DETERMINATION OF CROSS-LINKS IN ELASTIN

M. LEDVINA AND F. BARTOŠ
Department of Chemistry and Biochemistry and Department of General Biology, Medical Faculty, Charles University, Hradec Králové (Czechoslovakia)

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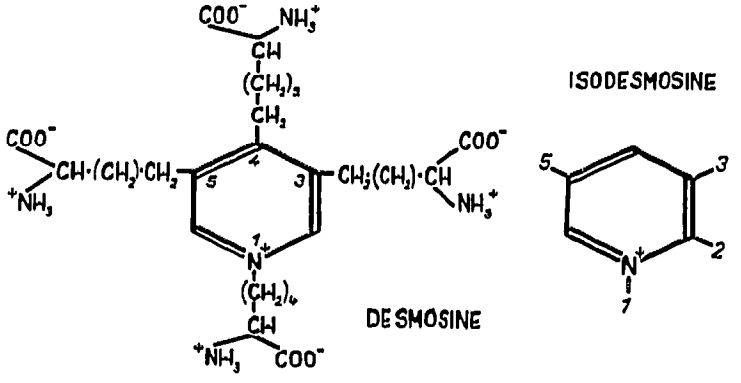
In 1963, during studies on the cross-links connecting the peptide chains in elastin, PARTRIDGE *et al.*[1,2] discovered two hitherto unknown amino acids with a relatively high molecular weight in the elastic fibres; they designated them desmosine and isodesmosine. Their structure is as follows:



Desmosine is a tetraamino-tetracarboxylic acid with a quaternary pyridinium nitrogen. The ring can be built up by combination of one unchanged lysine with three lysine molecules oxidized to α-aminoadipic semialdehyde. Isodesmosine is its isomer with the same substituents in the positions 1, 2, 3 and 5.

In this paper, a method for the determination of desmosine and isodesmosine in elastin hydrolysates is described and discussed.

EXPERIMENTAL

Preparation and hydrolysis of elastin

Ligamentum nuchae was obtained from freshly killed adult cows, cut into strips and dried at 37°. The hard mass was finely pulverized with the help of a file and delipidated with ether and acetone. Elastin was prepared by means of the procedure of PARTRIDGE *et al.*[3], and in some cases by the method of MCGAVACK AND KAO[4].

The isolated elastin was hydrolyzed in 6 *N* HCl under argon at 105° for 48–72 h. The hydrolysates were filtered and repeatedly evaporated to dryness under reduced pressure at 40°.

Ion-exchange chromatography

The animo acid mixture was separated on a 50 cm column (diameter 0.9 cm)

of the cation exchange resin Dowex 50 W X4, maintained at 50°. Before use, the resin was washed successively with 0.20 *M* and then with 0.38 *M* NaOH, and subsequently with the elution citrate buffer, to constant pH values. The vacuum-dried sample was dissolved in 0.4 ml of 0.20 or 0.38 *M* citrate buffer, pH 3.0, applied to the column, and elution was performed with citrate buffers with the following composition: (a) 0.20 *M*, pH 4.45, (b) 0.38 *M*, pH 4.35. In the course of the chromatography, both pH and ionic strength remained constant. The flow rate was 12.5 ml/h, and 1.4–2.0 ml fractions were collected in a Shandon fraction collector.

At the beginning the fractions were investigated using the ninhydrin reagent. Later the measurement of U.V. absorbance at 274 nm was found to be more suitable and simpler; the absorption curves of purified and desalted desmosine fractions are shown in Fig. 1.

