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Separation of elastin cross-links as phenylisothiocyanate derivatives

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ABSTRACT

A method has been developed for the separation and quantitation of desmosines in tissue samples. The tissue is treated with cold 10% trichloroacetic acid to remove collagen and hydrolysed in HCl vapours in sealed vials. Preseparation of desmosines from tissue acid hydrolysates is performed on a cellulose column, first eluted with *n*-butanol–acetic acid–water to wash out other amino acids and then with water to recover desmosines. Separated desmosines are then derivatized with phenylisothiocyanate and determined by reversed-phase high-performance liquid chromatography using a gradient system with sodium acetate pH 6.4 and acetonitrile. Desmosines were detected spectrophotometrically at 254 nm. The method was applied to the determination of desmosine in elastin, rat aorta and bovine ligamentum nuchae.

INTRODUCTION

Desmosine and isodesmosine, the elastin-specific cross-linking amino acids, are frequently used as markers of elastin metabolism and its content in tissues. The main problem in their determination is the low proportion of these cross-links relative to other amino acids in elastin (*ca.* two residues per 1000 residues). In elastin-containing tissues their content is even lower, which necessitates tedious enrichment procedures to achieve the necessary accuracy of the assay procedure.

In the first attempts to determine desmosine and isodesmosine, conventional ion-exchange chromatography [1–3], thin-layer chromatography [4,5] and immunoassay procedures [6,7] were used. High-performance liquid chromatography was exploited more recently both with and without precolumn derivatization [8–13]. Of these procedures, derivatization with dansyl chloride [8] or naphthalenedialdehyde cyanide [9] appeared promising, but were used only for the determination of desmosines in pure elastin samples and were not applied to elastin-containing tissues.

Recently Guida *et al.* [14] developed a general strategy for the latter purpose. The hydrolysed elastin or elastin-containing tissue sample was passed through a cellulose mini-column during which the bulk of amino acids were removed. During this operation desmosines were retained and were washed out from the cellulose bed using water as mobile phase. More precisely, after homogenization and extraction of collagen with hot 5% trichloroacetic acid and hydrolysis of the elastin-containing sample with 6 *M* hydrochloric acid, the desmosines were preseparated on a cellulose

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precolumn as mentioned above and were subsequently determined on a Spherisorb ODS-2 column after conversion into dansyl derivatives. The Spherisorb ODS-2 column was eluted with a linear gradient of acetonitrile–25 m*M* phosphate buffer (pH 7.2) from 15 to 60% of the organic modifier. Basically the desmosine enrichment step exploits the procedure described earlier by Skinner [15].

Although in principle the method is applicable to natural elastin-containing samples, there are some problems that require more detailed study. First, although the reported recovery of desmosines reached 87% with a relative standard deviation (R.S.D.) of 2.8% at 50 pmol injected, we have frequently obtained much lower recoveries depending on the batch and provenance of the cellulose sorbent used. Second, determination of the desmosines as dansyl derivatives requires a specialized set-up of the reversed-phase system, which cannot be used directly for conventional amino acid analysis. Therefore, we attempted to combine a commercially optimized amino acid analysis system based on separation of phenylisothiocyanate (PITC) derivatives (PICO-TAG) with an optimized cellulose precolumn enrichment step.

EXPERIMENTAL

Chemicals

Purified desmosines (a mixture of desmosine and isodesmosine) and elastin were obtained from Biotechnology Laboratory, Agricultural Cooperative Enterprises (Letohrad, Czechoslovakia). Standard set of amino acids was purchased from Serva (Heidelberg, Germany). Several batches of cellulose of different provenance were tested in preliminary experiments, and microcrystalline cellulose (Lachema, Brno, Czechoslovakia) was selected for use in further experiments. Acetonitrile (spectrometric grade) was purchased from Janssen (Beerse, Belgium) and *n*-butanol and acetic acid from Merck (Darmstadt, Germany). All other chemicals were obtained from Lachema. Doubly distilled water was used in all operations.

