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Carbohydrate Content of Insoluble Elastins Prepared from Adult Bovine and Calf Ligamentum Nuchae and Tropoelastin Isolated from Copper-Deficient Porcine Aorta

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1. Insoluble elastin has been prepared by several different methods from adult bovine and calf ligamentum nuchae. Highly purified tropoelastin has been prepared from copper-deficient porcine aorta. 2. Amino acid analyses indicated that all preparations, except that obtained from calf ligamentum nuchae by using an EDTA extraction followed by collagenase digestion (preparation E6), were typical of pure elastin having high concentrations of hydrophobic and low concentrations of hydrophilic amino acids. Preparation E6 was found to contain approx. 40% collagen. 3. The determination and composition of the carbohydrates associated with these preparations is reported. With the exception of preparation E6, the insoluble elastins contained only trace amounts of neutral sugars (0.13–0.35%, w/w) and amino sugars (0.01–0.06%, w/w). The porcine tropoelastin contained virtually no carbohydrate. 4. The results suggest that carbohydrate analyses can yield valuable information about the purity of elastin preparations.

The majority of methods used for the isolation and purification of insoluble elastin depend on the marked inertness of this protein to chemical treatments. The removal of the contaminating collagen and ground substance has been attempted by repetitive autoclaving (Partridge, Davis & Adair, 1955), by extraction in hot dilute alkali (Lansing, Rosenthal, Alex & Dempsey, 1952), by extraction in acidic solution (Haas, 1942) or by alternating enzymic digestion with trypsin and collagenase (Hospelhorn & Fitzpatrick, 1961). The success of these methods varies depending on the nature and age of the tissue (LaBella, Vivian & Thornhill, 1966) and with the severity of the extraction procedure (Gotte, Stern, Elsden & Partridge, 1963; Anwar, 1966; Jackson & Cleary, 1967). In all these procedures it is difficult not to envisage at least partial modification of the protein and the criterion of purity generally used has been the constancy of the amino acid composition of the protein isolated (Gotte *et al.* 1963).

The fact that the amino acid composition and properties of elastin may vary considerably depending on the method of extraction and upon the tissue being extracted has given rise to some doubts whether elastin does in fact represent a distinct chemical entity; and whether it is a unique protein common to various elastic connective tissues of any given species and closely related to elastin in other species. Partridge (1962) and Jackson & Cleary (1967) considered it likely that elastin is a single protein or family of related proteins and the application to adult and foetal bovine aorta and ligamentum nuchae of enzymic techniques designed specifically to remove non-elastin components (Steven & Jackson, 1968) has supported this view.

The isolation of purified tropoelastin, the soluble precursor of insoluble elastin (Sandberg, Weissman & Smith, 1969) has also supported this thesis, and has provided a standard amino acid analysis by which it may be possible to assess the purity of insoluble elastin preparations. This assessment may now be extended to judge purity on the basis of carbohydrate content also, for knowledge of any carbohydrate content of tropoelastin will allow a truer evaluation of whether carbohydrate present in insoluble elastin preparations (see review by Ayer, 1964) is an integral part of the molecule or a contaminant associated with the interfibrillar

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amorphous substance (Moret, Serafini-Fracassini & Gotte, 1964).

The present paper reports the determination and composition of carbohydrates associated with four insoluble elastin preparations from adult bovine ligamentum nuchae, two insoluble elastin preparations from calf ligamentum nuchae, and tropoelastin isolated from copper-deficient porcine aorta.

MATERIALS AND METHODS

Materials. Ligamentum nuchae were removed from 3-year-old cows and a 7-day-old calf soon after slaughter.

Crude α-amylase *ex. B. subtilis* (Batch No. F-2625-L) was purchased from Miles Chemical Co., Clifton, N.J., U.S.A. and collagenase from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Ion-exchange resins were purchased from Micro-Bio Laboratories Ltd., 46 Pembridge Rd., London W.11, U.K. Bio-Rad 50W-X8 (200–400 mesh) was cleaned and prepared in the H^+ form according to the method of Moore & Stein (1951) just before use. Bio-Rad AG2-X8 (200–400 mesh; Cl^- form) was converted into the HCO_3^- form by allowing the resin to stand in several changes of saturated $KHCO_3$ solution and then washing with water until Cl^- free.

