SOLID PHASE SEQUENCING: A NEW SUPPORT FOR THE HIGH SENSITIVITY DEGRADATION OF PEPTIDES AND PROTEINS

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1. Introduction

Since the introduction by Laursen [1] of the automated solid-phase Edman degradation, numerous modifications and improvements have been made to the original method. Of particular value have been the introduction of methods for coupling peptides containing lysine residues [2] and for coupling peptides derived from cyanogen bromide digests [3]. Recently, the use of derivatised porous glass for determining the N-terminal sequence of cytochrome *c* by a solid-phase procedure has been described [4]. I would like to describe an extension of this method using a new support, *N*-(2-aminoethyl)-3-aminopropyl glass, which contains activated amino groups suitable for coupling peptides and proteins by the diisothiocyanate method, as well as for coupling large and small cyanogen bromide fragments. The use of this method for the degradation of very small quantities of peptides and proteins is also described.

2. Experimental

2.1. *Preparation of support*

Controlled-pore glass beads (Corning CPG-10, 200–400 mesh, 75,240 or 700 Å mean pore diameter) were incubated with a solution of *N*-(2-aminoethyl)-3-aminopropyl-triethoxysilane (Pierce) in acetone (2% v/v) at 45°C for 18 hr [5] and then washed with several volumes of acetone and methanol on a sintered-glass filter. The product was dried in vacuo and stored at −20°C.

2.2. *Attachment of peptides and proteins*

For these experiments the B-chain of bovine insulin (0.1 mg, Boehringer) and superoxide dismutase from *Bacillus stearothermophilus* (50 nmol, 1 mg) a dimeric 40 000 mol.wt protein with a 200 residue sub-unit (E. Kolb and J. I. Harris, unpublished results) were attached by the diisothiocyanate method [2,6]. The glass support was activated by stirring with a solution of phenylene diisothiocyanate (DITC, Eastman) in dimethylformamide (DMF, Pierce). Normally, a 100-fold molar excess of DITC over NH_2 groups was added to minimise crosslinking and the mixture was then stirred under N_2 at 45°C for 40 min or at room temperature for 90 min. Excess DITC was removed by washing with DMF on a glass sinter until the filtrate was colourless or, if smaller quantities were being prepared, by repeated washing with DMF, centrifugation and removal of the supernatant. The peptide or protein was dissolved in *N*-methylmorpholine–H_2O (1/1,v/v) buffer adjusted to pH 9.0 with trifluoracetic acid and this was added to the freshly prepared support (2 nmols peptide or protein NH_2 group/mg support). The mixture was stirred at 45°C for 30 min, 200–300 µl ethanolamine were added, and incubation continued for a further 30 min. The support was then filtered, washed with several vol of methanol, and carefully dried under water-pump vacuum.

Cyanogen bromide fragments were coupled essentially as described by Horn and Laursen [3] except that the coupling was carried out in the *N*-methylmorpholine buffer described above.

2.3. *Automatic sequencing*

The dry supports were poured into columns (10 × 0.3 cm) and subjected to degradation in either an Anachem SPA 1200 Peptide/Protein Sequencer (Anachem, Ltd., Luton, U.K.), or a Sequemat 12K

sequencer (Sequemat Inc., Boston, U.S.A.). The programme was essentially as described by Laursen [1] except that [^{35}S]phenylisothiocyanate (Radiochemical Centre, Amersham, Bucks., U.K., 2 mCi/mmol PITC) was used for the coupling. Other sequencing chemicals were obtained from Pierce Chemical Co., Rockford, I11., U.S.A. and from Rathburn Chemicals, Walkerburn, Scotland. Liberated thiazolinones were converted to phenylthiohydantoins by incubation in N HCl at 80°C for 10 min.

The thiohydantoins were extracted with ethylacetate (2 X 0.7 ml), and these were identified by t.l.c. on silica gel plates (Merck 5554) using the solvent systems; chloroform:ethanol (98:2 v/v) followed by chloroform: ethanol:methanol (88:2:1:8:10, v/v). Marker spots were visualised under u.v. light and radioactive spots by autoradiography on Kodak 'Auto-process' film. Histidine and arginine were identified by amino acid analysis after HI hydrolysis of their phenylthiohydantoin derivatives [7].

3. Results and discussion

3.1. *Attachment of protein*

Of the three porosites of glass beads utilised, protein attached to the 75 Å pore diameter beads was found to sequence more efficiently giving rise to less overlap and, hence to higher repetitive yields than the higher porosity supports. All the results presented here were obtained with the 75 Å derivative supports.

