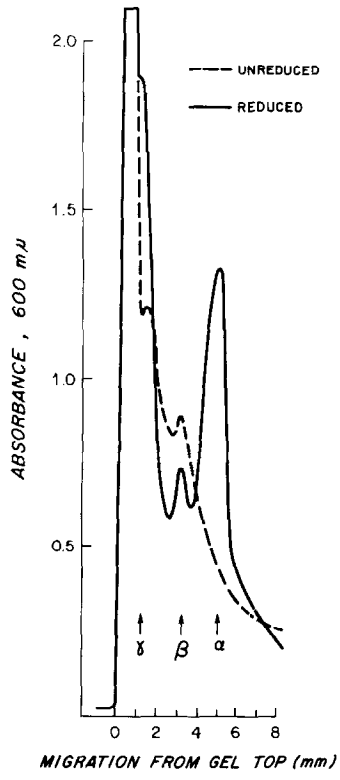

Figure 3: Densitometry scan of Coomassie blue stained SDS-polyacrylamide gels containing γ fraction (Figure 1) before and after DTT reduction.

analyzed by CM-cellulose chromatography and amino acid composition and indicated the presence of the two collagens found in the 300,000 MW fraction.

Chromatography of the 100,000 molecular weight species on CM-cellulose revealed three discrete peaks (Fig. 4) whose analyses are listed in Table I. The first chain to elute resembles the $\alpha 1$(I) described in human skin and the last peak resembles $\alpha 2$. The material eluting between $\alpha 1$ and $\alpha 2$ contains 1/2 cystine and has greater than 50% of the proline residues hydroxylated in the 4 position; thus by similarity of elution position and composition it is most like the material tentatively identified as type III collagen (Fig. 2, peak 2; Fig. 5, peak 3).

Chromatography of the aorta collagens on CM-cellulose immediately after reduction with DTT, but without prior separation on Agarose revealed four overlapping peaks

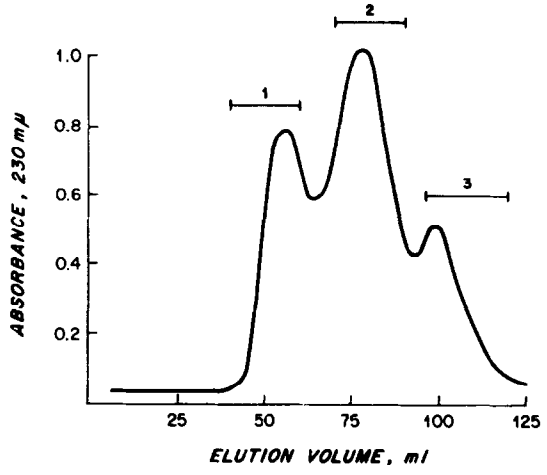

Figure 4: Chromatogram on CM-cellulose of α fraction from Figure 1. The three fractions appear to represent α1(I), α1(III) and α2 (Table I).

Table II

Amino Acid Composition of Aorta Collagens after DTT Reduction and Chromatography of CM Cellulose (Fig. 5)*

	Peak 1	Peak 2	Peak 3	Peak 4
3-Hydroxyproline	3	2	3	1
4-Hydroxyproline	112	106	112	104
Aspartic Acid	46	45	45	41
Threonine	17	17	16	18
Serine	33	33	33	38
Glutamic Acid	90	85	78	82
Proline	88	102	95	98
Glycine	326	335	350	335
Alanine	48	65	78	74
1/2 Cystine	3	3	2	0
Valine	26	26	21	29
Methionine	12	12	11	8
Isoleucine	26	20	20	23
Leucine	44	36	33	36
Tyrosine	10	7	6	6
Phenylalanine	23	19	15	13
Hydroxylysine	47	28	14	18
Lysine	11	17	21	23
Histidine	6	6	7	11
Arginine	29	36	40	39

* Residues/1000 Residues.

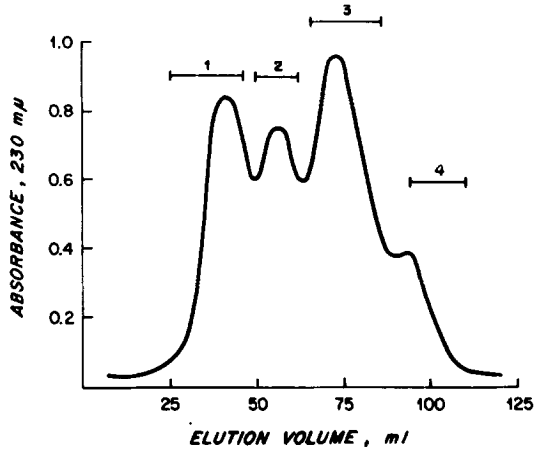

Figure 5: Chromatogram on CM-cellulose of purified aorta collagens after reduction with DTT. Fractions 1 and 3 can be identified as α1(IV) and α1(III) (Table II) despite the incomplete separation.

(Fig. 5). The amino acid composition of these fractions is given in Table II. The composition of the first material to elute (peak 1, Fig. 5) is quite similar to one of the high molecular weight collagens which also eluted first from CMC (peak 1, Fig. 2) and resembles type IV or basement membrane collagen (3). The prominent third peak eluted just before the usual position of α2, is distinctly similar to the other high molecular weight collagen (Fig. 2, peak 2) and closely resembles the recently isolated type III collagen (9, 10).

Complete purification and characterization of these three aorta collagens will presumably be possible by a variety of methods in addition to those used here including fractional salt precipitation, anion exchange chromatography and electron microscopy (11, 12, 13). From the present data, however it is apparent that the human aortic wall contains at least three different molecular species of collagen including type I, which presumably is present as 640 Å cross-striated fibrils; type IV which is probably the basement membrane surrounding the smooth muscle cells in the media; and a third species which represents type III collagen, first detected in human fetal skin by

cyanogen bromide digestion (14). The intact type III molecule isolated here chromatographs on CM-cellulose slightly before $\alpha 2$ and has a sufficiently different amino acid composition from previously described human α chains, to suggest that it represents a unique gene product. Of interest is its content of 1/2 cystine which, forming disulfide crosslinkages, would render it relatively insoluble even under the influence of lathyrogens. The location of this molecule within the tissue, its fibrillar or aggregated form and its biological function are unknown. The cell(s) of origin of these three aortic collagens is also unresolved at present, but it seems likely that medial and intimal smooth muscle cells are one of the principal sources (15). However other cells which should be considered are adventitial and medial fibroblasts as well as the endothelial cells which line the intimal surface.

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