A NEW APPROACH FOR THE SOLID PHASE SEQUENCE DETERMINATION OF PROTEINS

Jui Yoa CHANG, Ernest H. CREASER and Graham J. HUGHES
Protein Biochemistry Unit, Research School of Biological Sciences, The Australian National University, Canberra, ACT 2601, Australia

Received 14 March 1977

1. Introduction

The use of the modified Edman reagent, 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate, in the stepwise degradation of peptides and proteins has provided a useful method for sequence determination [1–3]. Released N-terminal amino acids are identified as 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoins directly by thin-layer chromatography (t.l.c.) on polyamide sheets, the intense colour of the amino acid derivatives allowing detection in subnanomol quantities. Low repetitive yields due to extraction losses and time-consuming operations at each drying step have reduced both sensitivity and efficiency of the manual sequencing work. However, the solid phase degradation of Laursen [4,5] whereby peptides are attached covalently to an insoluble support, eliminates extraction losses and shortens greatly the time needed for the drying of residues after the extractions.

A solid phase degradation procedure using 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate has been developed. The N-terminal sequence of native lysozyme (first 19 residues) and glucagon (first 10 residues) attached to controlled porosity glass beads activated by *p*-phenylene diisothiocyanate have been obtained. Reduced Histidinol dehydrogenase (mol. wt 40 000) from *Salmonella typhimurium*, of hitherto unknown sequence has been attached via cysteine residues to an iodoacetamide derivative of controlled porosity glass beads and the first eight amino acid residues determined.

This new solid phase sequenching method is sensitive and efficient, requiring practically, 2–10 nmol protein as the starting material. Each cycle of degradation takes approximately 130–140 min, needs no expensive equipment and thus should provide a general method for manual sequence determination.

2. Experimental

Crystalline histidinol dehydrogenase was prepared as described by Yourno [6]. 4-*N,N*-Dimethylaminoazobenzene 4′-isothiocyanate was synthesized according to our previous paper [1]. Trifluoroacetic acid and pyridine used in the coupling buffer were from Pierce. Other solvents were commercial analytical grade and used without further purification.

2.1. *Preparation of solid support (amino glass)*

Controlled porous glass beads (Corning CPG-250, 120-200 mesh) were reacted with 3-aminopropyl triethoxysilane according to Robinson et al. [7].

2.2. *Attachment of proteins to the glass beads activated by p-phenylene diisothiocyanate (DITC)*

The attachment of native lysozyme and glucagon (Sigma) via lysine residues to the amino glass activated by *p*-phenylene diisothiocyanate was following the procedure described by Bridgen [8]. The attachment of protein on to the activated glass beads is about 1 nmol protein/mg support.

2.3. *Preparation of glass beads activated by iodoacetic acid*

Amino glass (600 mg) was suspended in a solution of ethylacetate (4 ml) containing iodoacetic acid (90 mg) and dicyclohexylcarbodiimide (100 mg) and

stirred gently at room temperature for 2 h. The derivatized glass beads were then filtered and washed with large volumes of water and acetone. Excess solvent was removed in vacuo. The dried activated glass beads were stored in the dark at −20°C.

Quantitation of the functional iodoacetamide groups on the glass bead was achieved by stirring the iodoacetic acid activated glass beads with excess of reduced glutathione in pH 8.25, Tris-buffer ethanol (2 : 1, v/v) at room temperature for 1 h. The mixture was washed with large volumes of water, 1 N HCl, Tris/HCl buffer and acetone and dried in vacuo. After acid hydrolysis, the released glutamic acid, glycine and carboxylmethylcysteine were measured in a Beckman 120C amino acid analyser. About 0.14 nmol iodoacetamide/glass beads was obtained.

2.4. *Attachment of protein to the glass beads activated by iodoacetic acid*

Reduced enzyme (250 nmol), dissolved in 0.4 M Tris/HCl buffer (1.5 ml, pH 8.25) containing 8 M urea and 2 mM EDTA, was stirred under nitrogen and in the dark with 200 mg iodoacetamide derivatized glass beads. The reaction was carried out for 30 min at room temperature and then the excess iodoacetamide groups were reacted with mercaptoethanol (20 μl). The mixture was allowed to stand for an additional 30 min, then washed alternatively with 8 M urea Tris-buffer, 50% aq. pyridine and water. Excess solvent was removed in vacuo overnight. The efficiency of coupling, calculated from amino acid analysis is around 70%.

2.5. *Sequence determination of proteins attached on the glass beads*

The protein attached glass beads (10 nmol on 10 mg support) was placed in an acid washed tube, 1 cm (i.d.) × 5 cm fitted with a quickfit glass stopper and containing a 1/4 inch magnetic stirring bar. The following degradation cycle was performed:

(1) Add 400 μl aq. 50% pyridine and 200 μl 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate (10 nmol/μl in pyridine, freshly prepared).

(2) Flush with N_2, 30 s. Seal tightly with the glass stopper and place in a water bath (53–54°C) over magnetic stirrer for 1.5 h.

