MOLECULAR WEIGHT HETEROGENEITY OF TROPOELASTIN RESULTING FROM PROTEOLYSIS DURING PREPARATION

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1. Introduction

Tropoelastin, the soluble precursor to elastin, is isolated from copper deficient or lathyritic animals by extraction of aortas in neutral or acidic solvents followed by coacervation and fractionation using organic solvents [1–7]. These methods give rise to preparations varying in molecular size from 30 000 to 100 000 daltons [5–7]. The basis of the molecular weight heterogeneity has not previously been resolved. Our data show that proteolysis of tropoelastin by hydrolytic enzymes present and active in the extracts is the principal cause of the heterogeneity. A tropoelastin preparation with a uniform molecular size of 74 000 daltons is obtained under conditions in which the hydrolytic enzyme activity is inhibited.

2. Materials and methods

Aortas from 17-day old embryonic chick aortas were pulse labeled in the presence of β-aminopropionitrile for 30 min with DL-[4,5-^{3}H]lysine and extracts prepared as described elsewhere [4]. The presence of tropoelastin was determined by assay with lysyl oxidase [4]. Fifty μl of 10% sodium dodecyl sulfate (SDS) and 5 μl of mercaptoethanol were added to 0.5 ml of extract from 5 aortas and electrophoresis done in 5% polyacrylamide gels [8]. After electrophoresis the gels were destained, cut into 2 mm slices, digested in 0.25 ml of 30% H_2O_2 at 60°C and the radioactivity in each slice measured by liquid scintillation spectrometry [4]. The molecular weight of the material in peak A (figs. 1 and 3) was calculated from the relative mobilities of α-, β- and γ-chains of collagen and that of peak B from the migration of bovine serum albumin, ovalbumin and pepsin.

The radioactivity in hydroxyproline and hydroxylysine was measured using extracts of aortas labeled with L-[G-^{3}H]proline and L-[U-^{14}C]lysine after hydrolysis in 6 N HCl and separation on a Beckman 120 C amino acid analyzer [4].

3. Results

SDS gel electrophoresis of 0.85% NaCl extracts of

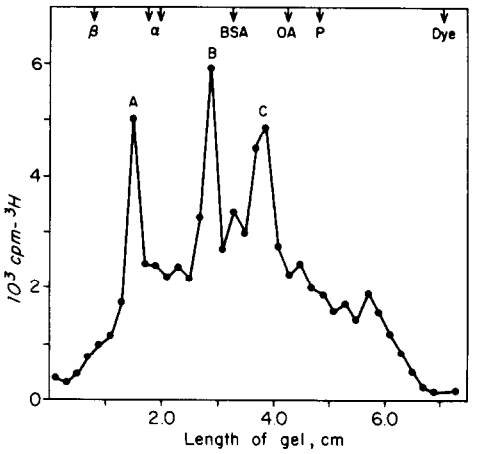

Fig. 1. SDS gel electrophoresis of 0.85% NaCl extract of embryonic chick aortas pulse labeled for 30 min with [4,5-^{3}H] lysine. In addition to the extract each gel contained 25 μg each of lathyritic rat skin collagen, bovine serum albumin (BSA), ovalbumin (OA) and pepsin (P). The mobilities of standard proteins are indicated by arrows.

Table 1
Hydroxyproline and hydroxylysine contents of peaks A, B and C obtained from 0.85% NaCl extracts

Fraction	cpm Hyp	cpm Pro	% Proline Hydroxylation	cpm Hyl	cpm Lys	% Lysine Hydroxylation
Peak A, fig. 1	4188	6320	39.9	193	1287	13.0
Peak B, fig. 1	968	6200	13.5	181	6632	2.7
Peak C, fig. 1	1076	17060	5.9	2	838	0.2
Chick tropoelastin*†	12	148	7.5	0	48	0
Chick skin collagen††	107	118	49.8	7	27	20.6

* Residues/1000.
† Value of Rucker and Goettlich-Riemann [13].
†† Value of Kang et al. [14].