Activated charcoal purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. was processed according to the method of Thomson (1958). Celite 560 was obtained from Koch–Light Laboratories Ltd. and all other chemicals were supplied by British Drug Houses Ltd., Poole, Dorset, U.K. and were of AnalaR grade whenever available.

Preparation of insoluble elastins. Adult bovine and calf ligamentum nuchae were cut into small pieces and disintegrated in a stainless-steel mill with liquid N_2 as refrigerant (Walser & Bodenloss, 1954). The disintegrated tissue was then defatted by soaking in acetone for 1h, the acetone was removed by centrifugation and the tissues were re-equilibrated with water at 4°C.

Samples of adult and calf tissues were treated with 0.1M-NaOH for 60min at 100°C by the procedure of Lansing *et al.* (1952). The insoluble residues were sedimented by centrifugation and re-extracted with 0.1M-NaOH for a further 20min at 100°C before again being collected by centrifugation. Exhaustive washing of the residues with cold water, then an excess of cold 0.1M-acetic acid and finally acetone drying yielded alkali-insoluble elastin from adult bovine ligamentum nuchae (preparation E1) and calf ligamentum nuchae (preparation E5).

A sample of the adult bovine ligamentum nuchae was autoclaved in water at a pressure of 15lb/in² for 2h. The insoluble material was collected by centrifugation, resuspended in water and autoclaved for a further 2h. The above procedure was repeated for a third 2h period before the insoluble elastin was exhaustively washed with cold 0.1M-NaOH, followed by cold water and finally acetone dried to yield preparation E2.

Samples of adult and calf tissues were extracted with 10% (w/w) EDTA, pH8.0, for 18h at 21°C to enhance the dispersion of collagen fibrils in acetic acid (Steven, 1967). The residues were washed with water and 0.2M-NaCl followed by dispersion of the collagen fibrils in 0.1M-acetic acid. Under these conditions the elastin remained insoluble and unswollen and was recovered by centrifugation at 300*g* for 20min. The insoluble elastin-rich residues were subjected to collagenase digestion employing an enzyme/substrate ratio of approx. 1:50 (w/w). Two further digestions each of 24h duration were carried out with collagenase to remove remaining collagen bound to the elastins. Exhaustive washing of these residues with cold 0.1M-NaOH, then water and finally acetone drying yielded preparation E3 from adult bovine ligamentum nuchae and preparation E6 from calf ligamentum nuchae.

A sample of adult bovine ligamentum nuchae was treated with crude bacterial α-amylase, the collagen fibrils dispersed in 0.1M-acetic acid and the elastin-rich residue twice digested with collagenase and further purified according to the method of Steven & Jackson (1968) to give preparation E4.

Preparation of tropoelastin. Soluble elastin believed to be the precursor of insoluble elastin was prepared from the thoracic aorta of copper-deficient pigs by the method described by Sandberg *et al.* (1969). The protein was further purified by the method of Sandberg (unpublished work). This process involves a series of eight steps that includes solubilization in a water–propan-1-ol–butan-1-ol solution, chromatography on Sephadex G-100 and finally subjection to ion-exchange chromatography in 8M-urea as described by Sandberg *et al.* (1969).

Resin hydrolysis of elastins. About 15–20mg of dried material was refluxed in a 50ml flask fitted with an air condenser (100cm in length) with 10ml of 20% (w/v) Bio-Rad 50W-X8 (H^+ form) suspension in 0.05M-HCl. After hydrolysis the flask and contents were cooled and the suspension quantitatively transferred to a column (40cm × 1.2cm diam.) with a sintered-glass disc at the base. The procedure described by Anastassiadis & Common (1958) for the separation of neutral sugars from the amino acids and amino sugars was followed but with double their volumes of water and 2M-HCl. Hence the neutral sugars were eluted in 50ml of water (eluate I) and the amino acids and amino sugars eluted in 50ml of HCl solution (eluate II).

The optimum time for hydrolysis of elastin was 24h when release of neutral sugar reached a maximum.

