Salt-Soluble Elastin from Lathyritic Chicks

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The isolation of a salt-soluble homogeneous elastin from the aortas of lathyritic chicks by chromatography on DEAE-cellulose and salt precipitation is described. These new techniques, as well as some previously published by other workers, were evaluated with the help of antiserum raised in sheep against insoluble chick elastin. The purified elastin was very basic and behaved in a predictable manner in coacervation studies. The protein migrated in sodium dodecyl sulphate–polyacrylamide gels as a single band moving slightly faster than pyruvate kinase (mol.wt. 57000).

Soluble elastin, an un-cross-linked monomeric form of the stable fibrous protein elastin, has been isolated in small amounts from the aortas of pigs (Smith *et al.*, 1968) and chicks (Rucker & Goettlich-Riemann, 1972) maintained on diets deficient in copper and also from chicks fed on the lathyrogen, β-aminopropionitrile (Sykes & Partridge, 1972). Both regimes are thought to decrease the enzyme-catalysed oxidation of peptide-bonded lysine residues and in this way prevent the formation of some of the lysine-derived covalent cross-links between adjacent polypeptide chains (Partridge, 1965; Pinnell & Martin, 1968). The soluble protein has been isolated from the aorta walls of copper-deficient or lathyritic animals by modifications of systems involving initial extraction of the tissue with either dilute formic acid or neutral low-salt buffers. The product prepared from acid-extracted pig aortas was heterogeneous in terms of molecular weight whereas the salt-soluble protein from the same tissue, prepared by milder procedures, was homogeneous by sedimentation-equilibrium centrifugation and polyacrylamide-gel electrophoresis (Smith *et al.*, 1972).

Though acid-extracted material from lathyritic chick aortas showed a degree of molecular-weight homogeneity on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, the protein stained poorly with Coomassie Blue or Naphthalene Black and required labelling with ^{125}I for effective location in the gel (Sykes & Partridge, 1972). This behaviour, coupled with the demonstration of a component in neutral salt extracts of lathyritic chick aortas that was serologically identical with purified soluble elastin (B. C. Sykes & J. W. Chidlow, unpublished work), encouraged the investigation of milder preparative techniques. Radial immunodiffusion (Fahey &

McKelvey, 1965) permitted quantitative evaluation of each step.

Experimental

Materials

β-Aminopropionitrile fumarate was obtained from Ralph N. Emmanuel (Wembley, Middx., U.K.). DEAE-cellulose was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.

Methods

Determinations of hydroxyproline were performed by using a Technicon automated system (Grant, 1964). Samples (0.2ml) of neutral salt extracts from lathyritic chick aortas were hydrolysed, neutralized and diluted by the method of Etherington (1972). The protein contents of soluble fractions were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the protein standard. Except where stated, all experiments were performed at +5°C.

Preparation of neutral salt extracts. Lathyritic and control chicks were raised and the aortas and major arteries removed as previously described (Sykes & Partridge, 1972). The cleaned and weighed tissues were homogenized (Polytron, Northern Media Supply Co., Brough, E. Yorks., U.K.) for 3 × 30s with 10vol. of neutral salt buffer [0.02M-sodium phosphate (pH7.4) – 0.5M-NaCl – 5mM-β-aminopropionitrile]. After 24h of continuous stirring, undissolved material was removed by centrifugation (1h at 40000g in an MSE Superspeed rotor no. 59596).

Preparation of insoluble elastin. Insoluble elastin was prepared from the pellet. The material was defatted with ethanol and acetone (Partridge *et al.*, 1955) and dried in a Büchner funnel. The dry material was added to 0.1M-NaOH (10ml/g) kept at 96°C in a boiling-water bath. After 10min the material was filtered and the process repeated twice before final

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washing with copious amounts of hot distilled water.

Amino acid analysis. Proteins were hydrolysed in sealed tubes with 6 M-HCl for 24 h at 105°C. Compositions were determined on a Locarte automatic amino acid analyser.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The migration of sodium dodecyl sulphate–elastin complexes was studied by using the apparatus and methods described by Sykes & Bailey (1971). Soluble elastin (100 μg) was run in an adjacent track to a mixture of proteins of known molecular weights.

