Journal of Chromatography, 285 (1984) 490–494
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,425

Note

Pre-column *o*-phthalaldehyde derivatization of amino acids and their separation using reversed-phase high-performance liquid chromatography

II*. Simultaneous determination of amino and imino acids in protein hydrolysates

J. D. H. COOPER*, M. T. LEWIS and D. C. TURNELL

Department of Biochemistry, Coventry, and Warwickshire Hospital, Stoney Stanton Road, Coventry CV1 4FH (U.K.)

(First received August 10th, 1983; revised manuscript received October 3rd, 1983)

Previously reported techniques[1,2] for the amino acid analysis of protein hydrolysates using *o*-phthalaldehyde–2-mercaptoethanol (OPA–MCE) derivatization have been unable to measure proline. As proline may be present in significant amounts in various proteins, its identification and quantitation are therefore of importance when investigating primary protein structure.

In Part I[3], a method for producing an imino acid product that reacts with OPA–MCE was described. This paper describes the application of this pre-column derivatization technique to protein hydrolysate analysis using reversed-phase high-performance liquid chromatography (HPLC). The chromatographic conditions for the rapid analysis of a hydrolysed sample of the enzyme α-chymotrypsin are presented. The procedure also incorporates the technique for the fluorescence detection of cysteine using the OPA–MCE reaction[4].

EXPERIMENTAL

Apparatus

The HPLC instrumentation used was as described in Part I[3].

Reagents

2-Mercaptoethanol and thiolactic acid were obtained from Aldrich (Gillingham, U.K.). Chloramine T, sodium borohydride, iodoacetic acid, amino acids, imino acids and α-chymotrypsin (obtained from bovine pancreas) were obtained from Sigma (Poole, U.K.). Unless otherwise stated, all chemicals were of analytical-reagent grade obtained from BDH (Poole, U.K.). Distilled, deionized water was used for all reagent preparations.

Amino acid standard solution. An aqueous solution containing 1 m*M* each of

* For Part I, see ref. 3.

0021-9673/84/$03.00 © 1984 Elsevier Science Publishers B.V.

proline, hydroxyproline, cystine and lysine and 500 μM of the other amino acids listed in Table I was prepared. Aliquots of this solution were stored at $-20°C$.

Internal standard solutions. An aqueous solution containing 1.5 mM each of methionine sulphone and aminocaproic acid was prepared and aliquots of this solution were stored at $-20°C$.

Chloramine T reagent. A 400-mg amount of chloramine T (N-chloro-*p*-toluenesulphonamide sodium trihydrate) was dissolved in 20 ml of dimethyl sulphoxide and diluted to 100 ml with 200 mM borate buffer (pH 8.5). This solution was stable for 24 h at room temperature.

Thiolactate reagent. A 20-ml volume of thiolactic acid was dissolved in approximately 80 ml of 400 mM borate buffer (pH 9.5) and the pH was adjusted to 9.5 with 4 M sodium hydroxide solution. The mixture was then diluted to 100 ml with water and was stable indefinitely at room temperature.

Borohydride–thiolactate reagent. A 500-mg amount of sodium borohydride was dissolved in 100 ml of 600 mM lithium hydroxide solution to which 1.0 ml of thiolactate reagent was added. This solution was stable for 24 h at room temperature.

Iodoacetic acid reagent. A 500-mg amount of iodoacetic acid was dissolved in approximately 50 ml of 400 mM borate buffer (pH 9.5), the pH was re-adjusted to 9.5 with 4 M sodium hydroxide solution and the solution was diluted to 100 ml with water. This was found stable for 1 month at room temperature.

OPA–MCE reagent. The OPA–MCE reagent was prepared according to Part I[3].

Chromatographic conditions

All solvents were filtered through a 0.45-μm filter (Anachem, Luton, U.K.) and de-gassed with helium before use. Stock aqueous solutions of 200 mM anhydrous disodium hydrogen orthophosphate and 500 mM propionic acid were prepared. A 500-ml volume of the stock phosphate solution was added to 500 ml of the stock propionic acid solution and the pH was then adjusted to 6.5 with 4 M sodium hydroxide solution. Solvent A was phosphate/propionate–methoxyethanol–acetonitrile–water (20:8:7:65) and solvent B was 2-methylpropan-2-ol–acetonitrile–water (12:38:50). The gradient programme used was as follows: at time 0, 0% B; at time 6 min, 10% B in 4 min; at time 10 min, 70% B in 9 min; at time 19 min, 100% B in 0.1 min; at time 22 min, 0% B in 0.5 min; solvent flow-rate, 2.5 ml/min.

Procedures

Hydrolysis. A 50-μl volume of internal standard solution was added to 50 μl of standard amino acid mixture and to 50 μl of a solution containing 1 mg/ml of α-chymotrypsin in water. A 100-μl volume of concentrated hydrochloric acid (Aristar grade; BDH) was added to each solution in a small screw-capped septum-sealed bottle. After heating the mixtures in an oven at 120°C for 24 h, the bottles were uncapped and transferred into a desiccator under vacuum for 48 h.