Total carbohydrate determination. Eluate I was dried by rotary evaporation at 30°C, dissolved in water (5ml) and uronic acids and their degradation products were removed by passage through a column (10cm × 0.6cm diam.) of 2g of Bio-Rad AG2-X8 (HCO_3^- form). The neutral sugars were eluted with water (20ml), concentrated by rotary evaporation and determined colorimetrically by a modification of Devor's method (Fuller & Northcote, 1956). Results are expressed in terms of mol of galactose which was run as standard.

Hexosamine determination. Eluate II was dried by rotary evaporation at 30°C and final traces of HCl were removed by leaving overnight over NaOH pellets. The residue was redissolved in water (4.5ml) and a 2ml portion (in duplicate) put through the procedure of Cessi & Piliego (1960). Results are expressed in terms of mol of glucosamine which was run as standard.

Sialic acid determination. Acetone-dried material (approx. 50mg) was equilibrated with water (1.5ml) before the addition of 1.5ml of 0.1M-H_2SO_4 followed by

incubation at 80°C for 1h. The residue was centrifuged and samples (0.5ml) of the supernatant were put through the thiobarbituric acid method of Aminoff (1961) with *N*-acetyl neuraminic acid as standard.

Chromatography. The sugars in eluate I were recovered after evaporation to dryness on a rotary evaporator at 30°C. Then 1ml of water was added and the solution passed through a column (10cm × 0.6cm diam.) of 0.5g of a mixture of Celite 560–activated charcoal (9:1, w/w) layered on top of 1g of Bio-Rad AG 50W-X8 (H^+ form) to remove traces of a water-soluble pigment arising from the resin hydrolysis. The sugars were eluted with water (20ml) and freeze-dried before chromatography.

Sugars from all the insoluble-elastin preparations were determined qualitatively by paper chromatography with the conditions described by Grant & Jackson (1968).

The calf elastin preparation E6 was sufficiently rich in sugars to permit quantitative determination of the monosaccharides by gas–liquid chromatography with the conditions described by Grant, Freeman, Schofield & Jackson (1969).

Amino acid analysis. Insoluble-elastin preparations were hydrolysed for 72h at 105°C under N_2 in 6M-HCl and analysed as described by Steven & Jackson (1968).

Tropoelastin was hydrolysed for 20h at 110°C *in vacuo* with redistilled 6.2M-HCl as described by Sandberg *et al.* (1969).

RESULTS AND DISCUSSION

Table 1 reports the amino acid compositions of insoluble elastins from four adult bovine ligamentum nuchae preparations (E1–E4) and two calf ligamentum nuchae preparations (E5 and E6), and also highly purified porcine aortic tropoelastin. The amino acid compositions of insoluble elastins (preparations E1–E5) show close correlation and compare well with those of other authors (Partridge, 1962; Gotte *et al.* 1963; Piez, Miller & Martin, 1965; Anwar, 1966; Serafini-Fracassini & Tristram, 1966). The tropoelastin has an amino acid composition rich in hydrophobic residues and is consistent with the amino acid compositions of the insoluble elastins with the exception of a high lysine content which is converted into the cross-linking desmosine and isodesmosine residues in the insoluble elastin (Partridge, Elsden & Thomas, 1963).

The successful application in preparation E3 of the EDTA–collagenase procedure (Steven, 1967) to facilitate removal of the collagen by dispersion in acetic acid offers obvious advantages over the more severe alkali and autoclaving methods. This procedure is also less time consuming than the crude bacterial α-amylase treatment and avoids the possibility of some non-specific proteolysis that may result from the use of some preparations of crude α-amylase (Bailey & Etherington, 1970).

Calf ligamentum nuchae elastin (preparation E6) prepared by EDTA extraction and collagenase digestion was impure, containing 40% residual collagen based on hydroxyproline analysis. This elastin preparation was subsequently treated again with EDTA and collagenase but the purity of the elastin was not significantly improved (35% residual collagen). The difficulty of purifying both foetal and calf elastins from residual collagen has been reported previously by Cleary, Sandberg & Jackson (1966) and Steven & Jackson (1968). The results of the present study indicate that EDTA is less effective than crude bacterial α-amylase (Steven & Jackson, 1968) as a pretreatment in the preparation of elastin from young bovine ligamentum nuchae.