Standards

Stock solutions of desmosines contained 2 μmol/l of the analyte in 0.01 *M* hydrochloric acid. Their precise concentration was determined spectrophotometrically ($\varepsilon = 4900$ at 268 nm for desmosine and $\varepsilon = 7850$ at 278 nm for isodesmosine). Aliquots of desmosines were diluted to 2 ml with 6 *M* hydrochloric acid mixed with a standard amino acid mixture prepared in 0.01 *M* hydrochloric acid (8 ml) and passed through a 4 × 0.5 cm I.D. cellulose minicolumn. This minicolumn was washed first with three 5-ml portions of *n*-butanol–acetic acid–water (4:1:1) and the washings were combined and lyophilized to be used (if necessary) for conventional amino acid determination and for the determination of desmosines in terms of residues per 1000 residues.

Next the cellulose minicolumn was eluted with three 5-ml portions of water, the eluates were combined and lyophilized, the residue was dissolved in 2 ml of 0.01 *M* hydrochloric acid and this solution was used as working standard for the chromatographic calibration.

Analysis of tissue samples

Dried samples of ligamentum nuchae or rat aorta (typically 30–100 mg) were

homogenized in water (1:5, w/v) with a Polytron PT 10-35 homogenizer (Brinkmann, Westbury, NY, U.S.A.). The homogenate was treated with cold 10% trichloroacetic acid and centrifuged at 10 000 *g* (4°C, 10 min). The supernatant was discarded and the residue was treated with 5 ml of 5% trichlororacetic acid at 90°C for 30 min in order to remove collagen (with other tissues this extract can be used for collagen determination by assaying it for its hydroxyproline content). The residue after trichloroacetic acid extraction was washed with acetone in a centrifuge and vacuum dried at 60°C. The dried material was suspended in 2 ml of 6 *M* hydrochloric acid, flushed with nitrogen and hydrolysed in sealed tubes at 110°C for 24 h. The hydrolysate was evaporated to dryness to remove excess of hydrochloric acid, dissolved in 2 ml of 0.01 *M* hydrochloric acid and processed through the cellulose minicolumn as described above.

Elastin samples

A sample of about 2 mg of the protein was dissolved in 2 ml of 6 *M* hydrochloric acid, flushed with nitrogen in a Pyrex tube, sealed and hydrolysed at 110°C for 48 h. The hydrolysed sample was processed as described under *Standards* and *Derivatization and chromatographic equipment.*

Derivatization and chromatographic equipment

Both the desmosines and standard amino acids were separated in the final step as phenylthiocarbamyl derivatives. Lyophilized hydrolysates after the cellulose step were derivatized in a Waters Work Station (Millipore, Milford, MA, U.S.A.) with phenyl isothiocyanate at room temperature for 25 min according to the procedure of Bidlingmeyer *et al.* [16]. Derivatized amino acids were analysed on a PICO-TAG amino acid analysis system (Millipore). Samples were separated by reversed-phase high-performance liquid chromatography using two eluents: eluent A (19.0 g/l sodium acetate trihydrate–0.5 ml/l triethylamine, titrated to pH 6.40 with glacial acetic acid, and subsequently 60 ml of acetonitrile being added to 940 ml of this buffer) and eluent B (400 ml of water added to 600 ml of acetonitrile). The column was conditioned with 100% eluent A for 18 min before every analysis at a flow-rate of 1.5 ml/min. During analysis the flow-rate was 1.0 ml/min. Elution was started with 100% eluent A followed by a linear gradient reaching 46% eluent B at 10 min and 100% eluent B at 10.5 min. Between 10.5 and 12.0 min elution was isocratic with 100% eluent B. The temperature was kept at 38°C and the effluent was monitored at 254 nm.

