Journal of Chromatography, 303 (1984) 429–432
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,086

Note

Determination of dityrosine in protein fractions isolated from merino wool using reversed-phase high-performance liquid chromatography

MICHAEL S. OTTERBURN* and PAUL E. GARGAN
The Queen's University of Belfast, Department of Chemical Engineering, 21 Chlorine Gardens, Belfast BT9 5DL (U.K.)
(Received July 24th, 1984)

Since the discovery of dityrosine by Andersen[1] in resilin, there have been many reports of the occurrence of the amino acid in a range of other structural proteins[2–14]. Approximate values for the dityrosine content of a number of proteins have been reported; the dityrosine was isolated from protein hydrolysates by ion-exchange chromatography and measured by means of molar absorption coefficients[1] or by spectrophotometric determination of the coloured complex formed between Folin's phosphomolybdic acid reagent[5] and the amino acid. More accurate determinations of the dityrosine content of various proteins have been made using automatic amino acid analysers with ninhydrin[4], fluorimetric and radiometric detection systems[2,7]. More recently, a method has been reported[13] which employs high-performance ion-exchange chromatography after preliminary chromatography of the hydrolysate on a Dowex column.

In earlier work[14] we reported the identification of dityrosine from tyrosine rich proteins isolated from oxidised wool using an amino acid analyser.

This paper describes a much simpler and more rapid method for the direct determination of dityrosine in keratin hydrolysate using reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials and reagents

All solvents and chemicals used were of Analar grade. All amino acids used to prepare standard solutions were purchased from Sigma. Dityrosine was prepared, as described in an earlier paper[15], by a procedure based on the method described by Gross and Sizer[16]. The tyrosine-rich protein fractions used in this work were extracted from a merino wool sample as described by Brunner and Brunner[17].

Chromatography apparatus

The HPLC system used consisted of a Perkin-Elmer Series 10 liquid chromatograph fitted with a Rheodyne Model 7010 sampling valve with a 20-μl loop, a Perkin-Elmer Model LC10 column oven and a Pye-Unicam Model SP6-550 variable-wavelength spectrophotometer, fitted with a Pye-Unicam flow-cell unit (8 μl). All

0021-9673/84/$03.00

experiments were carried out using a Spherisorb-5 ODS (octadecyl silica) column, 25 × 0.45 cm I.D., under isocratic conditions.

Procedure

Hydrolysates of the protein fractions were prepared by heating samples (20 mg) in hydrochloric acid (5.8 *M*) in sealed tubes under nitrogen at 110°C for 20 h. The hydrochloric acid was removed by evaporation *in vacuo* at 40°C. The hydrolysates were made up in the eluting solvents and were filtered using a 0.5-μm membrane filter (Millipore) before application to the column. Isocratic elution was performed with 0.1 *M* potassium dihydrogen phosphate at a constant flow-rate of 1.0 ml/min. Experimentation with the chromatographic conditions, to obtain optimum resolution of dityrosine from the other amino acids, involved variation of: (a) the pH of the phosphate elution buffer and (b) the column temperature. The column was equilibrated to new operating conditions for at least 30 min.

The dityrosine content of the keratin hydrolysates was determined using the method of standard additions.

RESULTS AND DISCUSSION

The most suitable wavelength for detection of dityrosine in the protein hydrolysates was found to be 222 nm. Higher sensitivity can be achieved at lower wavelengths but considerable instability of the baseline occurs.

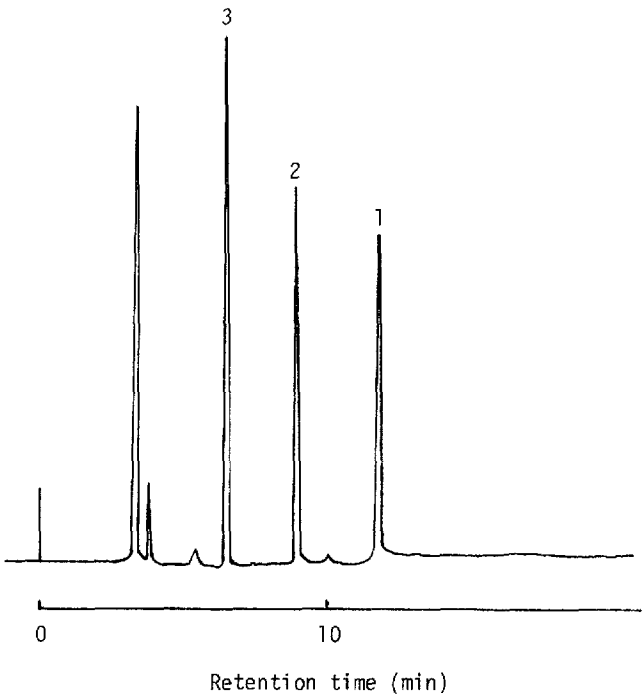

Fig. 1. Chromatogram of tyrosine-rich protein hydrolysate. Eluent, 0.1 *M* potassium dihydrogen phosphate (pH 4.5); column temperature, 33°C. Peaks: 1 = dityrosine; 2 = phenylalanine; 3 = tyrosine.

