Journal of Chromatography, 154 (1978) 336–339

CHROM. 10,937

Note

3-Chlorotyrosine can simulate galactosamine in automatic amino acid analysis

M. HAVLÍKOVÁ, P. SMOLEK, G. ENTLICHER and J. KOCOUREK
Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
(Received January 18th, 1978)

In glycoproteins and glycopeptides, amino sugars can be determined colorimetrically[1], by gas–liquid chromatography[2] or, simply, by ion-exchange chromatography on an amino acid analyzer. Amino sugars react readily with the ninhydrin reagent, and conventional two-column systems can be used directly for amino sugar analysis[3,4]. For specific amino sugar analyses, various modifications of the ion-exchange chromatographic method have been described[5–10]. However, under the mild conditions of hydrolysis used in these methods to ensure maximum yield of amino sugars, small peptides may be produced, and these can simulate the presence of hexosamines; for successful analysis, they must be removed from the analyzed sample[9,10].

During hydrolysis by hot hydrochloric acid under an atmosphere of air, some of the tyrosine of the protein or glycoprotein may be converted into 3-chlorotyrosine[11,12]. In this work, we show that the 3-chlorotyrosine thus formed can simulate the presence of galactosamine in analysis of a glycoprotein hydrolysate with an amino acid analyzer. The importance of exclusion of aerial oxygen during hydrolysis of peptides for amino acid analysis is well established, but the same precautions during liberation of amino sugars from glycoproteins are not commonly observed[13,14].

EXPERIMENTAL

Apparatus

A type AAA 881 amino acid analyzer (Mikrotechna, Czechoslovakia) was used; it was equipped with a conventional two-column system, with Ostion resins LG KS 0802 and 0803 in the short (0.7 × 6 cm) and long columns (0.8 × 62 cm), respectively[15] and was operated at 51°. The buffer system used was that of Benson and Patterson[4].

Hydrolysis of samples

For hexosamine analysis, the protein samples (1 mg) were hydrolysed for 8, 16 or 24 h at 100° with 0.5 ml of 4 *N* hydrochloric acid (prepared from redistilled hydrochloric acid) or with 0.5 ml of redistilled 6 *N* hydrochloric acid in sealed tubes that were not evacuated or filled with nitrogen. Alternatively, the samples were hydrolysed under the same conditions, but in tubes that were evacuated or flushed with nitrogen before sealing.

Preparation of tyrosine halogen derivatives

3-Chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine and 3,5-dibromotyrosine were prepared by slight modifications of known procedures[12,16,17]; the substances were purified by preparative paper chromatography with ethyl acetate–acetic acid–formic acid–water (9:1.5:0.5:2) (solvent A) as mobile phase[18]. The two melting-points of the prepared 3-chlorotyrosine hydrochloride hemiethanolate corresponded to reported values[16], and its elementary analysis was in accord with theory.

RESULTS AND DISCUSSION

In investigating the amino sugar content of pea lectin, we were unable to detect hexosamines in the acid hydrolysates colorimetrically[1] or by paper chromatography with solvent A. However, ion-exchange chromatography on an amino acid analyzer revealed the presence of *ca.* 3 moles of galactosamine per mole of lectin (M_r = 55,000). Further, gel filtration of a pronase lysate of the lectin yielded a fraction that, after acid hydrolysis, contained tyrosine and a substance in the position of galactosamine on the amino acid analyzer; before hydrolysis, this fraction behaved like pure tyrosine. Paper chromatography of the acid hydrolysate showed, in addition to tyrosine, the presence of some other ninhydrin-positive substance, the R_F value of which differed markedly from that of galactosamine. Thus, the lectin does not contain galactosamine, and the substance that behaves like galactosamine in amino acid analysis is formed from tyrosine by the hydrolysis.

Formation of a number of substances from tyrosine during treatment with hot dilute hydrochloric acid in the presence of aerial oxygen was observed by Sanger and Thompson[12], who identified these substances as 3-chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine and 3,5-dibromotyrosine (the bromo-derivatives are produced by the action of hydrobromic acid present as contaminant in the hydrochloric acid). We synthesized these four derivatives of tyrosine and established that 3-chlorotyrosine has an elution time identical with that of galactosamine on the long column of the amino acid analyzer (see Fig. 1). The elution times of the other halogen derivatives were much longer than those of glucosamine and galactosamine, so that there was no possibility of interference from these derivatives.

Under the conditions of amino acid analysis used by us, 3-chlorotyrosine was inseparable from galactosamine on the long column, and their mixture yielded a single peak on the chromatogram (see Fig. 1C). Small changes in pH of all the elution buffers had a much more pronounced effect on the behaviour of 3-chlorotyrosine than on that of other amino acids or amino sugars. On the short column, glucosamine, galactosamine and 3-chlorotyrosine were not separated from each other.

