A SOLID PHASE METHOD FOR PEPTIDE SEQUENCING FROM THE CARBOXYL TERMINUS

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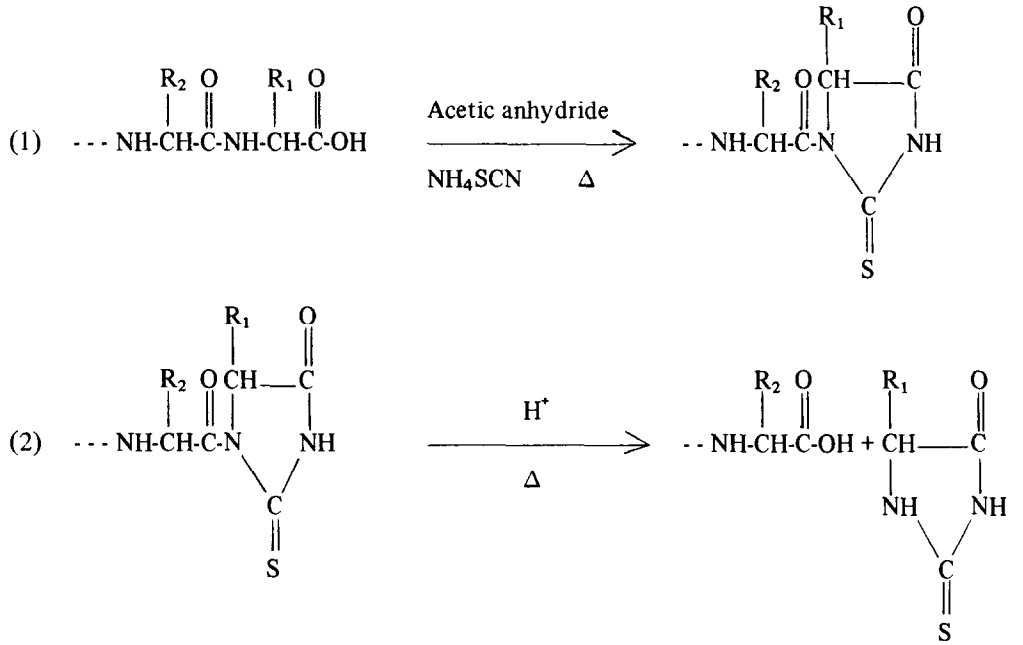
Received 1 April 1975

1. Introduction

It would be highly advantageous to have a method of sequencing peptides from the carboxyl end similar in sensitivity, quantitation and convenience to the Edman degradation from the amino terminus. The thiocyanate reaction [1] has been used [2–5] for consecutive determination of the carboxyl terminal amino acids as their thiohydantoins, but available methods are laborious. The present paper demonstrates the application of this reaction to a solid phase sequencing method. The use of a solid carrier has made this a simple and quantitative method, requiring at most one micromole of peptide; it has the possibility of development to an automated procedure.

The thiocyanate method [1] depends on the reactions shown in equations 1 and 2 [2,3].

Peptides are attached by their amino terminals to an activated porous glass support containing hydroxysuccinimide groups [6]. Reaction of side chain amino groups, when lysine is present, is prevented by carrying out one step of Edman degradation [7,8] before attachment of the peptide to the resin; this protects ε-amino groups as their phenylthiocarbamyl (PTC) derivatives and at the same time permits identification of the amino



terminal residue. After this step, the amino group of the second residue is the only amino group free for attachment. Amino acid analysis after acid hydrolysis of a small portion of the peptide-glass is used initially to determine the amount of peptide attached, and at each step of degradation for quantitative assay of the residue removed. The amino acid thiohydantoins removed may be confirmed by thin-layer chromatography [3,4] or by gas chromatography [9].

2. Materials and methods

2.1. *Apparatus*

The reaction vessel is similar to that used by Mross and Doolittle [10,11] for solid phase Edman degradation. It consists of a stoppered, water-jacketed vessel of 6.5 ml capacity, both stopper and vessel having coarse fritted glass plates; Luer joints are used at the bottom of the vessel and on the top of the stopper. Water is circulated at 50 or 60°C from an adjacent bath. The reaction vessel is clamped in a shaker [12] that rotates through 110°C at 30 rev/min. The peptide-glass and reagents are added by removing the stopper. Filtration is accomplished by applying nitrogen pressure from the top with the exit connected either to a collecting tube or to the waste. The shaker is adapted to attach multiple vessels; these are connected in series to the waterbath. For the various operations, which are brief, one vessel at a time is attached to the nitrogen at the top and to the exit tube at the bottom. Construction of an apparatus is in progress to connect the vessel by means of valves to the reaction bottles and to collection and waste receptacles.