(3) Remove supernatant by gentle centrifuging, wash the glass beads with pyridine (2 × 3 ml) and methanol (2 × 3 ml) and then dry in vacuo.

(4) Add 150 μl trifluoroacetic acid, flush with N_2, stopper and then incubate in the waterbath (53–54°C) over a magnetic stirrer for 10 min.

(5) Evaporate TFA in vacuo, extract the cleaved thiozolinone derivatives with 0.3 ml methanol.

(6) Wash the glass beads with 3 ml methanol, dry in vacuo (10 min) and then subject to the next cycle.

The thiozolinone extract obtained from (5) was dried in vacuo and treated with H_2O (20 μl) and acetic acid saturated with HCl (40 μl). The mixture was incubated in a separate waterbath or oven at 50–53°C for 45 min. After the reaction, the acid was dried thoroughly in vacuo and redissolved in 10–100 μl of ethanol for t.l.c. identification.

2.6. *Identification by thin-layer chromatography*

The technique for identification of 4-*N,N*-dimethylaminoazobenzene-4′-thiohydantoins derivatives of amino acids by t.l.c. on a micro-scale were essentially the same as those described in our previous report [2,3,9]. Two synthetic markers, 4-*N,N*-dimethylaminoazobenzene 4′-thiocarbamyol diethylamine (30 pmol) and 4-*N,N*-dimethylamino 4′-aminoazobenzene (30 pmol) were added to help clarify the identifications.

2.7. *Quantitation of the yield of cleaved amino acid thiohydantoins*

In the case when only one 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoin of an amino acid appeared (such as residue 2,3,11 of lysozyme), the yield could be determined by measurement of the optical density of a solution of the residue in ethanol. (ϵ_{max} about 3.4×10^4.) When the expected residue was contaminated by overlapping, it could be separated from the contaminating residues by t.l.c. on a polyamide sheet, scraped off and dissolved in 6 N HCl/ethanol (1 : 2, v/v) to measure the yield.

3. Results and discussion

Figure 1. shows the t.l.c. results of the first 19 amino acid residues of native lysozyme (attached via

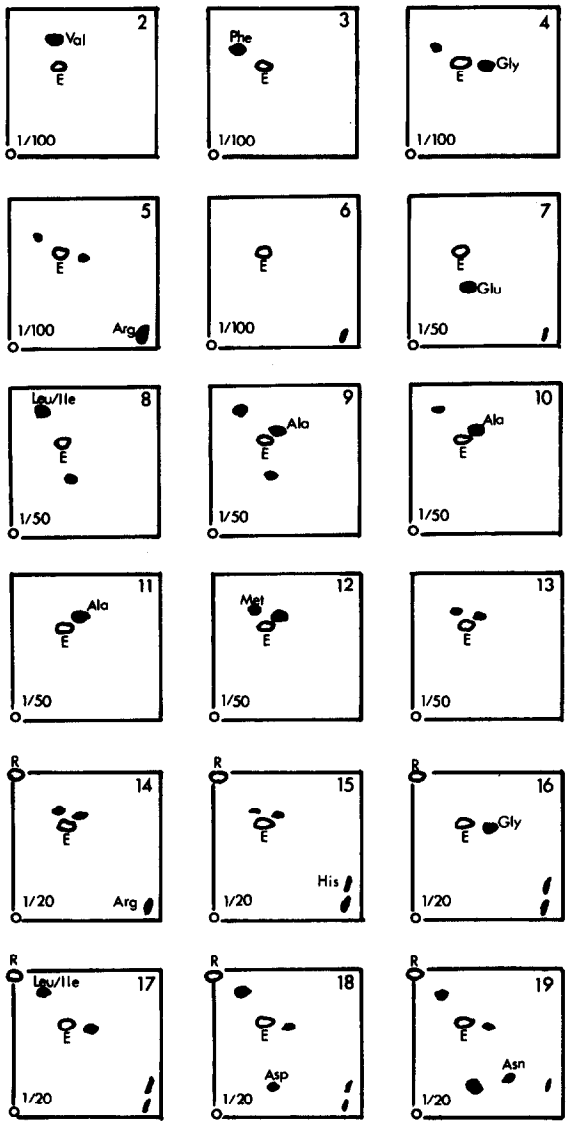

Fig.1. Schematic chromatograms of 4-*N,N*-dimethylamino-azobenzene 4′-thiohydantoins of amino acids (solid, red colour) on a polyamide sheet obtained from the sequencing of native lysozyme attached on the DITC-activated glass beads. The expected residues are denoted. The N-terminal residue and 13th residue (Lys) were attached on the glass beads. The 6th residue is cysteine. The area of the spots approximately represent the intensity of the residues appeared. A blue marker (E), 4-*N,N*-dimethylaminoazobenzene 4′-thiocarbamoyl diethylamine, was used on each t.l.c. sheet to help the identification of unknowns. R is the unextracted excess reagent (purple colour). The figures at the left bottom are the portions of ethanol extract applied on t.l.c. for identifications (starting with 10 nmol protein for sequencing).

lysine to the DITC activated glass beads) by the proposed sequenching method. It is apparent that overlapping is unavoidable. Although the expected residues appeared as the only or major spots on t.l.c. at the first 11 residues, they could appear as the minor spots after the 11 residue because of the heavy overlapping by their preceeding residues. It is, therefore, important, to notice the newly appearing spots when the heavy overlapping starts. According to this principle, one would be able to assign tentatively that the sequence of lysozyme from 16–19 is Gly–Leu/Ile–Asp–Asn. No improvements were obtained by either elevating the coupling temperature to 75°C or by the use of aqueous pyridine buffer containing dimethylallylamine/trifluoroacetic acid, pH 9.5.