aortas, pulse labeled with radioactive lysine, revealed three major peaks of molecular weights 128 000 (peak A), 74 000 (peak B), and 52 000 (peak C) (fig. 1). Peak A appears to be procollagen. It has a molecular weight comparable to that reported for procollagen [9], 13% of the lysine residues and 39.9% of the proline residues are hydroxylated (table 1) and the material in this peak is converted to collagen α-chains when pulse labeling is followed by incubation in medium containing [^{12}C]lysine (not shown). The material in peak B (fig. 1), with a molecular weight of 74 000, has an electrophoretic mobility comparable to authentic tropoelastin (fig. 3), although it contains hydroxylysine, an amino acid unique to collagen (table 1). Since 2.7% of the lysine residues are hydroxylated, approximately 10.2% of the lysine radioactivity in this fraction appears to derive from collagen (calculated from table 1 using the hydroxylysine and lysine contents of chick skin collagen). The hydroxyproline content of peak B also confirms that collagen components are present; while only 7.5% of the proline in tropoelastin is hydroxylated, 13.5% of the proline in peak B has been hydroxylated (table 1). Peak C has a molecular weight of 52 000. It appears to be free of collagen components and to derive from tropoelastin since it has no hydroxylysine (table 1) and 5.9% of the proline is hydroxylated as expected for tropoelastin.

In aorta extracts stored at 4°C for 24 hr, peak A disappears and the radioactivity in peak B and lower molecular weight components increases (not shown). This observation, along with the demonstration of

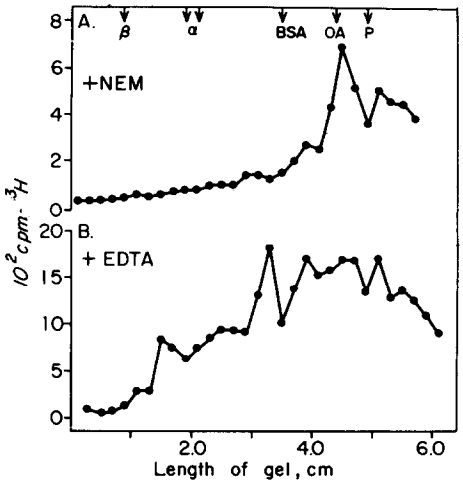

Fig. 2. SDS gel electrophoresis of aorta extracts incubated with (A) 0.001 M *N*-ethylmaleimide. (B) 0.01 M EDTA. The mobilities of standard proteins are indicated by arrows.

collagenous material in peaks A and B indicate the presence of collagenase and other proteinases in the aorta extracts. To study this possibility, extracts were adjusted to 0.05 M Tris (pH 7.5), and 0.005 M $CaCl_2$, and incubated at 37°C for 2 hr in the presence of 0.001 M *N*-ethylmaleimide to inhibit sulfhydryl proteinases [10]. Under these conditions peaks A and B are lost (fig. 2A). However, when incubated in the presence of 0.01 M EDTA, an inhibitor of collagenase, but without *N*-ethylmaleimide, these peaks are preserved (fig. 2B); thus it appears likely that col-

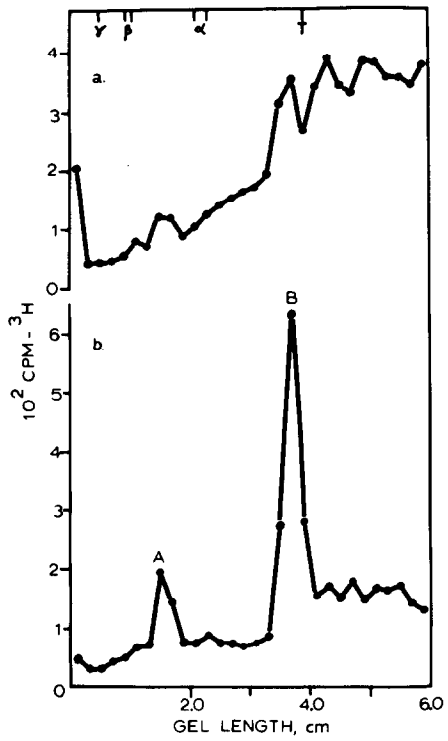

Fig. 3. SDS gel eclectrophoresis of pulse labeled aortas extracted in (a) 0.05 M Tris (pH 7.5) containing 1 M NaCl, 0.001 M DFP, 0.01 M *N*-ethylmaleimide and 0.025 M EDTA; (b) 0.5 M acetic acid. The migration of γ-, β- and α-chains of collagen and authentic chick tropoelastin are indicated.

lagenase is present in the extracts. In the latter case radioactive peaks of molecular weight less than 52 000 also appear, indicating the presence of other proteinases as well.