The carbohydrate contents of these elastin preparations are presented in Table 2. The values are expressed on a molar basis assuming a molecular weight of 67000 for the elastin monomer, tropoelastin (Sandberg *et al.* 1969). The adult bovine ligamentum nuchae preparations contain less than 1mol of neutral sugar/mol of elastin with the exception of preparation E4 which has 1.3mol/mol of elastin. The hexosamine contents of the adult bovine ligamentum nuchae preparations are all considerably less than 1mol of hexosamine/mol of elastin. These values represent neutral sugar contents in the range 0.13–0.35% (w/w) and amino sugar contents of the order of 0.01–0.06% (w/w) which are comparable with the lowest reported values for bovine ligamentum nuchae preparations (Loeven, 1965).

Calf ligamentum nuchae elastin (preparation E5) prepared by hot alkali treatment and known to be chemically pure by amino acid analysis (Table 1) contained only traces of hexoses and hexosamines (Table 2) and was comparable with adult bovine elastin (preparation E1) prepared by the same method. In marked contrast, calf elastin (preparation E6) was grossly contaminated with collagen and contained approximately 4.8 and 0.8 residues of hexose and hexosamine respectively per 67000g.

The possibility of the chemical cleavage of glycosidic linkages cannot be eliminated in the alkali preparatory procedures adopted for preparations E1 and E5 and enzymic cleavage is most likely in preparation E4 since the crude α-amylase is known to contain at least nine glycosidases (Muzzafar, 1968), but it seems unlikely that in the preparation of E2, E3 and E6 any such cleavage should occur.

The porcine tropoelastin contains virtually no carbohydrate, but it should be noted that great care is required in the purification of the tropoelastin for in preparations not subjected to the final ion-exchange procedure the neutral sugar content was found to be approx. 11 mol/mol of elastin. All this sugar was subsequently shown to be glucose which arose from hydrolysis of Sephadex G-100 carried over in the gel-filtration step. Introduction of the final ion-exchange chromatography step yielded a soluble elastin with a neutral sugar content of

Table 1. *Amino acid composition of elastin preparations*

Results are expressed as residues/1000 residues and corrected for hydrolytic losses.

	Adult bovine ligamentum nuchae				Calf ligamentum nuchae		
	E1 (NaOH)	E2 (autoclaved)	E3 (EDTA–collagenase)	E4 (α-amylase–collagenase)	E5 (NaOH)	E6 (EDTA–collagenase)	Porcine tropoelastin
Hydroxyproline	12.9	8.7	11.4	10.2	14.9	Impure elastin,	6.6
Aspartic acid	5.4	6.4	6.2	9.6	6.7	containing 40%	3.3
Threonine	5.3	7.4	7.7	8.7	5.0	collagen	13.2
Serine	6.6	8.7	8.8	10.4	8.1		9.2
Glutamic acid	11.9	15.7	14.3	20.2	14.8		15.8
Proline	96.4	118.4	117.0	117.3	108.0		101.1
Glycine	316.2	310.5	306.5	308.0	313.6		333.4
Alanine	243.1	238.6	242.1	221.9	219.7		237.0
Valine	154.3	143.9	142.9	139.9	149.2		125.4
Methionine	0.0	Trace	Trace	0.0	Trace		0.0
Isoleucine	24.6	23.3	24.5	26.2	26.2		16.1
Leucine	63.0	59.5	60.5	59.0	58.5		47.1
Tyrosine	13.0	10.7	12.9	17.2	18.2		14.1
Phenylalanine	32.1	28.6	31.7	29.9	28.1		28.3
*Isodesmosine	2.8	2.9	2.4	3.6	4.7		0.0
*Desmosine	4.9	5.5	4.0	6.5	5.9		0.0
*X_4	0.7	Trace	Trace	Trace	3.4		0.0
Ornithine	1.3	0.4	Trace	1.7	2.0		0.0
Lysine	3.1	3.1	2.5	3.6	7.5		45.1
Arginine	2.7	7.7	4.6	6.1	5.7		4.3
Total	1000.3	1000.0	1000.0	1000.0	1000.2		1000.0

* Calculated as lysine equivalents.

