DITYROSINE IN A NON-HYDROXYPROLINE, ALKALI-SOLUBLE PROTEIN ISOLATED FROM CHICK AORTA AND BOVINE LIGAMENT[1]

F.W. Keeley, Frank LaBella[2], and Gary Queen

Department of Pharmacology and Therapeutics, University of Manitoba
Faculty of Medicine, Winnipeg, Manitoba, Canada

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Dityrosine, a compound formed by two molecules of tyrosine in biphenyl linkage, was first found in nature by Andersen (1964; 1966) in acid hydrolysates of resilin, a rubber-like protein from the wing ligaments of locusts. Andersen estimated the dityrosine content of resilin to be approximately 8 residues per 1000 total amino acid residues. LaBella et al. (1967) and Keeley and LaBella (1968) identified dityrosine in hydrolysates of chick aortic elastin and estimated the content to be approximately 1 residue per 30,000 total amino acid residues. Using chick embryo aortic tissue cultures they showed that it was derived from tyrosine-^{14}C. Subsequently, LaBella et al. (1968) found traces of dityrosine in a few preparations of highly purified collagens; however, incubation of soluble collagens with horseradish peroxidase and hydrogen peroxide resulted in the rapid formation of rigid insoluble gels and the production of significant amounts of dityrosine, about 1 residue per 5000 total residues. Although dityrosine would appear to be a likely candidate for a cross-linking function in the fibrous proteins, in neither resilin nor elastin has it been established that the precursor tyrosine residues are located

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[2]Medical Research Associate, Medical Research Council of Canada

on different peptide chains. The physical data on peroxidase-treated collagen, however, were indicative of chain-to-chain linkage.

We now report on preparations of an alkali-soluble, non-hydroxyproline-containing protein from aorta and ligamentum nuchae which consistently contain dityrosine at approximately 1 residue per 10,000 and 1 per 40,000 total amino acid residues, respectively.

MATERIALS AND METHODS

Aortas from day-old chicks, cultured chick embryo aortas, or ligamentum nuchae from adult cattle was stripped of fat and fascia, defatted with chloroform:methanol (3:1), twice extracted for 24 hours with 0.2M NaCl at 4°, twice extracted for 24 hours with 0.5% acetic acid at 4°, and autoclaved in water at 110° for 24 hours. To isolate the alkali-soluble protein with which the present investigation is concerned, the residue was extracted four times with 0.1N NaOH at 100°. The first 2 extracts were pooled and exhaustively dialyzed to prepare purified alkali-soluble protein; the protein precipitated in the dialysis bag, but could be redissolved at alkaline pH. The tissue residue after the above extractions was shown by amino acid analysis to be highly purified elastin. Thoracic aortas from 12-day old chick embryos were fixed with blood clots to washed Millipore filters in test tubes containing 2 ml of Eagle's medium (Eagle, 1959). The tubes were incubated at 37° for 2 days, the medium replaced with 2 ml of fresh medium containing 1.4 μC of tyrosine-U-^{14}C (New England Nuclear Corp., 300 mC/mM), and incubated for 3 more days. The tubes were then cultured in the absence of ^{14}C-tyrosine for 6 days with a medium change after 3 days. Protein hydrolysates were analyzed for amino acids and monitored for fluorescence and ^{14}C (LaBella *et al.*, 1967, 1968). Authentic dityrosine was prepared as previously described (LaBella *et al.*, 1967). Protein hydrolysates were also examined by descending paper chromatography, and chromatograms were scanned for ^{14}C with a Nuclear-Chicago Actigraph III.

RESULTS

The four successive alkali extractions of the collagen-free aorta or ligamentum resulted in an exponentially diminishing yield of protein, although the amino acid compositions of the extracts were similar. Further extraction tended to solubilize elastin itself, as evidenced by the presence of hydroxyproline in the soluble fraction. Twenty-three per cent, by weight, of defatted, collagen-free chick aorta was solubilized by the four alkali extracts, but only 3% of similarly treated bovine ligamentum. The amino acid compositions of the alkali-soluble protein from aorta and ligament were very similar (Table I), indicating a common protein in these tissues.

Dityrosine in hydrolysates of the alkali-soluble protein was detected by its characteristic fluorescence and its chromatographic elution from Dowex-50 at various temperatures in comparison to authentic dityrosine (LaBella et al., 1968). There was an insufficient amount of dityrosine to yield a ninhydrin positive peak in column chromatograms. Further confirmation was obtained by showing that a radioactive component in hydrolysates of the tyrosine-^{14}C-labelled protein migrated on paper identically to authentic dityrosine (Figure 1). Estimates, from fluorescence data, of the dityrosine content of the alkali-soluble protein are shown in Table II.

