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Note

Determination of trace amounts of dityrosine in protein hydrolysates by means of an automatic amino acid analyser with spectrofluorimetric evaluation

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Dityrosine, formed from two tyrosine residues bound in peptide chains, was first discovered in connective tissue proteins in 1963 by Andersen[1]. The protein was resilin isolated from the ligaments of locusts. Dityrosine was shown to be a stabilizing element serving as a cross-link between two peptide chains. Dityrosine was isolated from acid hydrolysates of resilin by chromatography on a column of cellulose phosphate or DEAE-cellulose, and its physico-chemical parameters were examined.

Apart from resilin, which contains approximately eight residues of dityrosine per 1000 amino acid residues[2], dityrosine has also been found and determined in other connective tissue proteins, *e.g.*, in structural glycoproteins of the vessel wall and uterus[3,4], elastin[5], collagen[6], elastoidin[7] and other proteins. However, in these proteins the content of dityrosine is substantially lower than that in resilin, *e.g.*, only ten residues of dityrosine per 100,000 amino acid residues were found in structural glycoproteins of 1-day-old chick aortas[4].

To solve the problems connected with the determination of dityrosine in the hydrolysates of connective tissue proteins, we have proposed a sensitive technique for its determination that permits the measurement of even trace amounts. Literature data[8,9] indicate that the problem has been solved indirectly, *i.e.*, the decrease in tyrosine content after oxidation with hydrogen peroxide and peroxidase was examined. A direct determination of dityrosine by means of an amino acid analyser followed by a spectrofluorimetric or radiometric evaluation has been used in some instances to determine the dityrosine content of elastin and structural glycoproteins[3,4]. However, reproducible conditions for this determination were not specified[3,4]. Moreover, the conditions are strongly dependent on the type of amino acid analyzer used, on the sensitivity and the type of the spectrofluorimetric device. Therefore, we have developed a modification for our own amino acid analyser and spectrofluorimeter. In addition, we have improved the sensitivity of the measurement, so that radiometric methods have been shown to be unnecessary.

EXPERIMENTAL

Materials

As a standard, dityrosine prepared by oxidation of D,L-tyrosine with hydrogen peroxide and horseradish peroxidase[10] and isolated by a method suggested by us[11] was employed. The specific activity of the peroxidase preparation was 16.95 kat·kg^{-1} (*o*-dianisidine method). A standardized 10^{-8} *M* mixture of amino acids was used. Standards of isodesmosine and desmosine were prepared according to Starcher and Galione[12]. Dityrosine was separated on the longer column of the amino acid analyser (HD 1200E, manufactured by ZSNP, Žiar n. Hronom, Czechoslovakia), filled with Ostion LGKS 0803 cation-exchange resin (Spolek pro chem. a hutní výrobu, Ústí n. Labem, Czechoslovakia).

The dityrosine content in the individual fractions of the effluent was determined in 1-cm quartz optical cells on a CGA 3000/1 spectrofluorimeter (Ciampolini, Milan, Italy) or by the ninhydrin spectrophotometric method.

Determination of dityrosine

Dityrosine was dissolved in 0.04 *M* hydrochloric acid, then 0.1 ml of this solution, containing 0.36 μg of dityrosine (36 μg in the ninhydrin method), was placed on the top of the longer column (51 × 0.68 cm) of the amino acid analyser equilibrated with 0.2 *M* citrate buffer (pH 3.28). The temperature of the column was 52°, which gave the sharpest separation of dityrosine from other amino acids. The flow-rate of the eluting buffer was 0.424 ml·min^{-1}. The sample was eluted for 10 min with citrate buffer, pH 3.28, with 0.2 *M* citrate buffer (pH 4.25) for 45 min and subsequently with 0.35 *M* citrate buffer (pH 5.28).

If the concentration of dityrosine was high (10^{-3} *M*), the effluent was measured spectrophotometrically by means of the ninhydrin reaction, whereas a low dityrosine concentration necessitated a spectrofluorimetric determination. Then, 5-min fractions of the effluent were collected, using a fraction collector.