In our work on hexosamine analysis of pea lectin, 3-chlorotyrosine was always detected when samples were hydrolysed in 4 *N* hydrochloric acid, or with redistilled 6 *N* hydrochloric acid in sealed tubes that were not evacuated or filled with nitrogen before being sealed. Under these conditions, the amount of 3-chlorotyrosine formed increased with the time of hydrolysis and with the concentration of hydrochloric acid. Use of a nitrogen atmosphere or of hydrolysis *in vacuo* prevented formation of 3-chlorotyrosine. To eliminate loss of tyrosine due to formation of 3-chlorotyrosine, addition of phenol or thioglycollic acid during hydrolysis has also been recom-

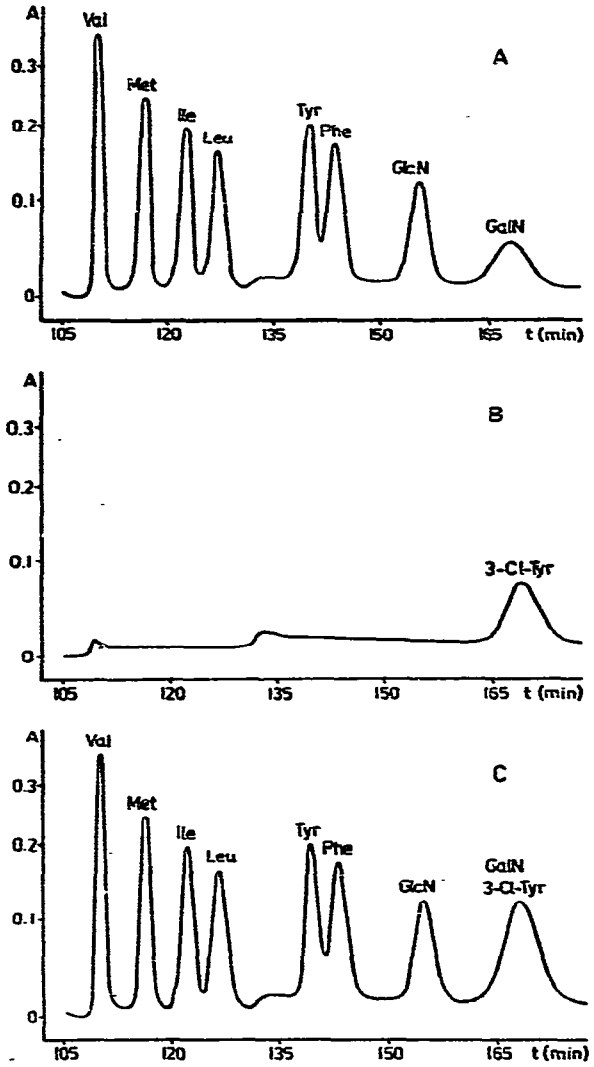

Fig. 1. Portions of chromatograms from the long column of the amino acid analyzer. A, Standard amino acid mixture supplemented with 50 nmoles each of glucosamine (GlcN) and galactosamine (GalN); B, 50 nmoles of 3-chlorotyrosine (3-Cl-Tyr); C, standard mixture of amino acids supplemented with 50 nmoles each of GlcN, GalN and 3-Cl-Tyr.

mended[12]; carefully selected conditions of hydrolysis are important also with respect to the reported occurrence of halogen derivatives of tyrosine in some proteins[19–21].

Our findings stress the necessity for exclusion of aerial oxygen and for the use of pure hydrochloric acid when hydrolysing glycoproteins for amino sugar estimation on an amino acid analyzer. Otherwise, misleading information can be obtained on the presence or amount of galactosamine. Unfortunately, even some recent review articles (see, for example, Spiro[13] and Lee[14]) dealing with conditions of amino sugar release from glycoproteins do not point out the necessity for observing these precautions.

REFERENCES

1 N. F. Boas, *J. Biol. Chem.*, 204 (1953) 553.
2 R. A. Laine, W. J. Esselman and C. C. Sweeley, *Methods Enzymol.*, 28 (1972) 159.
3 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
4 J. V. Benson, jun., and J. A. Patterson, *Anal. Chem.*, 37 (1965) 1108.
5 R. S. Steele, K. Brendel, E. Scheer and R. W. Wheat, *Anal. Biochem.*, 34 (1970) 206.
6 P. Guire, *Anal. Biochem.*, 42 (1971) 1.
7 K. Murayama, N. Shindo and H. Koide, *Anal. Biochem.*, 70 (1976) 537.
8 T. H. Plummer, jun., *Anal. Biochem.*, 73 (1976) 532.
9 M. W. Fanger and D. G. Smyth, *Anal. Biochem.*, 34 (1970) 494.
10 R. M. Zacharius, *J. Chromatogr.*, 125 (1976) 421.
11 R. Munier, *J. Chromatogr.*, 1 (1958) 524.
12 F. Sanger and E. O. P. Thompson, *Biochim. Biophys. Acta*, 71 (1963) 468.
13 R. G. Spiro, *Methods Enzymol.*, 28 (1972) 3.
14 Y. C. Lee, *Methods Enzymol.*, 28 (1972) 63.
15 Z. J. Zmrhal, J. G. Heathcote and R. J. Washington, in Z. Deyl, K. Macek and J. Janák (Editors), *Liquid Column Chromatography —A Survey of Modern Techniques and Applications*, Journal of Chromatography, Elsevier, Amsterdam, Oxford, New York, 1975, pp. 685, 690.
16 G. W. Kirby, *J. Chem. Soc.*, (1962) 3274.
17 E. O. P. Thompson, *Biochim. Biophys. Acta*, 15 (1954) 440.
18 L. Hough and J. K. N. Jones, *Methods Carbohyd. Chem.*, 1 (1962) 22.
19 J. Roche, *Experientia*, 8 (1952) 45.
20 S. O. Andersen, *Acta Chem. Scand.*, 26 (1972) 3097.
21 B. S. Welinder, *Biochim. Biophys. Acta*, 279 (1972) 491.