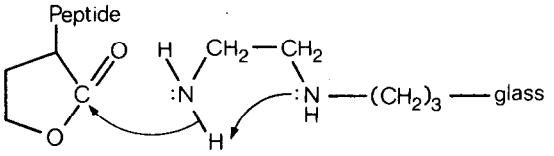
Using this support the N-terminal sequence of the 200 residue subunit of superoxide dismutase from *Bacillus stearothermophilus,* was determined and autoradiographs of t.l.c. plates for residues 1–18 are shown in fig.1. The sequence:

(Pro)–Phe–Glu–Leu–Pro–Ala–Leu–Pro–Tyr–
Pro–Tyr–Asp–Ala–Leu–Glu–Pro–(His)–Ile–

is clearly discernible. The histidine at position 17 was identified by amino acid analysis after HI hydrolysis of the acid phase remaining after extraction. This structure is in complete agreement with that found previously using a Beckman 890B Sequencer, (Bridgen, Harris and Northrop, unpublished results), and although fewer residues could be identified the amount of protein used was only 10% of that required for the spinning cup sequencer.

The sequence was confirmed by isolating a 28 residue N-terminal CNBr fragment (J. Bridgen, unpublished results), attaching this to the same support and sequencing as described above. This fragment could not be attached to the 3-aminopropyl glass described by Wachter et al. [6].

It is probable that the primary amine of the *N*-(2-aminoethyl)-3-aminopropyl glass is activated by intramolecular general base catalysis which facilitates proton removal from the attacking nitrogen atom [8].



This effect has also been exploited by Horn and Laursen [3] who have employed a triethylenetetramine derivative of polystyrene for coupling cyanogen bromide fragments. This resin has the disadvantages of being unstable and relatively difficult to prepare. Furthermore, it is necessary to perform the coupling reactions in organic solvent, which may give rise to solubility problems when working with large fragments. These problems are not encountered when using the glass derivative, although small peptides (less than 15 residues) do not always couple in good yield.

3.2. *Sensitivity of the method*

Since high sensitivity methods of amino acid sequence determination are becoming important in fields such as tumour immunology, hormone research, etc., it has become important to develop automatic methods capable of using small quantities of protein. Methods for use in the spinning cup type of sequencer have recently been described by Niall et al. [9], by Silver and Hood [10] and by McKean et al. [11]. This approach is not the best since the equipment is complex and expensive [12] and protein tends to be lost during the organic solvent extractions. The automatic solid-phase method suffers from neither of these disadvantages, and to test the sensitivity of this method the degradation of 10 μg (5 nmol) of insulin B-chain was attempted. The autoradiograph was developed after three days and the results for the first seven residues are shown in fig.2. The sequence (Phe)–Val–Asn–Gln–Leu–(His)–Gly could be