2.2. *Peptides*

Most of the peptides were purchased from Sigma Chemical Co., Cyclo Chemical Co. or Schwarz-Mann Biochemicals. Tyr–Phe–Tyr–Asn–Ala–Lys, Ile–Ile–Arg and Ala–Lys–Arg were derived from a tryptic digest of reduced carboxymethylated basic pancreatic trypsin inhibitor [13]. All peptides gave the expected amino acid analyses.

2.3. *Attachment of peptides to the activated glass support*

Peptides are initially dissolved in 0.1 M NaOH and adjusted to pH 8.3 with 1 M NaH_2PO_4. In a typical experiment 1 μmol of peptide in 1 ml of solution is added to 200 mg of activated hydroxysuccinimide porous glass (Pierce Chemical Co.) in a 3 ml 'Silli-Vial' (Pierce). The mixture is degassed for 10 min in a vacuum desiccator, covered with a screw cap, protected from light with aluminum foil, and shaken gently at 4°C for 24 hr [6]. The beads are transferred to a small fritted glass funnel, washed twice with 5 ml of 0.1 M sodium phosphate buffer, pH 8.3, once with 5 ml of water and sucked dry. A sample of the peptide-glass, about 20 mg dry weight, is hydrolyzed at 110°C for 24 hr with 5.7 M HCl for most peptides and with 3 M *p*-toluenesulfonic acid containing 0.2% tryptamine [14] for peptides containing tryptophan; amino acid analysis of this sample gives the yield of peptide attached to the glass. The remaining peptide-glass is transferred quantitatively to the reaction vessel with water and sucked dry.

For peptides containing lysine, one step of Edman degradation is carried out by conventional methods [e.g. 7,8]. The phenylthiohydantoin is extracted and the amino terminal residue is identified by thin-layer chromatography [15]. The peptide, which now has its lysine groups protected, is then attached to the porous glass.

2.4. *Sequential removal of carboxyl terminal residues (based on the method of Stark [5])*

To remove traces of water, the peptide glass is shaken for 3 min with 5 ml of glacial acetic acid and dried with nitrogen. For formation of the peptide thiohydantoin (equation 1), 1 ml of glacial acetic acid, 2 ml of acetic anhydride (redistilled) and 200 mg of NH_4SCN (recrystallized from absolute alcohol) are added. The vessel is closed by capping the Luer joints top and bottom and is shaken by inversion for 6 hr at 60°C. Reagents are removed by filtration and the peptide glass is washed with 50% acetic acid, then water, and dried with nitrogen. The thiohydantoin is cleaved (equation 2) by adding 2 ml of 0.1 M acetohydroxamic acid in 50% pyridine (redistilled) to the beads and shaking at 50°C for 2 hr. Product and excess reagents are collected by filtration and the peptide glass is washed with 50% pyridine and finally with water. The combined filtrate and washings are evaporated to identify the thiohydantoin, if desired. A sample of about 20 mg of the degraded peptide-glass is taken for subtractive

amino acid analysis. The remaining beads are ready to repeat the process for removal of the next amino acid.

3. Results and discussion

A number of different peptides were first tested under constant conditions to determine if the nature of the amino terminal residue is important for the attachment. The results of experiments 1–4, table 1, show that the percentage attachment does not depend on the peptide used. With four different peptides, attachment varied only from 49 to 57%. Subsequent peptides were used in smaller amounts with the same amount of activated porous glass and gave higher attachment yields (experiments 5–8). Experiments 6, 7 and 8 with the peptides for which the Edman degradation was performed first show that this is a practical method of protecting lysine for attachment of a peptide by its amino terminus only.

A systematic study was then conducted with Gly–Leu–Tyr. Fig.1 shows that as the amount of peptide attached to a given amount of glass was decreased, the attachment yield improved. Attachment up to 85% was achieved. A reasonable amount for degradation, 1.0 μmol of peptide with 200 mg of glass, gave almost 60% attachment.

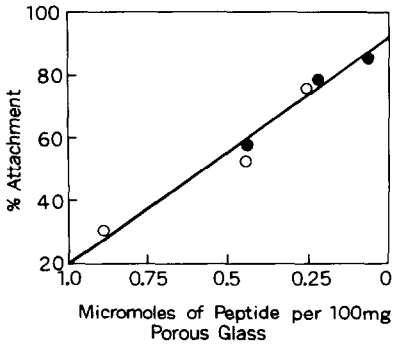


Fig.1. The effect of varying the ratio of peptide to glass on the percent of Gly–Leu–Tyr attached to activated hydroxysuccinimide porous glass. The reaction volume was 1 ml, pH 8.3; other conditions are described in the text. (○) 100 mg of porous glass; (●) 200 mg of porous glass.

Table 2 shows the stepwise removal of amino acids from several peptides. Subtractive analysis indicates that the degradative conditions developed by Stark [5] can be effectively applied to solid phase peptides for at least a few steps. The poor removal of asparagine in the third step of experiment 3 probably resulted from deamidation during the acid treatment of the previous steps; aspartic acid forms thiohydantoins with difficulty [2,4].