Isoelectric focusing. Focusing in a 110 ml capacity column (LKB no. 8101) was used to measure the isoelectric point of the purified soluble elastin. The protein focused very near the cathode with a wide-range ampholyte (pH 3–10) and a narrower range (pH 9–11) was used for most studies. The gradient was stabilized with sorbitol according to the manufacturer's instructions and the column cooled to 5°C with a water jacket. The cathode was at the base of the column. Fractions (1.5 ml) were collected and the pH of each fraction was determined by using a single-probe electrode (Pye no. 78).

pH-dependence of coacervation temperature. Soluble elastin (10 mg) was dissolved in 5 ml of an equal-volume mixture of 0.01 M-disodium phosphate and 0.01 M-acetic acid and the pH adjusted to 11.5 with the minimum quantity of 10 M-NaOH injected with a drawn-out Pasteur pipette into the protein solution. The cool solution was continuously stirred in a 10 ml beaker which was surrounded in ice and the pH monitored with a single-probe electrode meter (Pye model 58).

A small sample was drawn into a 20 μl capacity melting-point tube and both ends of the tube were sealed in a bunsen flame. The pH of the solution was lowered to 2.5 by adding 6 M-HCl at noted intervals of approx. 0.5 pH unit and the sealed melting-point tubes kept in ice. The remaining solution was re-adjusted to pH 11.5 with 10 M-NaOH and 5 ml was withdrawn. The salt concentration was increased to an ionic strength of approx. 0.1 by adding 26.3 mg of solid NaCl. The pH was lowered and samples were withdrawn as before.

The sealed tubes containing the samples of soluble elastin solution were fixed to a Perspex former with Plasticine and suspended in a water bath maintained at +2°C with a refrigerated 'cold-finger'. The glass walls were scrupulously cleaned to allow a clear view of the tubes illuminated with diffuse daylight. The cold-finger was removed and the water heated slowly with an immersed element. A stirring paddle kept the bath well mixed. The temperature at which the samples started to become opalescent was noted.

Preparation of soluble elastin. 1. Precipitation of collagen. Collagen was precipitated from the neutral salt-soluble extract of lathyritic chick aortas by acidi-

fication to pH 4 with acetic acid (Lewis & Piez, 1964). The flocculent precipitate was removed by centrifugation (10000 g for 1 h in an MSE 18 rotor no. 69179).
2. Coacervation. Soluble elastin was coacervated by neutralizing the supernatant from step 1 and raising the salt concentration to 1.0 M (Smith *et al.*, 1972). A precipitate formed after the mixture had been left for 1 h at 37°C, and it was removed by centrifugation (10000 g for 1 h at 37°C in an MSE 18 rotor no. 69179).
3. Salt precipitation. The cold supernatant from step 1 was neutralized and the salt concentration raised to 15% (w/v) by addition of solid NaCl. The flocculent precipitate, which formed after the mixture had been left standing, was collected by centrifugation (20 min at 2000 g in an MSE 18 rotor no. 69179).

The pellet was resuspended in cold neutral salt buffer [0.02 M-sodium phosphate (pH 7.4)–0.5 M-NaCl] and stirred in the cold overnight. Undissolved material was removed by centrifugation at 10000 g (MSE 18) and the precipitation step repeated twice. The pellet from the final precipitation step was dissolved in 0.01% (v/v) acetic acid, dialysed against the same solution and freeze-dried.

DEAE-cellulose chromatography. Portions (50 ml) of neutralized supernatant from step 1 were dialysed against 0.05 M-Tris–HCl buffer (pH 7.5)–0.2 M-NaCl–50 mM-β-aminopropionitrile and then concentrated fivefold with Carbowax 4 M (G. T. Gurr Ltd., Searle Scientific Services, High Wycombe, Bucks., U.K.). The non-diffusible material was applied to a column (20 cm × 2.2 cm) of DEAE-cellulose previously equilibrated with the same buffer. The column was developed with 150 ml of starting buffer. Acidic proteins were eluted by 100 ml of the same buffer with the salt concentration increased to 1.0 M. This is essentially the same method as that used by Miller (1971) to remove acidic glycoproteins from preparations of cartilage collagen.

Assay of soluble elastin. The concentrations of soluble elastin in neutral samples were determined by the radial diffusion method of Fahey & McKelvey (1965). Agarose (1%, w/v) was dispersed in neutral saline [0.02 M-sodium phosphate (pH 7.4)–0.5 M-NaCl–50 mM-β-aminopropionitrile] and 10 ml portions were cooled to 56°C. Volumes (1 ml) of 6 week antiserum (B. C. Sykes & J. W. Chidlow, unpublished work) were warmed to the same temperature and thoroughly mixed with the agarose suspension before pouring on to glass plates [7.6 cm × 7.6 cm (3 in × 3 in)] (Kodak Ltd., Kirby, Liverpool, U.K.). Holes (36, each 3 mm diam.) were cut in the hardened gel with a stainless-steel former. Standards were prepared by two times serial dilution of samples of purified soluble elastin. The wells were filled with duplicates both of the samples to be examined and the standards covering the required range of concentrations.