Phosphate elution buffer at pH 4.50 and a column temperature of 33°C produced the most satisfactory and practical resolution of dityrosine from the other aromatic amino acids present in the protein hydrolystes (Fig. 1).

At pH values lower than 4.5, the retention times of the aromatic amino acids, including dityrosine, are considerably increased, whilst the retention times of the amino acids with less hydrophobic side chains are more or less unaffected (Table I). Although resolution between dityrosine and the other aromatic amino acid is improved, peak broadening occurrs and tailing of peaks increases considerably at lower pH values. It was also observed that at lower pH values retention times and solute recovery were not as reproducible as at higher pH values.

TABLE I

RETENTION TIMES OF AROMATIC AMINO ACIDS AT VARIOUS pH VALUES OF ELUTING BUFFER

Eluting buffer pH	*Retention time (min)*		
	Dityrosine	*Tyrosine*	*Phenylalanine*
2.20	31.4	11.2	16.8
2.37	26.3	10.3	16.0
3.40	13.4	7.6	12.6
4.50	11.0	6.2	8.2

Retention times of the amino acids with relatively large hydrophobic groups decreased proportionally with increase in column temperature (Table II). Peak symmetry also improved with increasing temperature, but the resolution between dityrosine and phenylalanine decreased, being virtually irresolvable at 70°C. At temperatures below 33°C peak broadening increased significantly.

The content of dityrosine in the tyrosine-rich proteins ranged from 4.8 to 10.5 μmoles per gram of protein which is in good agreement with the values obtained using the amino acid analyser[14]. This work has shown that a reversed-phase HPLC system can be employed to provide a rapid and reproducible method for the determination of dityrosine in protein hydrolysates.

TABLE II

RETENTION TIMES OF AROMATIC AMINO ACIDS AT VARIOUS COLUMN TEMPERATURES

Column temp. (°C)	*Retention time (min)*		
	Dityrosine	*Tyrosine*	*Phenylalanine*
25	13.10	7.30	9.75
33	11.00	6.20	8.20
40	9.68	5.58	7.90
50	8.06	4.98	6.84
60	7.25	3.98	6.38
70	7.08	3.80	5.46

ACKNOWLEDGEMENT

P.E.G. acknowledges the grant of a scholarship from the Wool Foundation.

REFERENCES

1 S. O. Andersen, *Biochim. Biophys. Acta*, 93 (1964) 213.
2 F. LaBella, F. Keeley, S. Vivian and D. Thornhill, *Biochem. Biophys. Res. Commun.*, 26 (1967) 748.
3 A. Mullerova, I. Michlik and A. Blazej, *Leder*, 5 (1974) 85.
4 D. J. Raven, C. Earland and M. Little, *Biochim. Biophys. Acta*, 229 (1971) 96.
5 D. Fujimoto, *Comp. Biochem. Physiol.*, 51 (1975) 205.
6 F. LaBella, P. Waykole and G. Queen, *Biochem. Biophys. Res. Commun.*, 30 (1968) 333.
7 J. W. Downie, F. LaBella and M. West, *Biochim. Biophys. Acta*, 263 (1972) 604.
8 S. Garcia Castineiras, J. Dillon and A. Spector, *Science*, 199 (1977) 897.
9 B. S. Welinder, P. Roepstorff and S. O. Andersen, *Comp. Biochem. Physiol.*, 53 (1976) 529.
10 H. Kawaski, H. Sato and M. Suzuki, *Insect Biochem.*, 4 (1974) 99.
11 D. P. De Vore and R. J. Gruebel, *Biochem. Biophys. Res. Commun.*, 80 (1978) 993.
12 C. A. Foerder and B. M. Shapiro, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 4214.
13 K. Zaitsu, S. Eto and Y. Ohkura, *J. Chromatogr.*, 206 (1981) 621.
14 M. S. Otterburn and P. E. Gargan, *Biochim. Biophys. Acta*, submitted for publication.
15 M. S. Otterburn and P. E. Gargan, *J. Biol. Chem.*, submitted for publication.
16 A. J. Gross and I. W. Sizer, *J. Biol. Chem.*, 234 (1959) 1611.
17 H. Brunner and A. Brunner, *Eur. J. Biochem.*, 32 (1973) 350.