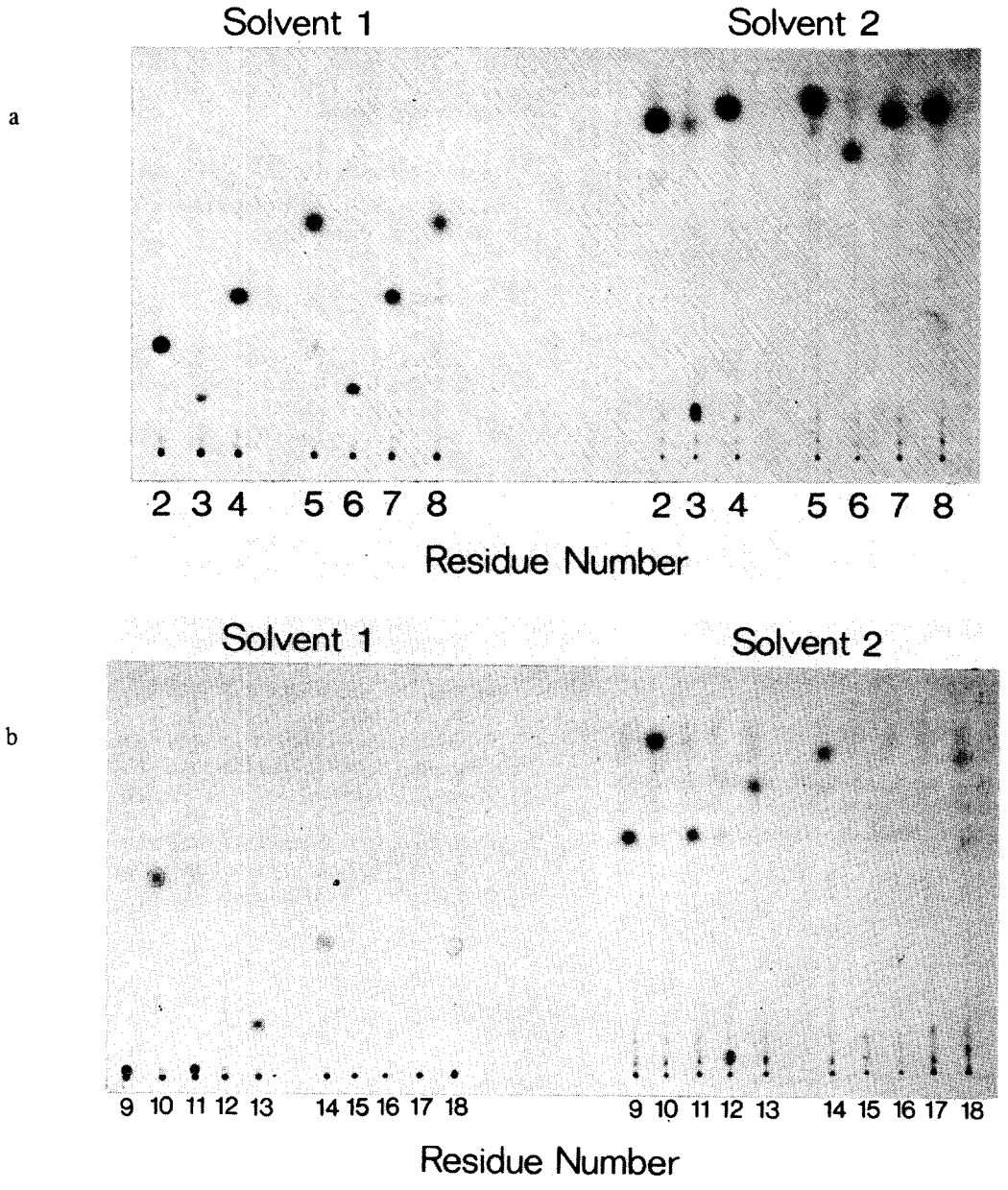


Fig. 1. Autoradiographs of t.l.c. plates showing the [^{35}S]phenyl-thiohydantoins derived from residues 1–18 of superoxide dismutase. The spots were identified by comparison with non-radioactive internal marker phenylthiohydantoins. Proline at residue 1 remains attached to the resin and was identified by dansylation of the intact protein. The faint spots seen at positions 3 and 12 after development in solvent 1 represent γ- and β-methyl esters of glutamic acid and aspartic acid, respectively.

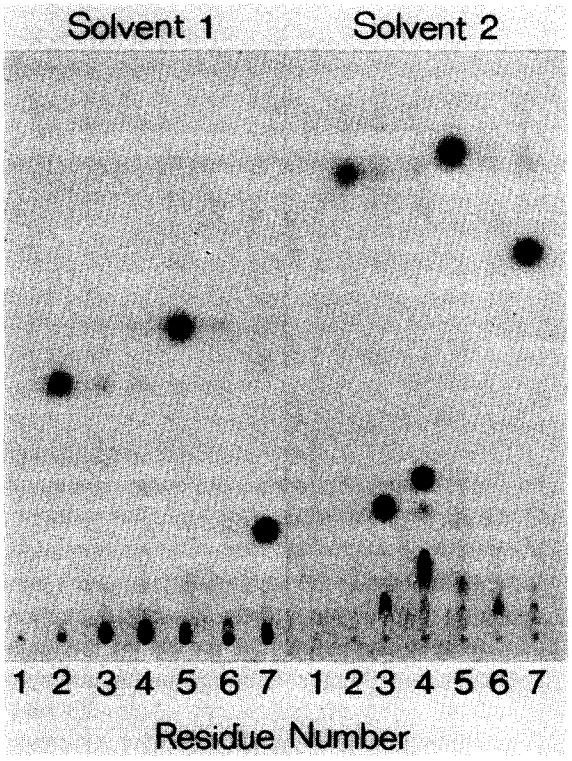


Fig. 2. Autoradiograph of t.l.c. plate showing the [^{35}S]phenylthiohydantoins derived from residues 1–7 of the β-chain of insulin (5 nmol). Residue 1 remains attached to the resin and is not identified. Residues 3 and 4, respectively asparagine and glutamine, remain at the origin in solvent 1 but give two spots in solvent 2. In both cases the slower spot corresponds to the free acid arising after deamidation side-reactions.

clearly identified. It appears, therefore, that limits of sensitivity for this method will probably arise from problems with the coupling manipulations rather than the sequencing and PTH identification.

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