After standing overnight rings developed around those wells which contained soluble elastin. Their

diameters were measured with a specially adapted rule while the gel was illuminated by diffuse light. The measured diameters of duplicates were generally within 0.1 mm and results outside this limit were disregarded. A standard curve was drawn on four-cycle logarithmic graph paper, plotting ring diameter on the linear scale and concentration on the log scale. The concentration of antigen in each sample was computed from the straight-line standard plot.

Results and Discussion

Fig. 1 shows the concentration of hydroxyproline and elastin in neutral salt extracts of aortas from chicks raised on control or lathyrogenic diets for different times. The pooled aortas from ten birds were used for each determination. The parallels between the two parameters in the lathyritic situation indicate the similarity between the optimum conditions for the accumulation of un-cross-linked collagen and elastin. The decrease in yield after 8 days coincides with the time when the chicks begin to lose weight. Subsequent experiments used tissue from chicks killed between 5 and 8 days.

The maximum yield of 7.5 mg/g of wet tissue was considerably higher than the yield (1–2 mg/g) that was obtained from neutral salt extracts by using the method of Smith et al. (1972) and this encouraged an investigation aimed at further improvements in yield. A neutral salt extract from 1068 aortas (57.6 g) was prepared and acidified to precipitate collagen. The yield and purification of this step was calculated from measurement of elastin and protein concentration and is shown in Scheme 1. The high yield indicated that very little soluble elastin was removed by this step and the subsequent operations described here used the neutralized supernatant as the starting material. When soluble elastin was purified from this supernatant either by coacervation (step 2) or salt precipitation (step 3) the yields were always low.

The amino acid compositions (Table 1) of the considerable amount of material precipitated by either method which did not redissolve in neutral low-salt buffers suggested by their high contents of valine, proline and glycine that they might contain soluble elastin. High values of aspartic acid, glutamic acid, threonine and serine suggested the additional presence of glycoproteins. Miller (1971) found that glycoproteins bound strongly to cartilage collagen and prevented the soluble collagen precipitated by salt during the purification procedure from redissolving in dilute acetic acid. Similarly, in this investigation, passage of the starting material through a column of DEAE-cellulose in low-salt buffers (Fig. 2) removed interfering acidic proteins and substantially increased the yield of purified soluble elastin obtained by coacervation or salt precipitation (Scheme 1). Moreover, the purified materials had lower contents of aspartic
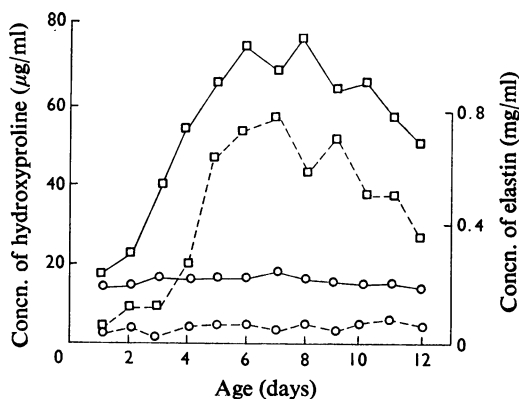

Fig. 1. *Hydroxyproline and elastin contents of saline extracts from aortas of lathyritic (□) and control (○) chicks of different ages*

——, Hydroxyproline and –––, elastin concentrations determined by radial immunodiffusion.

acid (Table 1), indicating a higher degree of purity of the end product. Scheme 1 summarizes the improvements in yield achieved by ion-exchange chromatography and salt precipitation. It was material purified from further batches by this method that was used in the experiments with salt-soluble elastin described below.