To each fraction, consisting of 2.12 ml of effluent, 0.08 ml of 2.5 *M* sodium hydroxide solution was added, giving a total volume of 2.20 ml and a pH of 11.5–12.5. The individual fractions were then measured against a blank solution on the CGA 3000/1 spectrofluorimeter at an excitation wavelength of 330 nm and an emission wavelength of 428 nm. Quartz optical cells (1 cm) were used. The dityrosine concentrations were read from a calibration graph and the sum of the dityrosine concentrations in the individual fractions gave the total content in the sample.

Influence of some factors on the determination of dityrosine

The following factors capable of influencing the results of the dityrosine determination were examined:

Influence of the presence of isodesmosine and desmosine on the fluorescence of dityrosine. A mixture of 10^{-5} *M* dityrosine and 10^{-3} *M* isodesmosine and desmosine (1:100) was prepared and poured on the top of the Ostion column (see above). The subsequent procedure was identical with that described under *Determination of dityrosine.*

Influence of acid hydrolysis in 6 N hydrochloric acid on the decomposition of dityrosine. Known amounts of dityrosine were subjected to acid hydrolysis with 6 *N*

hydrochloric acid (36 h, 115°, pressure 0.133 Pa). The vacuum-dried hydrolysate was dissolved in 2.0 ml of 0.2 *M* citrate buffer (pH 2.2) and the dityrosine content was determined in this solution.

Influence of acid hydrolysis on the decomposition of dityrosine in the presence of proteins (structural glycoproteins). Dityrosine (0.2 ml of a $1 \cdot 10^{-4}$ *M* solution) was hydrolysed together with 10 mg of structural glycoproteins isolated from rabbit aortas by the method of Rasmussen *et al.*[13].

RESULTS

The elution profile of dityrosine and other amino acids obtained in the presence of desmosine is shown in Fig. 1, which illustrates two examinations: (a) chromatography of a standard mixture of amino acids + isodesmosine + desmosine and (b) dityrosine alone; in both instances, the concentrations were determined by the ninhydrin method.

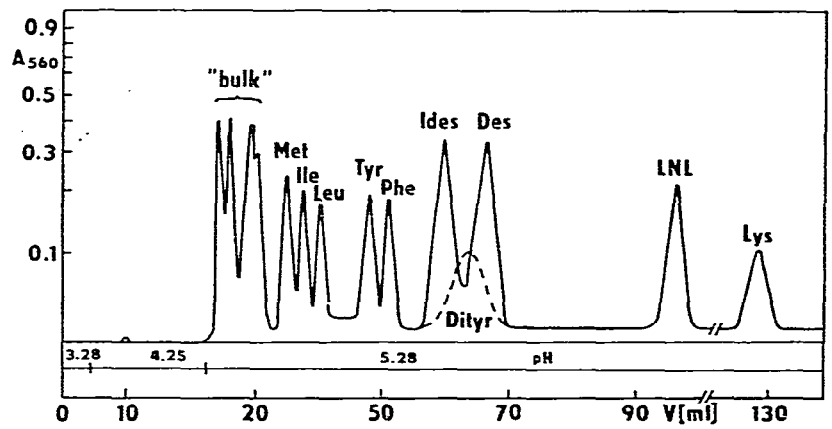

Fig. 1. Elution profile of dityrosine and other amino acids (0.1 μmole). Column (51 × 0.68 cm) of Ostion LGKS 0803 equilibrated with 0.2 *M* citrate buffer (pH 3.28) at 52°. Elution with 0.2 *M* citrate buffer (pH 4.25) and 0.35 *M* citrate buffer (pH 5.28); flow-rate, 2.15 ml per 5 min. Detection: ninhydrin method (560 nm). Broken line, dityrosine; LNL, lysinonorleucine.

It is clear that the elution zone of dityrosine is broader than that of isodesmosine and desmosine. This is due to its non-specific sorption on the cation exchange particles. The zone of dityrosine partially overlaps the regions of the elution of both isodesmosine and desmosine. This fact may be important when analysing elastin. Therefore, it was necessary to investigate whether or not isodesmosine and desmosine interfere in the spectrofluorimetric determination of dityrosine (see below). However, dityrosine was sharply separated from other amino acids by this technique.