Preliminary studies on this protein preparation extracted with hot 0.1N NaOH show that it is homogeneous by paper electrophoresis and that it has a molecular weight of approximately 12,000 as estimated by gel permeation chromatography.

DISCUSSION

The alkali-soluble protein which we have isolated from chick aorta and bovine ligamentum nuchae appears to be very similar in amino acid composition to glycoprotein preparations extracted in a variety of ways from calf-skin (Bowes et al., 1958), bovine cartilage (Partridge and Elsden, 1961), bovine ligamentum (Gotte et al., 1963), and rabbit skin (Timpl et al.

TABLE I

AMINO ACID COMPOSITION OF ALKALI-SOLUBLE PROTEIN

	bovine ligament	day-old chick aorta
	residues/1000 total residues	
HYPRO	0	0
ASP	102	103
THR	35	37
SER	51	60
GLU	139	125
PRO	87	61
GLY	103	115
ALA	75	85
VAL	73	69
CYS*	8	12
MET*	13	12
ILEU	38	43
LEU	81	93
TYR	31	29
PHE	42	39
LYS	62	54
HIS	17	16
ARG	37	40

* probably underestimates in these acid hydrolysates.

TABLE II

DITYROSINE CONTENT OF ALKALI-SOLUBLE PROTEIN AND ELASTIN

	bovine ligament	day-old chick aorta
	residues/100,000 total residues	
Alkali-soluble protein	2.5	10
Elastin	not detected	3.3

1968). Gotte and Serafini-Fracassini (1963) concluded from electron microscopical observations that the glycoprotein in bovine ligamentum nuchae

served as an amorphous cementing material between thin elastic fibrils which comprised large elastic bundles, and they suggested that the protein was a common constituent of the ground substance in various connective tissues.

Our significant finding concerns the presence of dityrosine, an unusual type of protein crosslink, in this apparently ubiquitous glycoprotein. Dityrosine has previously been identified only in resilin and elastin, two rubber-like proteins. Perhaps the glycoprotein, presumably part of the amorphous connective tissue matrix, also possesses elastic properties, in common with the other dityrosine-containing proteins.

It has been found that aortic elastin only from chick embryos or very young chicks contained detectable dityrosine and that, with time, the dityrosine was apparently converted to compounds which are strongly retained by the Dowex-50 during amino acid analysis (LaBella et al., 1967; Keeley and LaBella, 1968). In the present study, dityrosine was not detectable in elastin from adult bovine ligamentum. Furthermore, the observation that the alkali-soluble protein from the ligamentum contains only

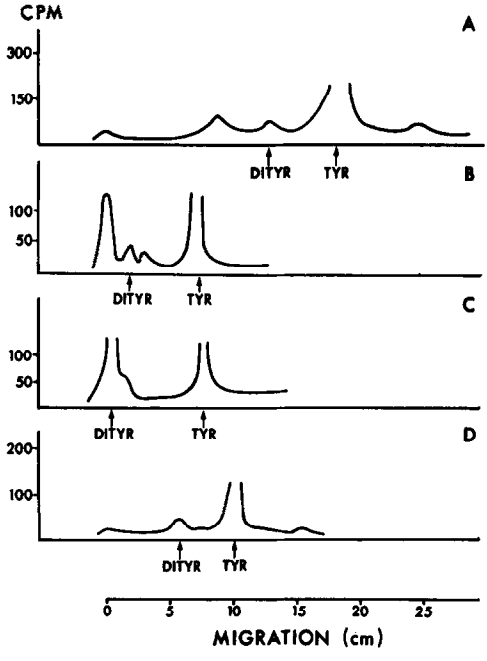

FIGURE 1. Paper chromatography of hydrolysates of alkali-soluble protein prepared from aortas cultured in the presence of tyrosine-C^{14}. Authentic dityrosine was chromatographed in parallel with the hydrolysate on the same paper strip.

A: n-butanol/acetic acid/water (35/30/35)
B: n-butanol/formic acid (88%)/ water (75/10/15)
C: iso-propanol/ammonia/water (8/1/1)
D: solvent C followed by solvent A

one-fourth as much dityrosine as that from day-old chick aorta suggests that a transformation of dityrosine, similar to that occurring in elastin, may occur in the alkali-soluble protein.

Our current investigations are concerned with the biosynthesis, characterization and fate of this interesting dityrosine-containing protein.

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