To increase the general applicability of the method, we tested the effect of a carboxypeptidase on a solid

Table 1
Attachment of various peptides to activated hydroxysuccinimide porous glass[a]

Experiment	Peptide	μmoles used	% Attached	
			Range	Average
1	Gly–Tyr	1.0	53–55	54
2	Trp–Val	1.0	–	49
3	Gly–Leu–Tyr	1.0	–	57
4	Met–Met–Ala	1.0	–	54
5	Ile–Ile–Arg	0.33	67–71	69
6	Phe–Tyr–Asn–Ala–(ε-PTC-) Lys[b]	0.27	59–72	66
7	ε-PTC–Lys–Arg[c]	0.63	–	68
8	Phe–Gly–(ε-PTC-) Lys[d]	0.11	–	71

[a] Attachment was conducted as described in Materials and methods with 100 mg of the porous glass in a volume of 0.5 ml at pH 8.3.
[b] This peptide was formed from Tyr–Phe–Tyr–Asn–Ala–Lys by one step of Edman degradation.
[c] This peptide was formed from Ala–Lys–Arg by one step of Edman degradation.
[d] This peptide was formed from Pro–Phe–Gly–Lys by one step of Edman degradation.

Table 2
Stepwise degradation of peptides by thiohydantoin formation and subtractive analysis

Peptide		Amino acid analysis (residues/mole)					
		Original peptide	After edman[a]	Step[b] 0	Step 1	Step 2	Step 3
Gly–Leu–Tyr	Gly	1	–	1.0	1.0	1.0	
	Leu	1.07	–	0.98	0.92	0.29	
	Tyr	1.12	–	1.06	0.18	0.16	
Leu–Tyr	Leu	1	-	1.0	1.0		
	Tyr	1.11	–	1.01	0.05		
Tyr–Phe–Tyr–	Phe	0.99	1.0	1.0	1.0	1.0	1.0
Asn–Ala–Lys	Tyr	1.89	0.96	0.91	1.00	0.98	0.96
	Asn	1.00	1.08	1.10	1.10	1.09	0.74
	Ala	1.00	0.92	0.92	0.71	0.27	0.12
	Lys	1.00	0.86[c]	0.66[c]	0.05	0	0
Pro–Phe–Gly–Lys	Pro	1.0	0				
	Phe	1.01	1.0	1.0	1.0	1.0	
	Gly	1.02	1.05	1.15	1.36	0.44	
	Lys	0.99	0.89[c]	0.75[c]	0	0	

[a] Removal of the amino terminal residue and conversion of the ε-amino group of lysine to the PTC-derivative.
[b] Analysis of the peptide attached to the glass..
[c] Partial reconversion of the PTC-derivative to lysine by acid hydrolysis.

phase peptide. A sample of porous glass with 0.36 μmol of Gly–Leu–Tyr attached was covered with 2 ml of 10 mM sodium phosphate buffer, pH 7.8, and incubated at 37°C with carboxypeptidase A (peptide: enzyme ratio, 50:1). Aliquots were taken at intervals from the supernatant solution, adjusted to pH 2.2, centrifuged and put on the amino acid analyzer. The results in table 3 show that tyrosine and leucine are readily liberated. Thus, this type of solid phase coupling does not prevent enzymatic digestion of the peptide.

The results demonstrate clearly the usefulness of a solid phase method for sequencing peptides from the carboxyl terminus by chemical degradation. A combination of a carboxypeptidase, such as carboxypeptidase C, with chemical degradation, should make it possible to continue past residues that are not very susceptible to chemical reaction, e.g. aspartic acid and proline [2,4]. The advantages of the solid phase method are elimination of laborious gel filtrations and evaporations to remove reagents, quantitative retention of the residual peptide-glass in the reaction vessel at each step of degradation and the possibility of automation. Further work is in progress to improve the conditions of the reactions so that degradation can proceed further.

After this work was well under way, an abstract was published [16] which also suggested a solid phase method for chemical degradation from the carboxyl terminus.

Table 3
Removal of amino acid residues from solid phase Gly–Leu–Tyr by carboxypeptidase A[a]

Reaction with carboxypeptidase A	Free amino acids in the supernatant (moles per mole of peptide)		
	Tyrosine	Leucine	Glycine
10 min	0.39	0.23	0
90 min	0.37	0.25	0
4 hr	0.45	0.37	0

[a] For experimental details, see text.

Acknowledgements

We thank Mr P. Sliney of Corning Glass Works, Biological Products Department, Medfield, Massachusetts for a sample of hydroxysuccinimide porous glass and for his advice regarding its use. We are grateful to Dr Russell F. Doolittle of the University of California, San Diego, for allowing one of us (B.K.) to see his appartus for solid phase Edman degradation. This work was supported by National Institutes of Health Grant AM-09826 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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