Only the yield of the first 12 residues of lysozyme are presented (fig.2). The presence of multiple spots and the unexpected retention of the excess reagent beyond those residues, make it difficult to separate and quantitate the individual residues. It should be pointed out that although the extraction losses are eliminated on solid phase sequencing, the use of a magnetic stirring bar could generate fines and result in physical losses of the beads during extractions. This could also account for the drop in yields (fig.2).

The sequence determination of lysozyme attached via cysteine to the iodoacetamide derivatized glass beads gave very similar results as those shown in fig.1.

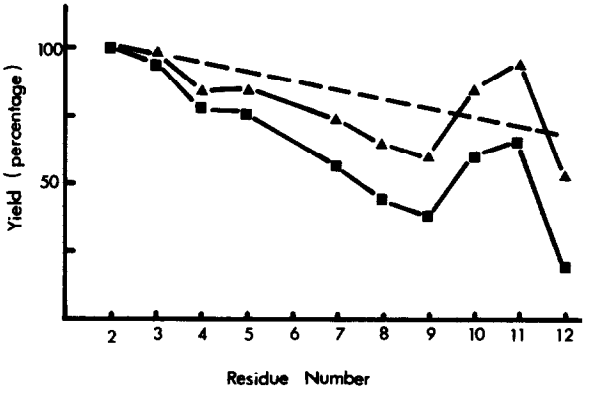

Fig.2. Yields of 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoin of amino acids of the first 12 residues of lysozyme obtained by the degradation method described (■–■). The broken line is the decrease of glass beads due to the mechanical loss. The yields/unit weight glass beads are also presented (–▲–▲).

Table 1
Thin-layer chromatography results of sequence determination of 10 nmol each of glucagon (attach via lysine to the DITC activated glass beads) and reduced histidinol dehydrogenase (attach via cysteine to the iodoacetic acid activated glass beads) by the described sequencing method

Glucagon	Histidinol dehydrogenase
1. Attach on the glass beads	1. Ser[a]
2. None	2. Phe[d]
3. Gln[d]	3. Asn[d] Phe[c]
4. Gly[d] Gln[b]	4. Thr[d] Asn[d] Phe[b]
5. Thr[b] Gly[b]	5. Leu/Ile[d] Thr[b] Asn[b]
6. Phe[d]	6. Leu/Ile[d]
7. Phe[b]	7. Leu/Ile[d] Asp[c]
8. None	8. Leu/Ile[d] Asp[b] Trp[b]
9. Asp[c]	
10. Tyr[c]	

[a] The N-terminal amino acid of histidinol dehydrogenase was confirmed as serine [10] by the liquid phase 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate method [3]
[b] Residue appeared with weak intensity
[c] Residue appeared with medium intensity
[d] Residue appeared with strong intensity

The N-terminal amino acid sequence of histidinol dehydrogenase deduced from the t.l.c. result is Ser–Phe–Asn–Thr–Leu/Ile–Leu/Ile–Asp–Trp.

The results of sequence determination of glucagon attached on the solid support (via lysine) (table 1) shows the much heavier destruction of threonine and serine residues compare to those obtained from the liquid phase sequencing method [3]. However, the results indicate that the proposed solid phase sequencing method is also suitable for the sequence determination of short peptides.

The N-terminal sequence of histidinol dehydrogenase has presented difficulty. The attachment of reduced carboxymethylated histidinol dehydrogenase via lysine to the DITC activated glass beads is unsatisfactory because of its extremely low solubility in aqueous pyridine buffer even in the presence of sodium dodecyl sulphate [8]. Sequence determination of the reduced carboxymethylated histidinol dehydrogenase by the liquid phase sequencing method [3] could only go through the first 3 residues with sharp drop of yield. However, we have been able to sequence the first eight amino acid residues of the reduced histidinol dehydrogenase attached via cysteine to the iodoacetamide activated glass beads (table 1).

The major advantages of the proposed manual solid phase sequencing method over the manual liquid phase method [3] are:

(i) Ease of extracting the by-products and excess reagent

(ii) Considerable time-saving at each drying step

(iii) Elimination of extraction losses.

The disadvantage is mainly the complete destruction of serine residues and much lower recovery of threonine residues, the reasons for which have not been determined.

Using the described method, we have been able to achieve four degradations along one polypeptide a day. Although 2–10 nmol protein is the practical amount to be sequenated, it could be reduced, as less than 0.1 nmol of the released 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoin is needed for identification by t.l.c.

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