The results of the investigation of the influence of the presence of isodesmosine and desmosine, and influence of acid hydrolysis are summarized in Table I.

Table I indicates that neither the presence of isodesmosine and desmosine nor acid hydrolysis influences substantially the determination of dityrosine. No alteration in the recovery of dityrosine was observed even in the presence of connective tissue glycoproteins.

TABLE I

RECOVERY OF DITYROSINE

Experiment No.	*Dityrosine added (μg)*	*Dityrosine found (μg)* *			
		A	*B*	*C*	*D*
1	0.360	0.335	0.339	0.340	0.336
2	0.360	0.339	0.342	0.340	0.342
3	0.360	0.340	0.335	0.336	0.342
4	0.360	0.343	0.341	0.339	0.340
5	0.360	0.343	0.339	0.336	0.339
Average standard	0.360	0.340	0.339	0.338	0.339
Deviation		0.0033	0.0025	0.0018	0.0025

* A = Analysis of dityrosine alone; B = analysis of a mixture of dityrosine, isodesmosine and desmosine (concentration ratio 1:100:100); C = analysis of dityrosine after acid hydrolysis; D = analysis of hydrolyzed mixture of dityrosine + structural glycoproteins. Lower values of dityrosine (recovery *ca.* 95%) were due to inaccuracy of the sample system of the analyser (volume only 0.095 ml).

The dependence of the elution volume of dityrosine on the temperature of the chromatographic column is shown in Fig. 2. This experiment was performed to establish the optimal temperature of the column in order to improve the separation of dityrosine from the other amino acids. The graph demonstrates that the temperature dependence is more pronounced with dityrosine than with other amino acids; this is due, of course, to the aromatic character of dityrosine, but the decrease in the elution volume with increasing temperature is greater than for tyrosine itself.

The results demonstrate that the fluorimetric method used for the determination of dityrosine is very sensitive. If it is connected with the separation of dityrosine, it is capable of determining 36 ng of dityrosine in 0.1 ml of protein hydrolysate (in

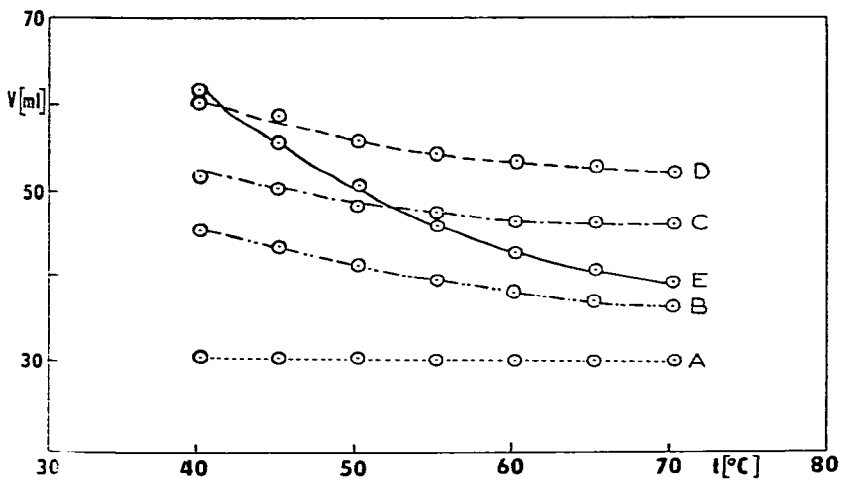

Fig. 2. Dependence of elution volumes of dityrosine and some amino acids on temperature. Column and conditions as in Fig. 1. A = Leucine; B = tyrosine; C = isodesmosine; D = desmosine; E = dityrosine.

pure solutions, $2 \cdot 10^{-8}$ M of dityrosine can be determined fluorimetrically under the given conditions). The determination is specific, and is not affected by either other amino acids (especially desmosines with similar elution volumes) or by acid hydrolysis of the sample.

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