N-AMINOETHYL POLYACRYLAMIDE AS SUPPORT FOR SOLID-PHASE SEQUENCING OF PROTEINS

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1. Introduction

The automatic sequencing of proteins starting from N-terminus by solid-phase technique should be performed following the same principles as for peptides [1]. In this case the solid-phase sequencing could entirely cover the sequencing field from smaller to larger peptides and proteins and became an alternative rather than a complementary route for automatic sequence analysis if comparated to the homogenous phase technique.

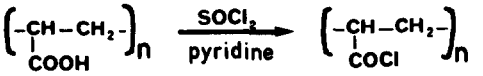
However, the serious difficulties which arise when large peptides are to be linked to a solid support for their subsequent sequential analysis constitute the principal limit for routine work.

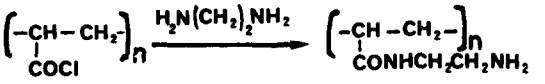
Nevertheless the chemical and physical problems associated with the insolubilization step seems to be eliminated or minimised as shown by the successful sequencing of proteins linked to aminated porous glass [2].

Despite some inconveniencies, essentially a low degree of substitution [3] and the potential lability of the –Si–O–C linkage, the aminated porous glass possesses the general properties of an ideal support for large polypeptide sequencing.

In other words the best results should be obtained using an inert and rigid support possessing a high concentration of suitable functional groups on its surface.

On the basis of these considerations we synthetised *N*-aminoethyl polyacrylamide starting from a commercial polyacrylic resin:





Some proteins were coupled to this aminated support in good yields using DITC as linking reagent [4]. Better yields were obtained when lysine-containing proteins were activated with DITC and then allowed to react with the aminated resin rather than by the inverse procedure involving the activation of the resin.

The N-terminal portion of the resin-bonded proteins was then automatically sequenced by phenyl isothiocyanate [5] as well as by methyl dithioacetate [6] entirely within non-aqueous media [7].

The results reported in the present paper show that the proposed aminated resin possesses chemical and physical properties favorable for the coupling of proteins in aqueous media as well as for sequencing reactions in organic solvents. These properties should widen the choice of solvents required for chemical reasons when reagents other than phenyl isothiocyanate are utilised [6,8].

Abbreviations: TFA, trifluoroacetic acid; DMF, dimethylformamide; DITC, *p*-phenylenediisothiocyanate.

2. Experimental

2.1. N-*aminoethyl polyacrylamide*

10 g of commercial Biorex 70 resin (200–400 mesh) were suspended in about 50 ml dioxane/2 N NaOH (3/1, v/v) and heated at about 50°C for 2 h. The resin, collected by filtration was suspended in dioxane/2 N HCl (3/1, v/v) heated at 50°C for 2 h, collected by filtration, washed with water, aceton, ethyl ether and dried under vacuum.

The purified resin (5 g) was cautiously suspended in thionyl chloride (25 ml) containing pyridine (5%) and then gently refluxed for 3 h with moisture protection.

The chlorinated resin was collected by filtration over a sintered glass filter, washed with benzene, dry ether and allowed to stand overnight under vacuum over KOH pellets.

An aliquot of the chlorinated polymer was treated few minutes with boiling 1 N NaOH: the hydrolisable chlorine content, determined by Volhard method [9] was about 5 meq./g of dry resin and this represent the half of the carboxy groups present in the starting resin.

These groups are probably the most exposed and then the most efficient, since more prolonged times of reaction or the use of other chlorinating reagents do not improve the reaction yield.

Chlorinated resin (1 g) was treated with ethylene diamine (10 ml) and allowed to stand at 40°C with occasional shaking. After 2 h the aminated polyacrylic polymer was collected by filtration, washed several times with water, acetone trifluoroacetic acid, ethyl ether and stocked as trifluoroacetate.

Chlorine determination on the separated excess of ethylene diamine and washing water account for a complete aminolysis of the chlorinated resin.

2.2. *Attachment of proteins*

1 ml of solution of lysine containing protein (300–500 nM) in pyridine/water (1/1) was mixed with 2 ml of DMF containing DITC (30–50-fold molar excess over protein amino groups and allowed to stand 2 h at 40°C under nitrogen atmosphere. The reagent concentration and the solvent composition are not critical and can be modulated in order to assure a better compromise of solubility between all the components.

Table 1
Percent insolubilization of some proteins by attachment on aminoethyl polyacrylamide resin and its suitability for sequence analysis; 10 to 20 residues were degraded for each protein derivative in order to extrapolate the amount of analysable material

Protein	Attached %	Degraded %
Chicken lyzosyme	80	75[a b]
Horse apomyoglobin	68	58[b]
Human globin α-chain	60	58[a]
Immunoglobulin κ-chain (Boyer)	60	50[a]

[a] Degraded with phenylisothiocyanate.
[b] Degraded with methyldithioacetate.