RESULTS AND DISCUSSION

Cellulose precolumn sample enrichment

As demonstrated in Fig. 1A–C, the bulk of common amino acids are eluted with the organic mobile phase [*n*-butanol–acetic acid–water (4:1:1)]. Even with different batches of cellulose we have never observed leakage of desmosines into the first three organic phase washings. The third organic phase washings of the cellulose precolumn are virtually always devoid or contain very little of any of the common amino acids (less than 2.0 mol% of originally loaded Asp, Glu, Hypro, Ser, Gly, His, Thr, Ala, Pro, Tyr, Val, Phe and Lys and less than 5.0 mol% of originally loaded Arg).

Fig. 1D–F demonstrate typical amino acid profiles obtained with subsequent elution with water. Particularly the first aqueous washings contain considerable

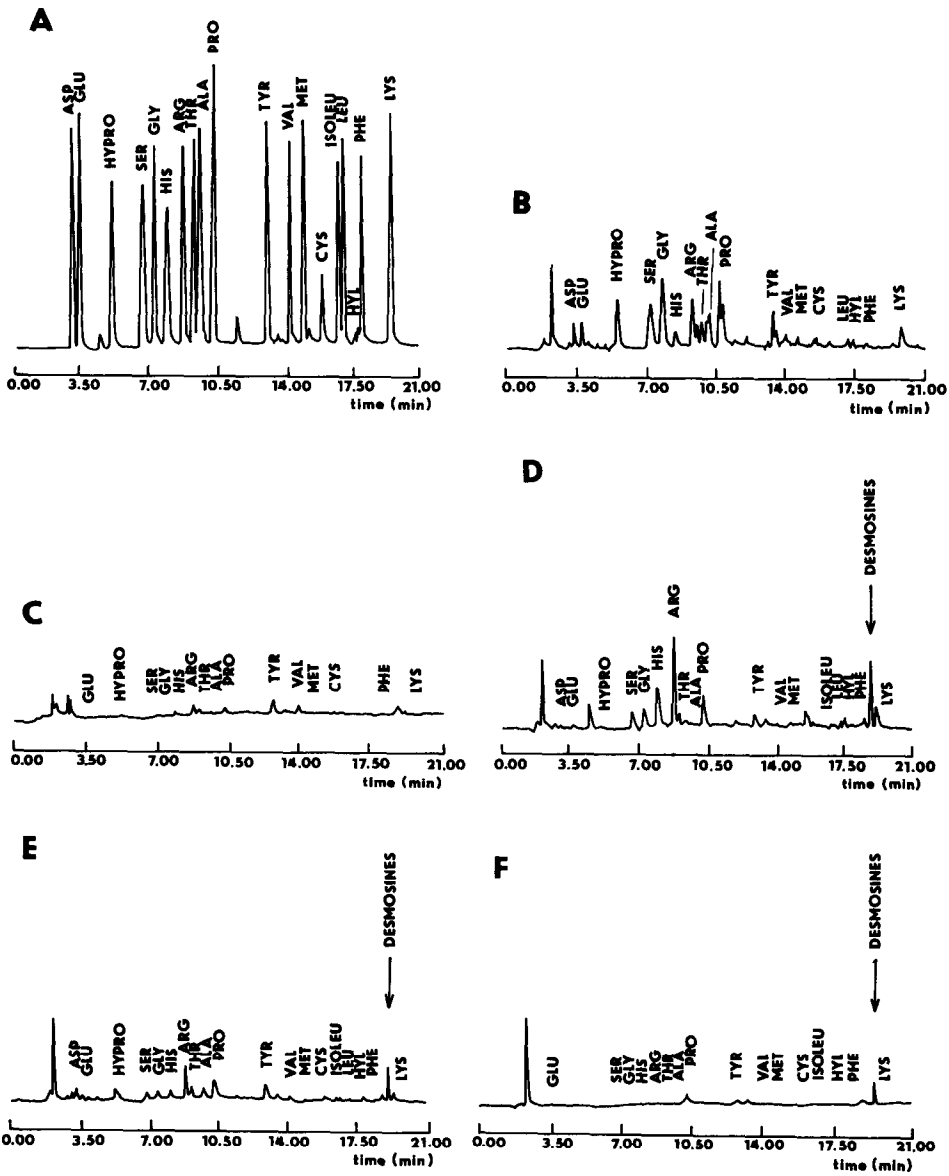

Fig. 1. Cellulose precolumn sample enrichment. A–C: amino acid analysis of the first three fractions eluted with *n*-butanol–acetic acid–water (4:1:1); note that the third organic phase washings are devoid of common amino acids. No washing out of desmosines can be observed. D–F: stepwise elution of desmosines with water, amino acid analysis of the three eluted fractions.

amounts of His (8.24 ± 1.23 mol%), Arg (16.23 ± 2.8 mol%) and Pro (6.32 ± 0.76 mol%) (±S.D., $n = 8$) of the amount loaded (2500 pmol).

The second aqueous washings contain only traces of the originally loaded

amount of contaminating common amino acids and the third wash is completed only to increase further the recovery of desmosines.

As has been mentioned, no desmosines are recovered with the *n*-butanol–acetic acid–water elution. In the first aqueous washings 80.03 ± 2.20 mol% of desmosines were recovered; an additional 6.24 ± 0.31 mol% were recovered in the second and 2.04 ± 0.7 mol% in the third washings (±S.D., 250 pmol injected), thus reaching a total recovery of 88.31 mol%. The within-day and day-to-day reproducibility (R.S.D.) of the method was 2.2 and 5.1%, respectively (with 25 pmol of desmosines injected).

By cellulose precolumn treatment an enrichment factor of $5 \cdot 10^3$ can be easily achieved with a limit of detection of 2.5 pmol of desmosines (signal-to-noise ratio 3 at attenuation × 1).