In additional experiments, aortas were extracted under conditions inactivating collagenase and other proteinases. After pulse labeling, the aortas were immediately homogenized and extracted in either cold, freshly made 0.05 M Tris (pH 7.5) containing 1 M NaCl, 0.001 M DFP, 0.01 M *N*-ethylmaleimide and 0.025 M EDTA [11] or cold 0.5 M acetic acid [9,12]; the extracts were separated and lyophilized. When analyzed by SDS gel electrophoresis the distribution of radioactive material in the extracts containing proteinase inhibitors was very similar to that shown in fig. 1 (fig. 3a). In the acid extract, only two radioactive fractions were present (fig. 3b). Peak A of the acid extract appears to be procollagen because it has molecular weight

Table 2
Hydroxylysine contents of peak A and B obtained from acetic acid extracts

Fraction	cpm Hyl	cpm Lys	% Hydroxylation
Peak A, fig. 3	185	658	21.9
Peak B, fig. 3	0	1882	0.0

138 000, it is converted to collagen α-chains during a cold chase (not shown) and 21.9% of the lysine residues are hydroxylated (table 2). Peak B (fig. 3b) has molecular weight 74 000 and exhibits electrophoretic identity with purified tropoelastin. This peak does not contain collagenous material as demonstrated by the absence of hydroxylysine (table 2). The radioactivity of peak B (fig. 3b) represents 83% of the total of A and B.

4. Discussion

We have shown previously that embryonic chick aortas pulse labeled for 30 min with radioactive lysine in the presence of β-aminopropionitrile synthesize proteins of which tropoelastin is the major component. We have used the radioactive tropoelastin so obtained to investigate the discrepancy in molecular weight of tropoelastin observed by Sandberg et al. [5,6] and Sykes and Partridge [7].

The 0.85% NaCl extract of pulse labeled aorta contains three radioactive proteins. On the basis of the molecular weight of 128 000, the hydroxylysine and hydroxyproline content and the conversion to α-chains during cold chase, peak A appears to be procollagen. Peak B has molecular weight of 74 000 and its electrophoretic mobility corresponds to that of tropoelastin. However, 10.2% of the radioactivity of this peak is collagenous as indicated by the hydroxylysine content and susceptibility to collagenase digestion. From the hydroxyproline content and absence of hydroxylysine, peak C appears to contain mostly tropoelastin-derived material, indicating that in the salt extract of aortas tropoelastin exists in at least two molecular sizes of 74 000 and 52 000. However, when the aortas are extracted in 0.5 M acetic acid, a reagent not conducive to proteinase activity and hence used to

extract procollagen [9,12], only two peaks are present. Peak A (mol. wt. 138 000, fig. 3b) is procollagen. Peak B (mol. wt. 74 000, fig. 3b) comigrates with tropoelastin from other sources; it does not have hydroxylysine and is therefore devoid of collagen components. Smaller molecular weight components are absent. In the acid extract, the radioactivity of peak B constitutes 83% of that of peaks A and B (fig. 3b); this value is in agreement with that which we obtained in our earlier studies showing that 87% of the lysyl oxidase substrate activity present in the aorta extracts is from tropoelastin and the rest from collagen [4].

The above results show that proteinases are present in the aorta extracts and they are able to degrade tropoelastin molecules and lead to the heterogeneity observed by others [5–7]. Buffers containing the proteinase inhibitors DFP, *N*-ethylmaleimide and EDTA have been used to inactivate proteinases when isolating procollagen [11] but these are not as effective as 0.5 M acetic acid for the extraction of tropoelastin. Therefore the use of extractants that inhibit proteinases, coupled with short extraction and purification methods are necessary in order to obtain undegraded tropoelastin.

Acknowledgements

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