CARDIOVASCULAR STUDIES ON COPPER DEFICIENT SWINE. XII. PARTIAL PURIFICATION OF A SOLUBLE PROTEIN RESEMBLING ELASTIN

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Defective elastogenesis in copper deficiency is indicated by reduction in the elastin content of blood vessels and by alteration in the mechanical properties, increase in solubility and alteration of the amino acid content of the isolated elastin (Kimball et al., 1964; Weissman et al., 1963; O'Dell et al., 1966; Weissman et al., 1965). At the same time there is accumulation of an excess of salt-soluble protein. A hitherto unreported property of the soluble protein is its reversible coacervation by warming to room temperature. Advantage has been taken of this property to separate and partially purify a cold salt-soluble protein which has an amino acid composition that indicates its probable relationship to elastin. Inasmuch as it lacks the known cross-linkages of elastin it may represent a soluble elastin precursor.

MATERIALS AND METHODS

Copper deficiency was induced in pigs as described earlier (Weissman et al., 1965), and controls were reared on the same basal diet.

Preparation of Coacervated Protein. The aortas of 5 deficient pigs sacrificed at 96-98 days were dissected free of adventitia, and 48.4 gms of the combined tissue were ground in a Latapie grinder and stirred at 4°C for 15 hrs. in 290 ml of 0.015 M phosphate buffer (pH 7.4) containing 0.985 M NaCl. The mixture was filtered through 400 mesh nylon netting and the filtrate was centrifuged at 100,000 x g for 30 minutes at 5°C.

The supernate was filtered and allowed to stand until it reached 23°C. A fine coacervate was collected by centrifuging at 22,000 x g for 1 hour at

23°C. The precipitate was resuspended in 290 ml of the buffer and redissolved by stirring at 4°C overnight. The solution was again warmed to 23°C and centrifuged again at 20,000 x g for one hour. The precipitate was redissolved in 15 ml of 0.3 M KCl containing 0.05 M $MgCl_2$ and 0.05 M phosphate buffer at pH 7.4. The protein concentration of this solution was 5 mg/ml.

Preparation of Elastin. Insoluble elastin was prepared from control and copper deficient aortas by autoclaving (Weissman et al., 1963). Alpha elastin was prepared by partial hydrolysis of control insoluble elastin with 0.25 M oxalic acid (Partridge et al., 1955) followed by desalting on a G-10 Sephadex column (1.5 x 30 cm). The desalted α-elastin was then lyophilized.

Chemical Analyses. Protein was estimated by the Lowry method using α-elastin as a standard (Lowry et al., 1951). Aliquots of the protein solution in 6 N HCl were hydrolyzed in vacuo in sealed tubes for 20,40 and 70 hours at 110°C and a fourth aliquot was hydrolyzed at 140°C for 3½ hours. The amino acid composition was determined with an automatic amino acid analyzer, Spinco Model 120B, employing the method of Spackman et al. (1958), utilizing the physiological columns. The amino acid contents of the different proteins are expressed as residues per thousand residues including isodesmosine and desmosine which are reported as quarter lysine residues.

Disc electrophoresis was done on the first supernate, the first coacervate and the supernate after coacervation. The method of Reisfeld, et al. (1962) was modified by using 6 M urea in the gels and Safranin O as a tracking dye (Racusen, 1967). The coacervate was resuspended in the original volume of starting buffer.

RESULTS

Table 1 compares the amino acid contents of two insoluble aortic elastins from animals on control and copper deficient diets with that of

Table I Comparison of Amino Acid Contents of Soluble Coacervate and Insoluble Aortic Elastins (Residues per 1000).

