Journal of Chromatography, 174 (1979) 474–477

CHROM. 11,823

Note

Enzymic hydrolysis of peptides and their analysis on a gradient-operated Chromaspek amino acid analyser*

A. CASTEL, J. M. DE GRAAF and B. KRAAL

Department of Biochemistry, State University of Leiden, P.O. Box 9505, 2300 RA Leiden (The Netherlands)

(First received December 27th, 1978; revised manuscript received February 26th, 1979)

In the course of investigations on the elucidation of the primary structure of the coat protein of alfalfa mosaic virus strain VRU we used enzymic hydrolysis with aminopeptidase M and carboxypeptidases A and B. The digestion was performed by standard methods[1,2] in 0.2 *M* sodium hydrogen carbonate buffer and was stopped by the addition of an equal volume of 14% (w/v) trichloroacetic acid containing 0.2 *M* sodium acetate[3]. After centrifugation the supernatant used to be directly applied to a Unichrom amino acid analyser (Beckman, Munich, G.F.R.) based on a stepwise elution buffer system. More recently, when using a gradient-operated Chromaspek amino acid analyser (Rank Hilger, Margate, Great Britain), we faced the problem of very bad resolution of peaks, especially at the beginning of the run (Fig. 1A). This was caused by a serious disturbance of the programmed pH gradient in the elution buffer, owing to the strongly acidic character and high salt concentration of the sample applied. The pH gradient on the column is produced by mixing two buffers of pH 2.2 and 11.5, controlled by a profile on a programme drum[4].

At first we tried to solve this problem by titrating the samples with 1 *N* sodium hydroxide solution, but the results were unsatisfactory. The high salt concentration caused serious broadening of the peaks, especially for Asp, Thr, Ser, Glu, Gly and Ala, which resulted in a bad separation. Although we succeeded in designing a programme that gave a better separation of amino acids at the beginning of the run, it was not possible to separate clearly all of the amino acids within the same total run time.

Therefore, we decided to develop an alternative procedure, in which the enzymic digestion takes place in 0.1 *M* ammonium hydrogen carbonate solution. To avoid disturbances to the analysis by trichloroacetic acid or sodium acetate in the sample, the incubation with the enzyme was stopped by deproteinization with acetone. Experimental details of this method are as follows.

In digestion with the carboxypeptidases, the reaction mixtures contained 10–20 nmoles of peptide and in all instances 20 nmoles of norleucine were added as an internal standard. Carboxypeptidase A (E.C. 3.4.12.1) was an aqueous suspension

* Dedicated to Prof. E. Havinga on the occasion of his 70th birthday and his retirement from the chair of Organic Chemistry at Leiden University.

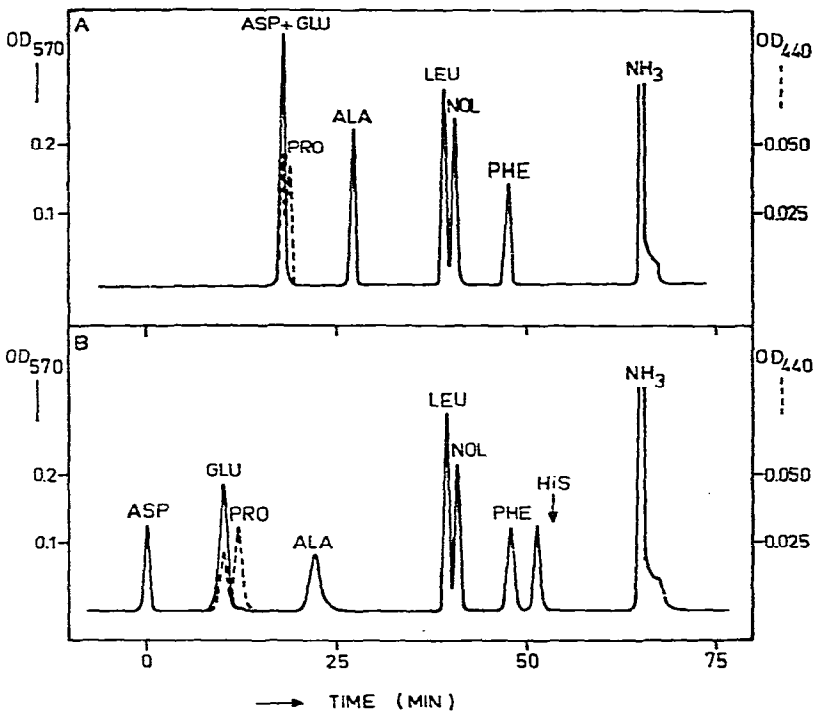

Fig. 1. Results of an aminopeptidase M digestion of the peptide Phe–Asp–Ala–Leu–Pro–Glu on a Chromaspek amino acid analyser. (A) Result with conventional procedure; (B) result with the proposed method. Experimental details are given in the text. The extra peak in B between phenylalanine and histidine is a result of the triethylamine treatment to remove excess of ammonia from the sample.

