AMINOPROPYL GLASS AND ITS *p*-PHENYLENE DIISOTHIOCYANATE DERIVATIVE, A NEW SUPPORT IN SOLID-PHASE EDMAN DEGRADATION OF PEPTIDES AND PROTEINS

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1. Introduction

Solid-phase Edman degradation has been used with appreciable success for quenching peptides containing up to 30 amino acid residues. In almost all cases, the solid support in these degradations was aminopolystyrene [1–3]. The coupling yield of peptides strongly depends on the quality of the resin preparations [1]. The varying quality of resin preparations may be one of the main reasons for failures of the solid-phase method. To overcome this difficulty, we experimented with 3-aminopropyl glass (APG) as a solid support which is easy to prepare and had a constant binding capacity. Our initial experiments suggest that AGP is well suited for attaching peptides and even proteins, such as cytochrome *c*. Moreover, APG exhibits excellent flow rate properties in the reaction column of the sequencer.

2. Experimental

2.1. *Preparation of 3-aminopropyl glass and its p-phenylene diisothiocyanate derivative*

Controlled-pore glass beads (Corning CPG-10, 200–400 mesh; 75, 120 or 170 Å mean pore diameter) were reacted with 3-aminopropyltriethoxysilane (Pierce) according to Robinson et al. [4]. For attaching smaller peptides, this product (APG) was used as such. For attachment of cytochrome *c*, the APG amino groups were converted to isothiocyanate groups by treatment with *p*-phenylene diisothiocyanate (DITC; fig. 1). DITC (Eastman) was used in a 25-fold excess over NH_2-groups to prevent crosslinking. APG was added in small portions to a solution of DITC in dimethylformamide (DMF) under slight stirring within 1 hr. The reaction mixture was allowed to stand for another 2 hr at room temp. To remove excess DITC, the final product was washed thoroughly on a fritted glass filter with DMF and methanol, dried *in vacuo* and stored under nitrogen in a freezer.

2.2. *Attachment of peptides and cytochrome c*

The attachment of shorter peptides to APG was studied with tryptic peptides of the protease inhibitor from dog submandibular glands [5], which have already been sequenced by manual Edman degradation [6]. Peptide T-I, Gly–Pro–Pro–Pro–Ala–Ile–Gly–Arg, (200 nmol) was blocked with t-butyloxycarbonylazide (Pierce) according to Levy and Carpenter [7]. The blocked peptide was activated with *N,N'*-dicyclohexylcarbodiimide (Roth; 0.05 ml of a 10% solution in DMF for 6 hr at 37°C). The solution containing the activated peptide was mixed with 100 mg APG suspended in 0.2 ml DMF and kept at 37°C overnight. Peptide T-VIII, CMCys–Ser–Phe–CMCys–Asp–Ala–Val–Lys, (170 nmol) was attached to APG by the DITC method described by Laursen [3].

Melittin [8], the toxic peptide of bee venom (Serva), was used without further purification. 200 nmol of melittin were coupled by a slight modification of the DITC method. In this case 50 mg of dry APG were suspended in the DITC-activated peptide solution; another 50 mg of APG were added 1 hr later.

Cytochrome *c* from horse heart (Boehringer; 200 nmol) was dissolved in 0.5 ml of pyridine–water