Amino Acid	Control Diet: Insoluble Elastin 3½ hr 140°C* n=3 Mean d	Copper Deficient Diet: Insoluble Elastin 3½ hr 140°C n=4 Mean d	Soluble Coacervates** 3½ hr 140°C	20 hr 110°C	40 hr 110°C	70 hr 110°C
Hydroxyproline	11.7 ± 0.6	9.8 ± 0.8	11.8	14.6	16.2	14.6
Aspartic Acid	11.2 ± 2.1	20.5 ± 2.1	8.2	7.8	8.0	8.1
Threonine	15.6 ± 1.1	20.6 ± 1.0	10.8	14.3	13.8	13.8
Serine	13.8 ± 1.1	19.7 ± 1.3	6.0	11.5	11.6	11.8
Proline	108 ± 2.0	101 ± 2.0	113	112	111	110
Glutamic Acid	24.5 ± 2.8	35.0 ± 1.9	22.2	21.2	21.1	21.0
Glycine	318 ± 5.0	293 ± 7.0	321	319	315	317
Alanine	226 ± 1.7	205 ± 4.0	212	211	214	215
Valine	121 ± 5.7	116 ± 2.0	127	124	122	124
Methionine	2.1 ± 0.4	3.8 ± 0.2	1.7	1.2	1.1	1.2
Isoleucine	19.8 ± 0.9	21.6 ± 0.9	16.8	17.2	17.1	17.2
Leucine	55.1 ± 0.8	58.5 ± 1.9	48.2	47.5	48.0	47.7
Tyrosine	17.7 ± 0.1	19.8 ± 0.5	17.0	16.8	16.7	16.6
Phenylalanine	32.5 ± 0.4	32.9 ± 0.4	28.9	28.5	28.2	28.3
Isodesmosine	5.3 ± 0.2	4.0 ± 0.8	--	--	--	--
Desmosine	6.7 ± 0.0	3.5 ± 0.9	--	--	--	--
Hydroxylysine	----	----	0.9	0.9	0.8	0.8
Lysine	8.6 ± 1.8	22.2 ± 3.1	45.2	42.6	45.1	42.4
Histidine	1.6 ± 0.3	3.9 ± 0.6	1.0	1.0	0.9	1.3
Arginine	9.6 ± 1.1	15.4 ± 1.1	8.6	8.5	8.7	8.6

* The values for the insoluble elastins are arithmetic means ± the deviation from the mean. n=number of different animals analyzed.
** Soluble coacervate analyses - see methods.

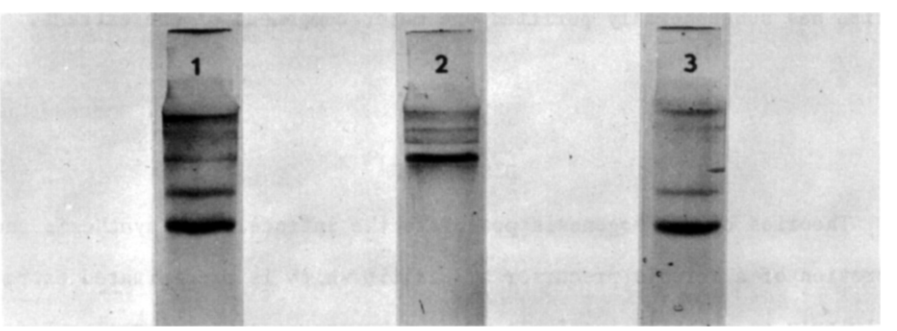

Figure 1. Disc electrophoretic patterns of buffered 1 M NaCl extracts of copper deficient aortas. (1) Supernate before first coacervation; (2) Coacervated material; (3) Supernate after first coacervation.

coacervated protein from the copper deficient. The insoluble elastin of the deficients contains less desmosine and isodesmosine, more lysine and a slightly higher ratio of polar to non-polar amino acids than the control. No hydroxylysine is detected in either one.

The soluble coacervate has the composition of an elastin. Thus, the non-polar amino acids constitute 86% of its total amino acids compared to 88% for the insoluble control elastin. It differs in lacking desmosine and isodesmosine and in its much higher content of lysine. It contains a small amount of hydroxylysine. There were lower amounts of several minor constituents (Asp, Thr, Ser, Isoleu, Leu, ϕAla) when the hydrolysis of the coacervate was carried out at 140°C for 3½ hours. When the hydrolysis was carried out at 110°C the lowering of threonine and serine did not occur and the hydroxyproline was higher than that of the insoluble elastins. Comparing the 20 hr. hydrolysate of the coacervate with that of the insoluble control elastin, we see that four of the major amino acid constituents of elastin (Pro, Gly, Ala, Val) equal to 77% of the total residues, agree within the experimental error of the analyses.