(21 mg/ml) and obtained from Sigma (St. Louis, Mo., U.S.A.). Carboxypeptidase B (E.C. 3.4.12.3) was a suspension (2 mg/ml) in 0.1 *M* sodium chloride solution, also obtained from Sigma. All other reagents, except for triethylamine [Pierce, (Rockford, Ill., U.S.A.), "sequenal grade"], were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.). Digestion was carried out as follows: 2 μl of a suspension of carboxypeptidase A and 10 μl of a suspension of carboxypeptidase B were added to a solution of 10–20 nmoles of peptide in 0.25 ml of 0.2 *M* ammonium hydrogen carbonate buffer (pH 8.5). The mixtures were incubated at 37° for 2–24 h, after which they were placed in ice and 0.75 ml of acetone was added. They were thoroughly mixed and left in ice for at least 1 h. Centrifugation of the samples was carried out for 10 min at 9000 *g* in the cold and a clear supernatant appeared. The supernatant was freed of acetone in a stream of nitrogen and dried *in vacuo*, after which 4% (v/v) triethylamine was added and the sample was dried again. This procedure was repeated once more with pure triethylamine to remove all of the ammonia. Finally, the sample was taken up in 200 μl of 0.025 *M* hydrochloric acid and applied to the Chromaspek instrument for amino acid analysis by means of the citrate–borate buffer gradient system[5].

Aminopeptidase M (E.C. 3.4.11.2) was a crystalline suspension in a solution containing 3.2 *M* ammonium sulphate and 10 m*M* magnesium chloride (pH 6.0) and was obtained from Boehringer (Mannheim, G.F.R.). Digestion was carried out by

adding 20 μl of the enzyme suspension to a solution of 10–20 nmoles of peptide in 0.2 ml of 0.1 *M* ammonium hydrogen carbonate buffer (pH 8.5). Incubation took place at 37° for 24 h, after which the samples were prepared for amino acid analysis as described for the carboxypeptidase digestions.

In control experiments using a standard calibration mixture the recoveries of different amino acids in this deproteinization procedure were tested. From Table II, it is clear that the recoveries are very good: amino acids seem not to be trapped in the precipitate. In another control experiment it was established that 42 μg of carboxypeptidase A and 20 μg of carboxypeptidase B could be precipitated in the final volume (1.0 ml) of 75% (v/v) acetone solution to the extent of more than 99%. To avoid a disturbance of the chromatogram in the region of arginine, the excess of ammonia in the samples was removed by repeated addition of triethylamine and drying *in vacuo*.

TABLE I

SOME EXAMPLES OF ENZYMIC DIGESTIONS WITH CARBOXYPEPTIDASE A AND B OR AMINOPEPTIDASE M

Enzyme	*Substrate*	*Amino acids released (residues/molecule)*
Carboxypeptidase A and B	N-Acetyl-Ser–Ser–Ser–Gln–Lys	Lys (0.7)
	Lys–Ala–Gln–Leu–Pro–Lys–Pro–Pro–Ala–Leu–Lys	Lys (1.0), Leu (1.0), Ala (<0.1)
Aminopeptidase M	Met–Ala–Ser	Met (1.0), Ala (1.0), Ser (1.0)
	Phe–Asp–Ala–Leu–Pro–Glu	Phe (0.9), Asp (0.7), Ala (0.9) Leu (1.2), Pro (1.0), Glu (1.1)

TABLE II

RECOVERY OF AMINO ACIDS AFTER ACETONE PRECIPITATION

A standard calibration mixture, containing 10 nmoles of each amino acid, was taken up in 0.2 ml of 0.1 *M* ammonium hydrogen carbonate buffer (pH 8.5) containing 20 μl of enzyme suspension. The mixture was placed in ice and 0.6 ml of acetone was added. Amino acid analysis of the supernatant was performed as described in the text.

Amino acid	*Recovery (%)**	*Amino acid*	*Recovery (%)**
Aspartic acid	97	Methionine	100
Threonine	100	Isoleucine	99
Serine	104	Leucine	95
Glutamic acid	105	Norleucine	100
Proline	98	Tyrosine	103
Glycine	100	Phenylalanine	95
Alanine	103	Histidine	100
Cystine (half)	102	Lysine	98
Valine	99	Arginine	97

* Mean values of triplicate experiments.

As a result of the triethylamine treatment an extra peak was seen in the chromatogram in a position between phenylalanine and histidine; this was not investigated further, however, because it did not interfere with the analysis.

Some examples of enzymic digestions carried out according to this new method are listed in Table I. Although digestion with aminopeptidase M in a

ammonium hydrogen carbonate buffer has been reported before[6], this procedure is not very commonly used.

We found that in this buffer the enzyme can hydrolyse peptides very efficiently (Table I). In the fragment Phe–Asp–Ala–Leu–Pro–Glu even proline is split off quantitatively. A typical example of a Chromaspek run of a peptide hydrolysed by aminopeptidase M by means of the new procedure is shown in Fig. 1B. Throughout the chromatogram the separation of amino acids is satisfactory and allows a straightforward determination of the result. In a number of duplicate experiments the results were essentially the same.

In conclusion, the proposed micro-scale method of analysis of an enzymic hydrolysate is convenient and reproducible. Digestion in a volatile buffer and deproteinization of the samples by acetone precipitation allows an easy interpretation of the results obtained on a gradient-operated high-sensitivity instrument such as the Chromaspek amino acid analyser.

REFERENCES

1 R. P. Ambler, *Methods Enzymol.*, 25 (1972) 262.
2 A. Light, *Methods Enzymol.*, 25 (1972) 253.
3 B. Kraal, J. M. de Graaf, T. A. Bakker, G. M. A. van Beynum, M. Goedhart and L. Bosch, *Eur. J. Biochem.*, 28 (1972) 20.
4 A. J. Thomas, in A. Baillie and R. J. Gilbert (Editors), *Microbiology of Applied Bacteriology*, Vol. 4, Academic Press, London, 1970, p. 107.
5 C. Murren, D. Stelling and G. Felstead, *J. Chromatogr.*, 115 (1975) 236.
6 D. Wouters-Tyrou, P. Sautière and G. Biserte, *FEBS Lett.*, 65 (1976) 225.