Calibration graphs for 20–2500 pmol of phenylisothiocyanate derivatives of desmosines exhibited good linearity of plots of peak area *vs.* amount of analyte injected with a correlation coefficient of $r = 0.997$.

With some batches of cellulose (particularly from other sources) the data may vary and it is therefore recommended to use for a series of experiments a single batch of cellulose that has been adequately tested before being put into routine use.

Analysis of elastin containing tissues

Typical examples of the determination of desmosines in elastin hydrolysate, rat aorta and bovine ligamentum nuchae are presented in Fig. 2A–C. With standard addition of desmosines the recovery of these cross-linking amino acids was 88.24 ± 4.7% (±S.D., $n = 20$, ligamentum nuchae), which is similar to the value obtained with an artificial amino acid mixture. Also, the amount of desmosines found increases linearly with the amount of tissue taken for analysis ($n = 20$, $r = 0.996$). The minimum amount of tissue needed for reliable determination of desmosines in elastin-rich tissues was 30 mg for ligamentum nuchae and 50–100 mg for rat aorta. With separate samples of dried aorta the R.S.D. obtained was 6.3% ($n = 20$). The proportion of contaminating common amino acids present in the combined aqueous eluates differs considerably from the contaminants seen when working with standard amino acid mixtures, which obviously reflects the amino acid composition of the proteinous material analysed.

In comparison with the method published by Guida *et al.* [14], the present procedure has similar analytical parameters; its advantage is the exploitation of commercially available equipment for amino acid analysis. The unsatisfactory results we obtained at the beginning with the above-mentioned procedure are obviously due to the differences in the properties of the cellulose used. With some batches of cellulose we observed considerable retention of common amino acids even after repeated elution of the cellulose precolumn with *n*-butanol–acetic acid–water (4:1:1). Two, or better three, washes (as described in this paper) are necessary to eliminate as much of the common amino acids as possible. No leaking of desmosines into the organic phase washings was observed, thus allowing multiple elution of the cellulose precolumn. Also, repeated elution with water is needed to obtain good recoveries of desmosines.