Disc electrophoresis (Fig. 1) of the first supernate shows 3 major bands and one minor below the boundary between the stacking gel and separating gel. The coacervate has one major band which is lacking from the supernate after coacervation and it contains the minor band. It is evident that the coacervation has substantially purified one major component of the extract.

DISCUSSION

Theories of elastogenesis postulate the intracellular synthesis and secretion of a soluble precursor of elastin which is precipitated extracellularly as a highly insoluble protein by progressive intermolecular cross-linking during maturation (Piez et al., 1965). The dominant cross-links are represented by desmosine and isodesmosine which have been shown to be

derived from a reaction believed to take place between 3 aldehyde intermediates produced by the oxidative deamination of the ε-amino groups of lysine and an ε-amino group of the 4th lysine (Partridge, 1965a). The altered properties of elastin in copper deficiency have been attributed to failure of the oxidative step in this reaction, due perhaps to lack of a copper-containing amine oxidase (Partridge, 1965b).

Until now the postulated soluble precursor of elastin has not been isolated. The presence of an excess of soluble protein with a high proline: hydroxyproline ratio in the aortas of copper deficient swine (Weissman et al., 1963) suggested that an elastin precursor might accumulate in that condition. The further investigation of this protein in the present study lends support to that suggestion. The diminution of desmosine cross links and excess of lysine residues in the insoluble elastin of copper deficient pig aorta has been confirmed. The coacervated protein separated from the soluble extract of these aortas lacks desmosines entirely and has a still greater excess of lysine. The content of its four major amino acid components is identical to that of elastin.

The composition of the coacervated protein explains its peculiar solubility properties. This protein has seven non-polar amino acids (Gly, Ala, Pro, Val, IsoLeu, Leu, ϕAla) which constitute 86% of the total. These would be expected to engage in hydrophobic bonding (Kauzmann, 1959). We may assume that the hydrophobic bonds interact with one another in the interior of the molecules at low temperatures, leaving the polar groups on the surface to react with the solvent and bring the molecules into solution. In this state the free lysine groups would aid solubilization. When the temperature is raised the molecule unfolds and then intermolecular bonding occurs between the non-polar side chains, causing a reversible coacervation.

Partridge and coworkers (1955) first observed reversible coacervation of a soluble degradation product of elastin from ox ligamentum nuchae produced by oxalic acid hydrolysis. The coacervated protein, which they

called α-elastin, was a polydisperse product with a molecular weight range of 60,000 to 130,000. It contained 84% of non-polar amino acids. An important difference between these coacervates is the presence of desmosine and isodesmosine in α-elastin and its lower lysine content, owing to its derivation from an extensively cross-linked product.

The excess of lysine residues in the copper deficient insoluble elastin cannot be accounted for entirely by desmosine and isodesmosine. This indicates that there may be a significant number of other crosslinks involving lysine derivatives in normal elastin. The identification of lysinonorleucine in elastin hydrolysates (Franzblau et al., 1965) supports this hypothesis. The presence of an even greater excess of lysine in the coacervate suggests that other cross links may be even more abundant than those already identified. It is most interesting in this connection that Petruska and Sandberg (1968) have predicted the lysine content of the porcine elastin monomer theoretically to be 46 residues per thousand which agrees very well with that determined for the coacervated protein.

The significantly higher hydroxyproline and small amount of hydroxylysine in the coacervate indicate a small but significant contamination of this fraction by soluble collagen. This has indeed been confirmed by electronmicroscopic examination of the coacervate. It may be related to the minor band in the disc electrophoresis pattern (Fig. 1) which has not yet been identified. The presence of soluble collagen in the copper deficient aortic extract would not be unexpected since the cross-linking of collagen may also involve the ε-amino group of lysine (Bornstein et al., 1966). In fact, O'Dell et al. (1966) have shown a sufficiently high hydroxyproline in the salt soluble extract of copper deficient chick aortas to suggest a significant increase in soluble collagen.

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