Fig. 2 demonstrates a typical elution diagram of a hydrolysate of elastin

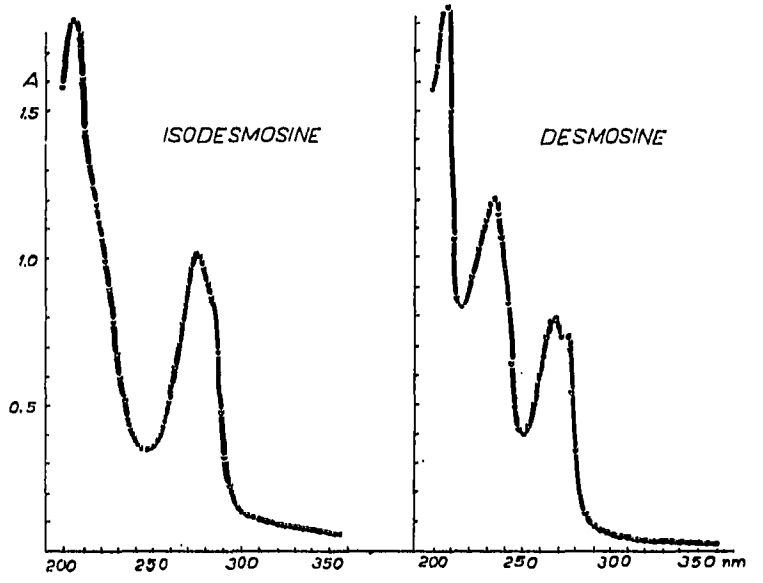


Fig. 1. Absorption curves of isodesmosine and desmosine isolated as described in the text.

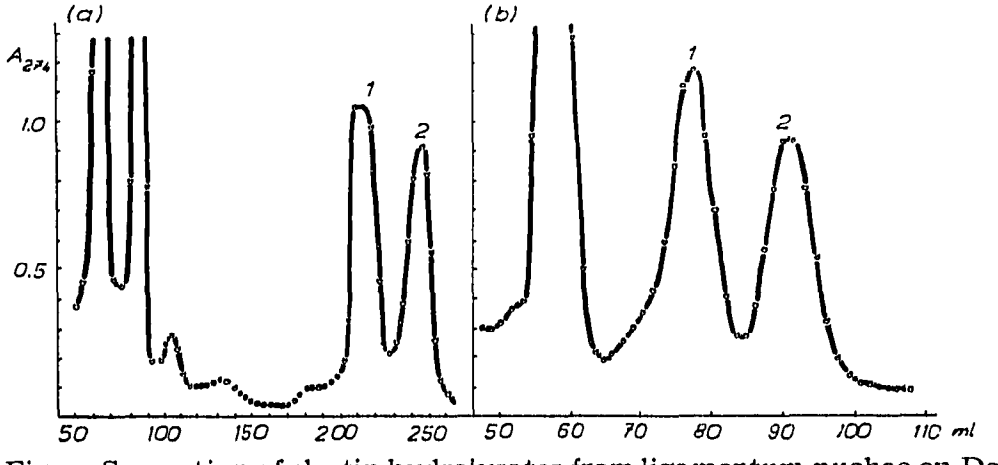


Fig. 2. Separation of elastin hydrolysates from ligamentum nuchae on Dowex 50 W X4. (a) Elution with 0.20 *M* citrate buffer pH 4.45; (b) elution with 0.38 *M* citrate buffer pH 4.35. Note the different volume scale on the abscissa. Elastin amounts: (a) 320.8 mg; (b) 271.5 mg. 1 = Isodesmosine; 2 = desmosine.

from ligamentum nuchae of an adult cow in both the citrate buffers indicated above. At lower elution volumes small quantities of acidic amino acids emerged and afterwards considerable quantities of neutral ones. The position of the peaks of the desmosines is similar in both buffers. In the more dilute buffer the distance between the peaks of the desmosines and the group of neutral and aromatic amino acids is greater. However, the elution volumes are much greater in this case, and consequently the chromatography lasts longer. As the purity of the fractions is not improved in comparison with the more concentrated citrate buffer, the 0.38 *M* buffer was given preference in later investigations.

When the individual effluents were analyzed, the fractions were found to lack homogeneity, especially the isodesmosine fraction; spectral curves did not correspond to literature data, and paper chromatography in the solvent system butanol–acetic acid–water (4:1:5) showed, in addition to isodesmosine with zero mobility, a further eight ninhydrin-positive spots, though in most cases only in trace amounts. The separation of desmosine and isodesmosine effluents from Dowex by means of molecular exclusion chromatography on Sephadex G-15 confirmed similar heterogeneity of both fractions; in addition to the main component (desmosine and isodesmosine) which is the largest molecule, some further substances were found which absorbed at 274 nm. The possibility cannot be ruled out completely that part of these substances were artifacts of the isolation procedure.