Derivatization. After removal of the hydrochloric acid, the residue was reconstituted with 100 μl of 200 mmol/l borate buffer (pH 8.5). A 200-μl volume of chloramine T reagent was pre-heated to 60°C in a thermostated water-bath and 50 μl of the reconstituted hydrolysed enzyme or standard were added. Exactly 1 min later, 200 μl of thiolactate–borohydride reagent were added. Exactly 10 min later, the mix-

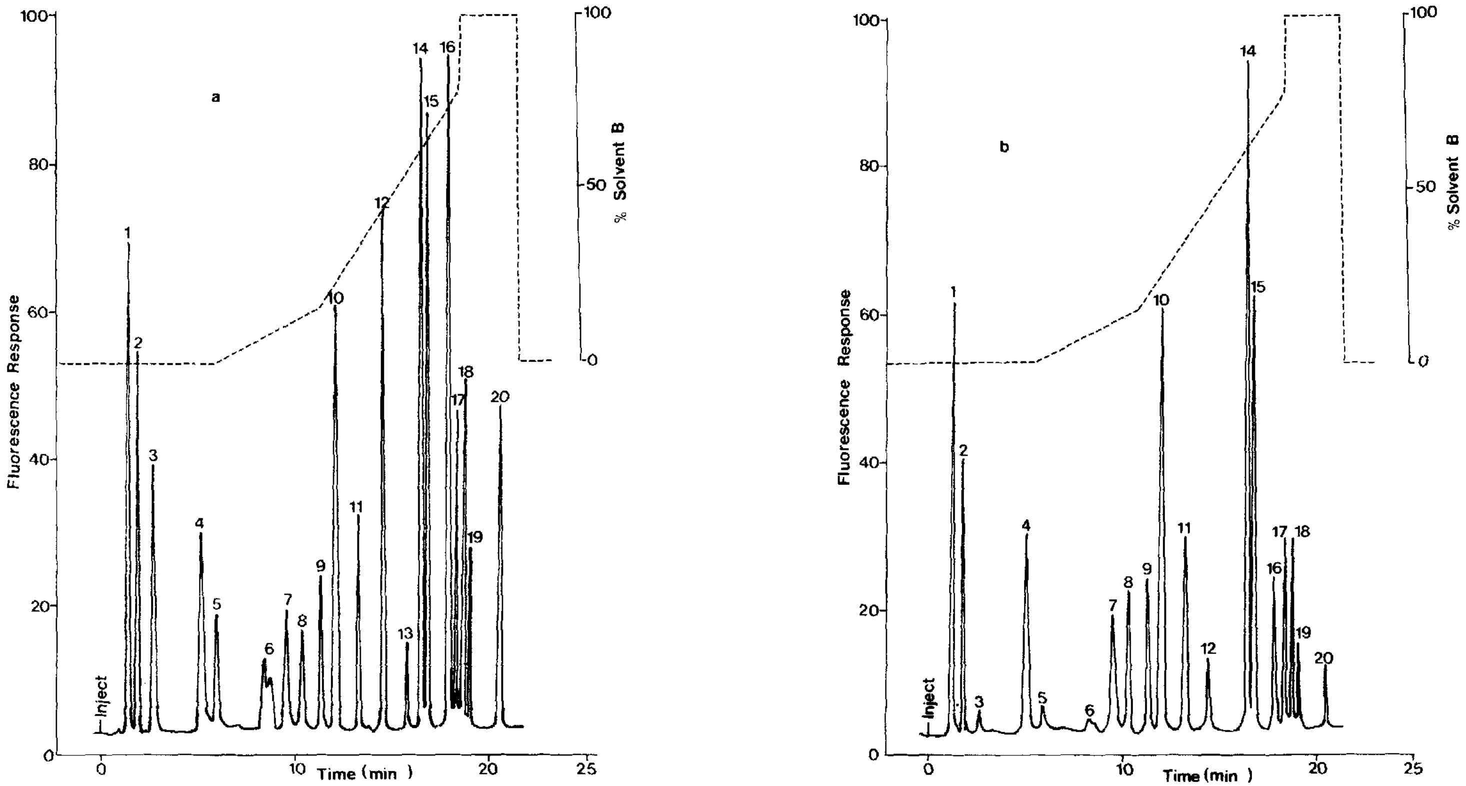

Fig. 1. Chromatograms of (a) the standard amino acid solution and (b) a hydrolysate of α-chymotrypsin (1 mg/ml). The standard and chymotrypsin solutions were prepared as described in the text. The amino acid peaks are numbered as in Table I. The broken lines indicate solvent gradient composition.

ture was removed from the water-bath and 200 μl of the iodoacetic acid reagent were added. A 1-ml volume of OPA–MCE reagent was added and 20 μl of the mixture were injected immediately on to the HPLC column using the filled loop technique.