Before passing to description of the physical properties of the purified salt-soluble elastin a comparison of its composition with that of fibrous elastin from the aortas of control and lathyritic chicks is of interest. Two features of the data in Table 1 require comment: (1) the hydroxyproline contents estimated for the samples of fibrous elastin are considerably higher than those for salt-soluble elastin; (2) the contents of free lysine plus the quarter-desmosine residues contained in the cross-linking amino acids (Partridge, 1965) are together equivalent to about 10–15 lysine residues/1000 residues in mature fibrous elastin, whereas in the salt-soluble protein the lysine content is about 45 residues/1000 residues. It is clear that more than 30 residues of lysine remain unaccounted for if the salt-soluble protein is the direct precursor of fibrous elastin. Both these features were also observed during the study of soluble elastin from copper-deficient chicks reported by Rucker & Goettlich-Riemann (1972) and satisfactory explanations of either the high hydroxyproline content of fibrous elastin or the discrepancy in the number of lysine residues recovered are not yet forthcoming.

It is clear that the purification of soluble elastin is now a much more satisfactory process than present methods for the purification of the insoluble cross-linked protein from elastin fibres. The isolation of

Lathyritic aortas

Extraction in neutral salt buffer [0.02 M-sodium phosphate (pH 7.4)–0.5 M-NaCl– 5 mM- β-aminopropiononitrile] at 5°C. Centrifugation at 40 000 g for 1 h

Precipitate   Supernatant

Acidification to pH 4.0
Centrifugation at 40 000 g for 1 h

Precipitate   Supernatant

Yield = 85%
Purification = 3.2-fold

Passage through DEAE-cellulose
in 0.2 M-NaCl buffer

Yield = 78%
Purification = 1.1-fold

	Salt precipitation	Coacervation	Salt precipitation	Coacervation
	Yield = 62%	Yield = 32%	Yield = 22%	Yield = 19%
	Purification = 3.6-fold	Purification = 3.7-fold	Purification = 3.8-fold	Purification = 3.9-fold
Yield from 1 g of tissue (mg) ...	3.8	2.0	1.7	1.5

Scheme 1. *Summary of preparative procedures giving yield and purification at each step*

fibrous elastin requires the use of hot dilute NaOH or other vigorous procedures which may result in degradation and loss of some of the lysine-derived cross-linking amino acids. Degradation during acid hydrolysis may account for further losses of lysine-derived amino acids and appears to be accompanied by the formation of coloured substances which tend to interfere with the estimation of hydroxyproline. It should also be observed that mature fibrous elastin, but not salt-soluble elastin, is yellow in colour in its native condition and has a marked blue–white fluorescence. It is possible that the fluorescent pigments may arise by side reactions of the process of condensation of free lysine and lysine-derived aldehydes that is responsible for the biosynthesis of stable intermolecular cross-linkages in both elastin and collagen.

The electrophoretic migration of soluble elastin complexed with the anionic detergent sodium dodecyl sulphate was studied by using the procedures already given. A single band, migrating a little faster than pyruvate kinase, was stained by Coomassie Blue. The clearly visible band contrasted with the staining behaviour of the material purified by the acid–alcohol method (Sykes & Partridge, 1972) which could be located only by labelling with ^{125}I before electrophoresis. The reference proteins were: (1) phosphorylase, 100000; (2) pyruvate kinase, 57000; (3) creatine kinase, 41000; (4) triose phosphate isomerase, 27000; (5) myoglobin, 17000. The molecular weight of the soluble elastin estimated by using the standard semilog plot was 56000.

The demonstration of the salt-soluble elastin as a single band visible on staining of the gel showed that a single molecular species had been isolated with no detectable contamination from covalently cross-linked aggregates which would have migrated less far into the gel.

Table 1. *Amino acid compositions (residues/1000 residues) of soluble and insoluble elastin fractions prepared by different procedures*

See the text for details.

Amino acid compositions (residues/1000 residues)