Ion-exchange chromatography combined with molecular exclusion chromatography

From the above experiments a combination of ion-exchange chromatography on Dowex 50 W X4 with molecular exclusion chromatography appeared promising. We examined the separation of whole elastin hydrolysates on Sephadex columns G-10 to G-25. The dried hydrolysates were dissolved in 0.05 *M* pyridine–formate buffer pH 3.0, and eluted from Sephadex with the same buffer. The following patterns were found (Fig. 3):

The less retarded fraction, *i.e.* the mixture of desmosine and isodesmosine, has the strongest absorbance at 274 nm, and it is distinctly separated from other low-molecular amino acids. Sephadex G-15 appeared to be most suitable. Attempts to separate both isomers on Sephadex in acid or alkaline buffers were not successful.

The fastest fraction from Sephadex G-15 was dried, dissolved in 0.38 *M* citrate buffer, pH 3.0, applied to the column of Dowex 50 W X4 and eluted with 0.38 *M* buffer, pH 4.35, as described above. An elution diagram was obtained (Fig. 4), which is rather similar to that shown in Fig. 1 for the separation of the whole hydrolysate on the Dowex column. Fig. 4 shows both desmosine isomers, but the tyrosine peak is absent. In this way we could prepare pure fractions, whose absorption curves are shown in Fig. 2. The fractions were desalted on a short column of Dowex 50 W X8 in the H^+ form according to ANWAR[9], elution was with 1 *N* ammonia. The use of more concentrated ammonia (3 *N*) resulted in partial decomposition of the desmosines with the loss of the ring structure.

DISCUSSION

It was found by other authors[1,11] that the cross-linking elements of elastin, desmosine and isodesmosine, are absent from all proteins with the only exception

of elastin. It follows from this fact that the determination of desmosines in tissues containing elastin can assume the same significance as the determination of collagen on the basis of its hydroxyproline content. According to present knowledge, the determination of elastin on the basis of desmosines seems to be even more specific. Several authors[5–8] have found (and we could confirm it) that there are relatively high amounts of hydroxyproline in elastin; therefore it is not possible to calculate the collagen content from analytical figures for hydroxyproline in the tissues rich in elastin.

We attempted to determine the desmosines with currently available means, using Dowex 50 W X4. This approach proved to be successful. Citrate buffers have been used for the elution of elastin hydrolysates in some previous publications[1,9,12].