Quantitation. Amino and imino acid OPA–MCE derivatives were identified by their retention times relative to the reference peaks produced by methionine sulphone and aminocaproic acid. The analyte concentrations were quantified by comparing their peak areas with that of methionine sulphone (internal standard).

RESULTS AND DISCUSSION

Fig. 1a and b show the chromatograms obtained for an aqueous standard amino acid mixture and a hydrolysed α-chymotrypsin sample using the pre-column derivatization procedure described. Although hydroxyproline is not usually found in standards for amino acid measurements from protein hydrolysate, it was included to identify its capacity factor using the chromatographic conditions described. The solvent compositions for the gradient chromatography were based on a previous publication[5]. However, the inclusion of 2-methoxyethanol in solvent A was found to be necessary to resolve methionine sulphoxide and glycine. Addition of 2-methylpropan-2-ol to solvent B was also necessary to resolve proline from phenylalanine.

Chloramine T, in addition to oxidizing the proline ring, also oxidizes methionine to methionine sulphoxide, which chromatographically eluted as two partially resolved peaks (Fig. 1a). A commercial preparation of L-methionine sulphoxide (obtained from Koch-Light, Slough, U.K.) showed an identical chromatographic pattern. The reason for the formation of two products from the oxidation of methionine is not understood at present.

Although borohydride is known to reduce cystine to cysteine[6], poor recoveries of the OPA–MCE cysteine–iodoacetic acid derivative were obtained. Thiolactate was added to improve the yield of this OPA–MCE derivative. MCE, which has been used previously for cysteine measurement using the OPA–MCE reaction[4], was found to react slowly with borohydride and could not be incorporated into the reagent.

Precision and accuracy

The within-batch precision was determined by replicate analyses of three different concentrations of α-chymotrypsin in water using the procedure described. The between-batch precision was determined by analysing aliquots of a 1 mg/ml solution of chymotrypsin on ten different days. The amino acid concentrations in the 1 mg/ml solution of α-chymotrypsin ranged from 44 to 438 μM and the results are shown in Table I.

The mean analytical recovery was 99.9% (standard deviation 6.9%), calculated from the analysis of an α-chymotrypsin hydrolysate supplemented with known amounts of amino acids and proline.

Using the procedures described, the relationship between peak area and concentration was linear up to 1500 μM for each amino acid.

Sensitivity

A zero standard was analysed using the fluorimeter setting and chromatographic conditions described but with the integrator peak threshold set at 1 (usually

TABLE I

WITHIN-RUN AND BETWEEN-RUN PRECISION OF THE ESTIMATED AMINO ACID CONCENTRATIONS IN THE α-CHYMOTRYPSIN HYDROLYSATE

Peaks 10 and 14 are methionine sulphone and aminocaproate (internal standards), respectively.

No.	*Amino acid*	*Within-run (n = 10) C.V. (%) Chymotrypsin range (mg/ml)*			*Between-run (n = 10) C.V. (%)*
		0.5	*1.0*	*2.0*	
1	Aspartate	3.3	2.9	2.5	4.1
2	Glutamate	3.9	1.7	1.6	2.6
3	Cystine*	5.7	3.0	2.9	4.5
4	Serine	5.1	2.5	2.4	3.7
5	Histidine	7.5	3.4	4.2	4.8
6	Methionine	6.8	5.9	5.5	8.5
7	Glycine	7.6	6.1	3.8	8.6
8	Threonine	4.5	4.6	3.8	6.5
9	Arginine	8.2	4.9	4.2	6.9
11	Alanine	10.0	3.5	3.3	6.8
12	Tyrosine	10.7	1.5	2.3	2.3
13	Hydroxyproline**				
15	Valine	4.7	1.9	2.3	2.8
16	Phenylalanine	14.5	5.6	4.3	7.9
17	Isoleucine	10.6	8.7	4.2	12.4
18	Leucine	6.4	2.5	2.0	3.5
19	Proline	7.7	4.8	3.5	6.7
20	Lysine	10.8	6.8	5.5	9.6

* Calculated as the iodoacetic acid derivative.

** Imino acid not present.

set at 12). Comparison of the peak areas that resulted from baseline noise with that of the internal standard allowed the calculation of the limit of detection. The limit of detection on-column for each amino acid was 70 fmol, estimated as the mean + 2 standard deviations concentration of the zero standard peaks ($n = 143$).

REFERENCES

1 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667.
2 B. R. Larsen and F. G. West, *J. Chromatogr. Sci.*, 19 (1981) 259.
3 J. D. H. Cooper, M. T. Lewis and D. C. Turnell, *J. Chromatogr.*, 285 (1984) 484.
4 J. D. H. Cooper and D. C. Turnell, *J. Chromatogr.*, 227 (1982) 158.
5 D. C. Turnell and J. D. H. Cooper, *Clin. Chem.*, 28 (1982) 527.
6 H. R. Mahler and E. H. Cordes (Editors), *Biological Chemistry*, Harper and Row, New York, 1967.