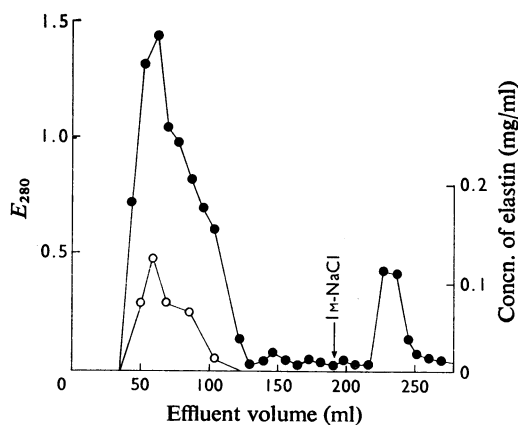|	Coacervation			Salt precipitation				
	Without DEAE-cellulose step		With DEAE-cellulose step	Without DEAE-cellulose step		With DEAE-cellulose step	Insoluble elastin	
	Soluble material	Insoluble material		Soluble material	Insoluble material		Lathyritic	Control
Hydroxyproline	15	9	12	12	16	12	24	25
Aspartic acid	7	27	4	11	42	3	4	3
Threonine	11	47	11	13	30	9	9	7
Serine	13	52	15	15	39	11	7	5
Glutamic acid	16	37	17	18	38	14	14	13
Proline	130	110	127	121	80	129	121	123
Glycine	317	214	321	315	213	322	340	346
Alanine	168	132	173	167	151	173	175	176
Cystine	0	2	0	0	4	0	0	0
Valine	170	109	173	171	112	178	163	170
Methionine	0	3	0	0	5	0	0	0
Isoleucine	18	41	20	22	36	19	19	20
Leucine	49	88	51	53	70	49	55	59
Tyrosine	9	16	8	9	16	8	14	14
Phenylalanine	17	42	18	19	56	20	21	21
Desmosine	0	0	0	0	0	0	2	2
Histidine	0	11	0	0	10	0	0	0
Lysine	43	40	45	44	55	46	14	3
Arginine	6	22	6	7	31	6	6	5
Total	989	1003	995	997	1004	999	988	990

Fig. 2. *Elution profile of neutralized extract after precipitation of collagen*

Stepwise elution with first 0.2M- and then 1.0M-NaCl (change at arrow). ●, E_{280}; ○, elastin concentration determined by radial immunodiffusion.

pH-dependence of coacervation temperature

The phenomenon of reversible coacervation was demonstrated in oxalic acid-solubilized elastin by Partridge *et al.* (1955). When the pH-dependence of coacervation temperature was investigated in buffers of two different ionic strengths two differently shaped curves resulted. These are reproduced in Fig. 3. At low ionic strength ($I = 0.01$) a definite minimum was observed at pH 5.2, whereas at higher salt concentrations ($I = 0.1$) the curve was flattened and displaced.

The equivalent curves for purified salt-soluble elastin show a large shift of the minimum in low salt to the region of pH 10. The pI of purified material was determined as 9.8 by isoelectric focusing, a reflexion of the marked preponderance of basic amino acids, especially lysine, in the soluble elastin. The coincidence of the minimum with the isoelectric pH is explained well by the proposed mechanism of coacervation. This postulates the aggregation of polypeptide chains owing to the increase of hydrophobic interaction as the temperature rises and the simultaneous decrease in the intensity of the hydrophilic
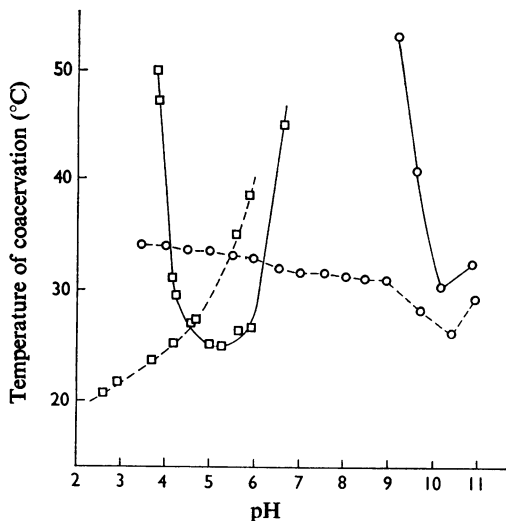
Fig. 3. *pH-dependence of coacervation temperature in low* (——) *and high* (————) *ionic strength buffers of soluble elastin* (○) *and oxalic acid-solubilized elastin* (□) *from bovine ligamentum nuchae*

interaction between charged residues and the solvent. The latter interaction being minimal when the net charge is zero, the temperature at which hydrophobic interaction between adjacent chains is sufficient for a visible aggregation is lowest at the isoelectric pH.

Equally, when the hydrophilic interactions between the protein and solvent are decreased by higher salt concentration, the coacervation temperature is decreased throughout the whole range of pH and the minimum becomes less marked. This observation provided the basis for raising the salt concentration in order to decrease the coacervation temperature to below 5°C and precipitate elastin in the cold.

An aspect of the method is that the judgement of the first appearance of opalescence is subjective and, because of this, only limited importance can be attached to the exact temperatures quoted. However, the order in which the solutions in different tubes coacervated, which is the important factor governing the shapes of the curves, is relatively easy to determine by visual inspection.

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