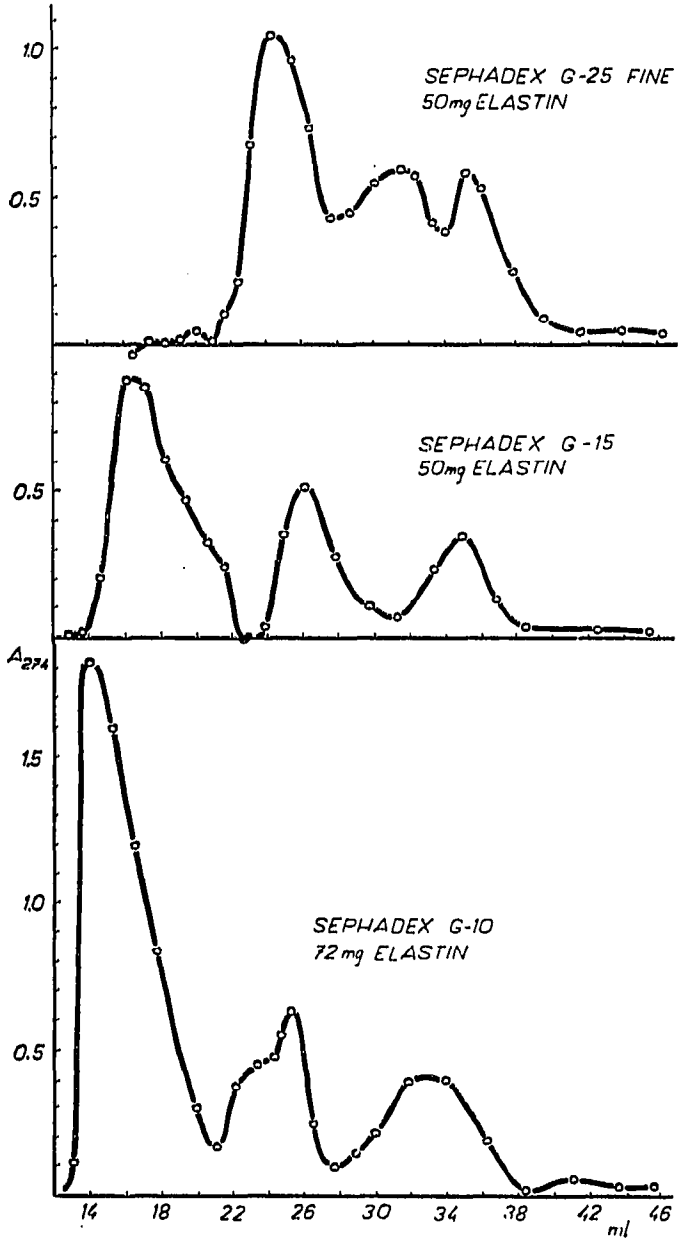


Fig. 3. Analysis of elastin hydrolysates from ligamentum nuchae on Sephadex G-25, G-15 and G-10. Elution with 0.05 *M* pyridine–formate buffer, pH 3.0.

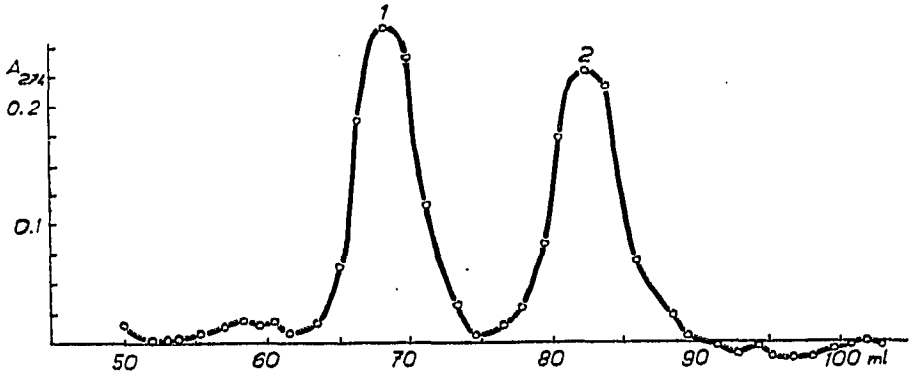


Fig. 4. Separation of the less retarded fraction from Sephadex G-15 on Dowex 50W X4; elution with 0.38 *M* citrate buffer, pH 4.35. 1 = Isodesmosine; 2 = desmosine.

The composition of the buffers used in this laboratory is similar to those used by ANWAR[9], with the only exception that the pH of the 0.38 *M* buffer is slightly more basic in our experiments. All hitherto published papers report the use of automatic amino acid analyzers[1,2,9,10] for the determination of the ninhydrin reaction products. Only an abstract of a recent paper by KELLER AND MANDL[13] mentions a determination of U.V.-absorbing substances of elastin hydrolysates separated on Bio-Gel P-2. For routine determinations of the content of cross-linking elements a complete amino acid analysis and an automatic analyzer are not necessary. In this case it seems to be sufficient to collect only small effluent volumes in the vicinity of the peaks of the desmosines, and to analyze them more easily by U.V. absorption at the wavelength of 274 nm. This value does not represent the exact maximum of both absorption curves, but the differences are almost negligible.

The method worked out here permits routine investigations of cross-linking not only in isolated elastin, but also determinations of the elastin content in various connective tissues, even in those poor in elastin, from which the protein can be isolated with very low yields. It is possible to analyze much more tissue material, because the capacity of the Sephadex column in molecular exclusion chromatography is considerably higher than the capacity of the ion-exchange column chromatography.

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SUMMARY

A method for the determination of the cross-linking elements in elastin, desmosine and isodesmosine has been developed. It is based on the combination of ion-exchange chromatography with molecular exclusion chromatography (Sephadex G-15, and then Dowex). The fractions of the desmosines are detected by measurement of the U.V. absorbance at 274 nm. The chromatography on a column of Dowex 50 W alone does not yield pure fractions of the desmosines. The method described also permits the determination of the total elastin content in connective tissues poor in elastin fibres.

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