The aminated resin (300–500 mg) was added to the reaction mixture and the suspension maintained 2 h at 40°C under gentle shaking.

Methyl isothiocyanate (200 mg) was finally added to block the unreacted amino group of the resin.

The resin-bonded protein was then collected by filtration under nitrogen, washed with DMF, 50% acetic acid, water, methanol and then dried under vacuum.

The amount of insolubilized protein was determined by amino analysis after acid hydrolysis.

2.3. *Automatic sequence analysis of proteins*

The resin protein derivative was introduced into the column of an automatic sequencer [1] and

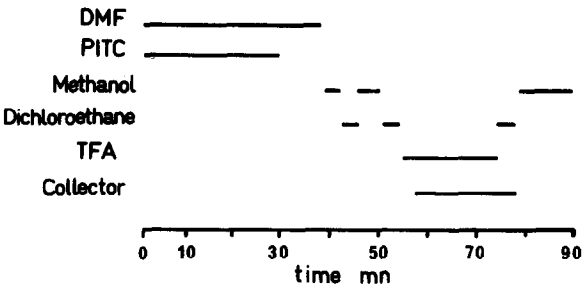


Fig.1. Sequencer program. DMF = dimethylformamide: *N*-ethylmorpholine (9:1), flow rate = 2.4 ml/h. PITC = phenylisothiocyanate 10% in dimethylformamide, flow rate = 1.2 ml/h. Methanol and Dichloroethane: flow rate = 50 ml/h. TFA = flow rate = 2.4 ml/h., t = 45°C.

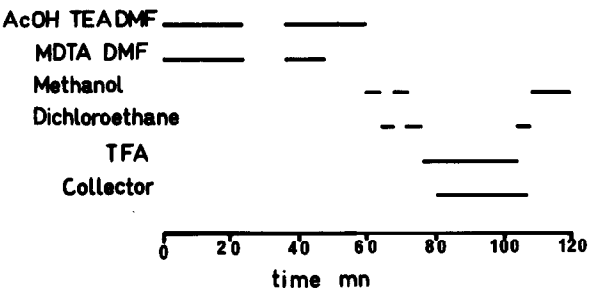


Fig.2. AcOH/TEA/DMF = 0.45 M acetic acid, 0.75 M triethylamine in dimethylformamide, flow rate = 2.4 ml/h. MDTA/DMF = methyldithioacetate 30% in dimethylformamide, flow rate = 1.2 ml/h. Methanol = mercaptoethanol 0.2% in methanol, flow rate = 50 ml/h. t = 45°C.

degraded step by step with phenyl isothiocyanate following the operation program of fig.1 or with methyl dithioacetate as in fig.2.

PTH derivatives were identified by thin-layer chromatography. Plates were examinated in u.v. light and also developed with ninhydrin coloration [10]. When favorable amino acid derivatives were encountered, the free amino acid was regenerated by alkaline hydrolysis [11] and automatically analysed.

Methyl thiazolinones deriving from methyl dithioacetate degradation were identified by acid hydrolysis (2 N HCl, 100°C, 3 h) and automatically analysed [6].

Acknowledgements

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References

[1] Laursen, R. A. (1971) Eur. J. Biochem. 20, 89–102.
[2] Wachter, E., Machleidt, W., Hofner, H. and Otto, J. (1973) FEBS Lett. 35, 98–102.
[3] Laursen, R. A. (1975) in: Immobilised Enzymes, Antigens, Antibodies and Peptides, (Weetall, H. H., ed.) M. Dekker, Inc. New York pp. 567–634.
[4] Laursen, R. A., Horn, M. J. and Bonner, A. G. (1972) FEBS Lett. 12, 62–70.
[5] Edman, P., Acta Chem. Scand. (1950) 4, 277–283.
[6] Previero, A. and Cavadore, J. C., (1975) in: Solid Phase Methods in Protein Sequence Analysis (Laursen, R. A., ed.) Pierce, Ch. Com., Rockford pp. 63–72.
[7] Previero, A., Derancourt, J., Coletti-Previero, M. A. and Laursen, R. A. (1973) FEBS Lett. 33, 135–138.
[8] Previero, A., Gourdol, A., Derancourt, J. and Coletti-Previero, M. A. (1975) FEBS Lett. 51, 68–72.
[9] Stewart, J. M. and Young, J. D. (1969) in: Solid phase Peptide Synthesis (W. H. Freeman, San Francisco) pp. 55–56.
[10] Roseau, G. and Pantel, P. (1969) J. of Chromatog. 44, 392–395.
[11] Africa, B. and Carpenter, F. M. (1966) Biochem. Biophys. Res. Comm. 24, 113–119.