Even with the high sample enrichment achieved by using the cellulose precolumn, in naturally occurring samples removal of non-collagenous and collagenous proteins by trichloroacetic acid treatment is inevitable as otherwise the content of desmosines even in elastin-rich tissues is for too low to allow direct analysis of the tissue.

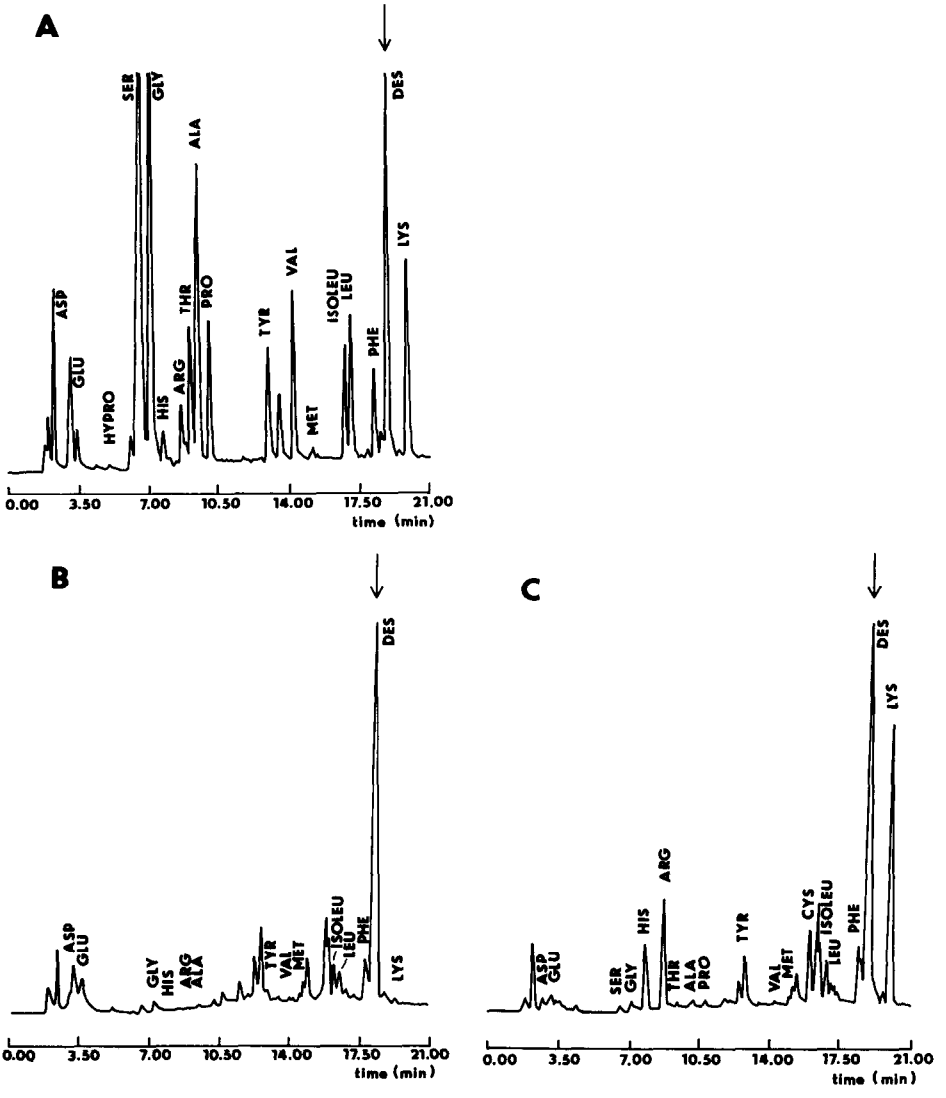

Fig. 2. Typical examples of the determination of desmosines in (A) elastin hydrolysate, (B) ligamentum nuchae and (C) rat aorta.

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