Table 2. *Carbohydrates associated with elastin preparations*

Neutral sugar values are expressed in terms of a galactose standard and hexosamine values in terms of a glucosamine standard.

Preparation	Mol of hexose/mol of elastin*	Mol of hexosamine/mol of elastin*
Adult bovine ligamentum nuchae		
E1 (NaOH)	0.49	0.05
E2 (autoclaved)	0.56	0.06
E3 (EDTA–collagenase)	0.92	0.37
E4 (α-amylase–collagenase)	1.30	0.23
Calf ligamentum nuchae		
E5 (NaOH)	0.24	0.04
E6 (EDTA–collagenase)	4.77	0.78
Porcine tropoelastin	0.15	0.10

* Calculated assuming a mol.wt. of 67000 for tropoelastin (Sandberg *et al.* 1969).

0.15mol and amino-sugar content of 0.10mol/mol of tropoelastin. Expressed on a dry weight basis these values represent contents of $<0.05\%$ (w/w) and $<0.03\%$ (w/w) of neutral and amino sugar respectively. Thus tropoelastin can be said to have no carbohydrate as an integral part of the tropoelastin molecule.

The identity and relative concentrations of sugars found to be associated with the insoluble elastin preparations E1–E5 were determined by paper chromatography. Galactose and glucose were present in all five preparations as the major components. Mannose was found in all except preparation E1 and traces of fucose were detected in all five preparations. These sugar analyses show a close correlation with the sugars of the sodium chloride-soluble glycoprotein associated with autoclaved elastin of young ox ligamentum nuchae (Moret *et al.* 1964).

Calf ligamentum nuchae preparation E6 was sufficiently rich in carbohydrate to permit quantitation of the individual monosaccharides by gas–liquid chromatography. The molar proportions of glucose:galactose:mannose:fucose were found to be 1.00:1.09:0.32:0.20 respectively. Preparation E6 also contained 0.40mol of sialic acid/mol of elastin. Amino acid analysis indicated that this preparation contained approx. 40% collagen and since previous work (Grant *et al.* 1969) has shown foetal calf ligamentum nuchae collagen preparations to contain considerable amounts of all these sugars and in similar molar proportions to those found in preparation E6, it is possible to attribute all the sugar of preparation E6 to the contaminating collagen.

The above observations and the fact that tropoelastin has been shown to contain no carbohydrate suggest that any carbohydrate associated with insoluble elastin is likely to be contamination of the elastin fibrils by the ground substance and/or collagen.

The ease of purification of elastin obviously depends on the success of particular methods in removing the non-elastin components and the relative proportions of elastin and non-elastin components in the tissue being extracted. Adult bovine ligamentum nuchae with its high elastin content of 60–70% (w/w) yields a purer elastin preparation than extraction of calf tissue which has a higher proportion of collagen and ground substance (Cleary, Sandberg & Jackson, 1967); and the problem increases when even younger foetal elastins are purified (Cleary *et al.* 1966). However, the major difficulty is in assessing purity and establishing criteria by which to judge the purity of the elastin preparation (Jackson & Cleary, 1967). It may be that the comparison of the amino acid content of unknown elastins with the amino acid composition of the purified porcine tropoelastin will become one criterion, although the possibility of species variation remains to be investigated. The absence of any carbohydrate in the porcine tropoelastin and the very low carbohydrate contents of insoluble elastins isolated by four different methods from adult bovine ligamentum nuchae suggest that carbohydrate analyses can also yield valuable information on the purity of elastin preparations.

The fact that tropoelastin contains no carbohydrate also raises interesting questions about the general applicability of the theory that secreted proteins require a carbohydrate moiety to facilitate transport of the protein through the cell membrane (Eylar, 1965). It is unlikely that any of the procedures used in the isolation of the tropoelastin would cleave any glycosidic linkages. Most of the porcine tropoelastin is probably extracted from the extracellular space so that the possibility of extracellular enzymic removal of a carbohydrate moiety cannot

be eliminated. Alternatively the secretion of tropoelastin may be facilitated by its close association with glycoprotein.

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