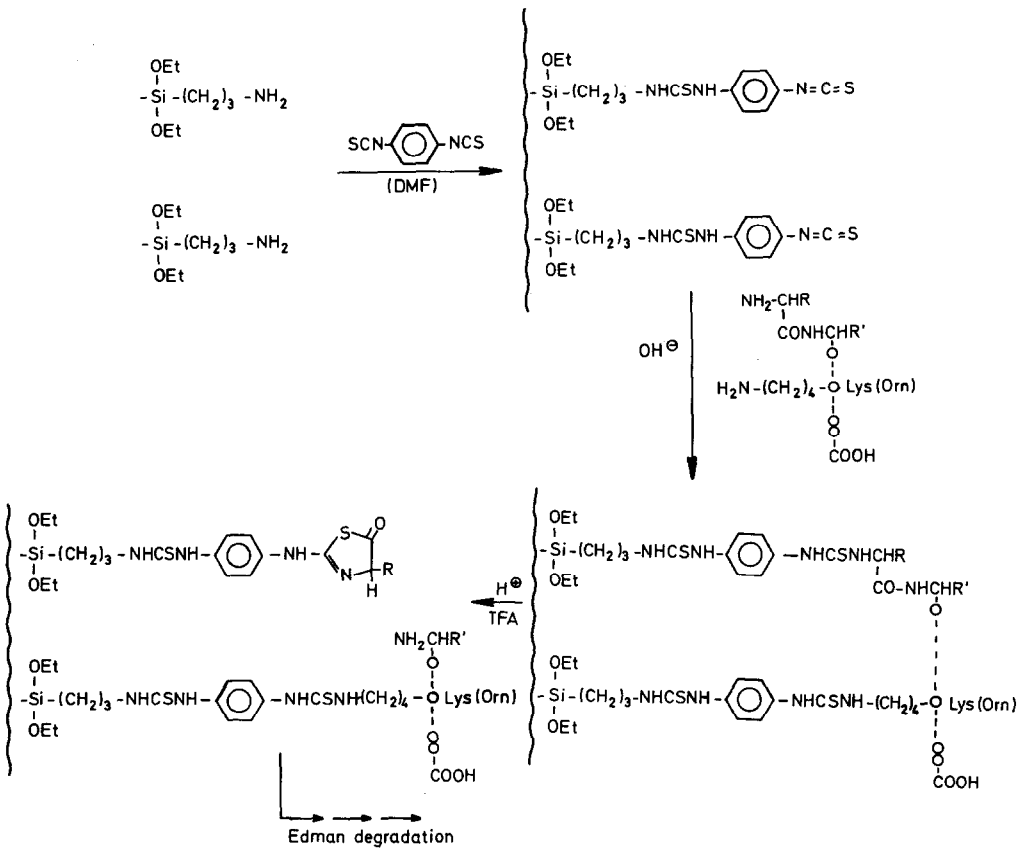

Fig. 1. Reaction scheme of the coupling of *p*-phenylene diisothiocyanate (DITC) to 3-aminopropyl glass (APG) and attachment of a peptide.

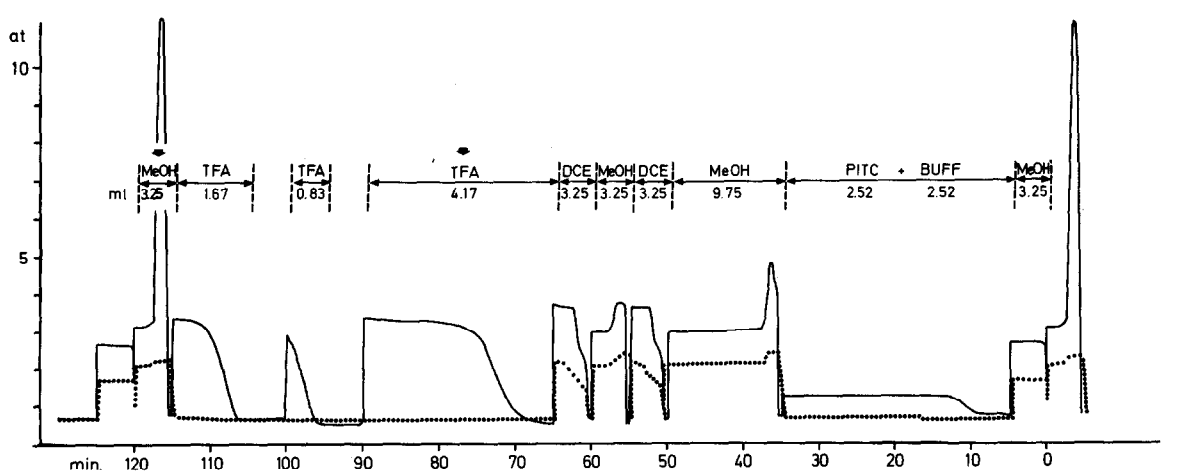

Fig. 2. Pressure profile and sequencer program for one degradation cycle with 200 mg of the *p*-phenylene diisothiocyanate derivative of 3-aminopropyl glass (·····) and 25 mg aminopolystyrene (——). Symbols at top indicate PITC: phenyl isothiocyanate (10% in acetonitrile); BUFF: sequencing buffer [1]; MeOH: methanol; TFA: trifluoroacetic acid. Numbers at top indicate ml reagent or solvent. Time from right to left. Samples are collected between 78 and 118 min.

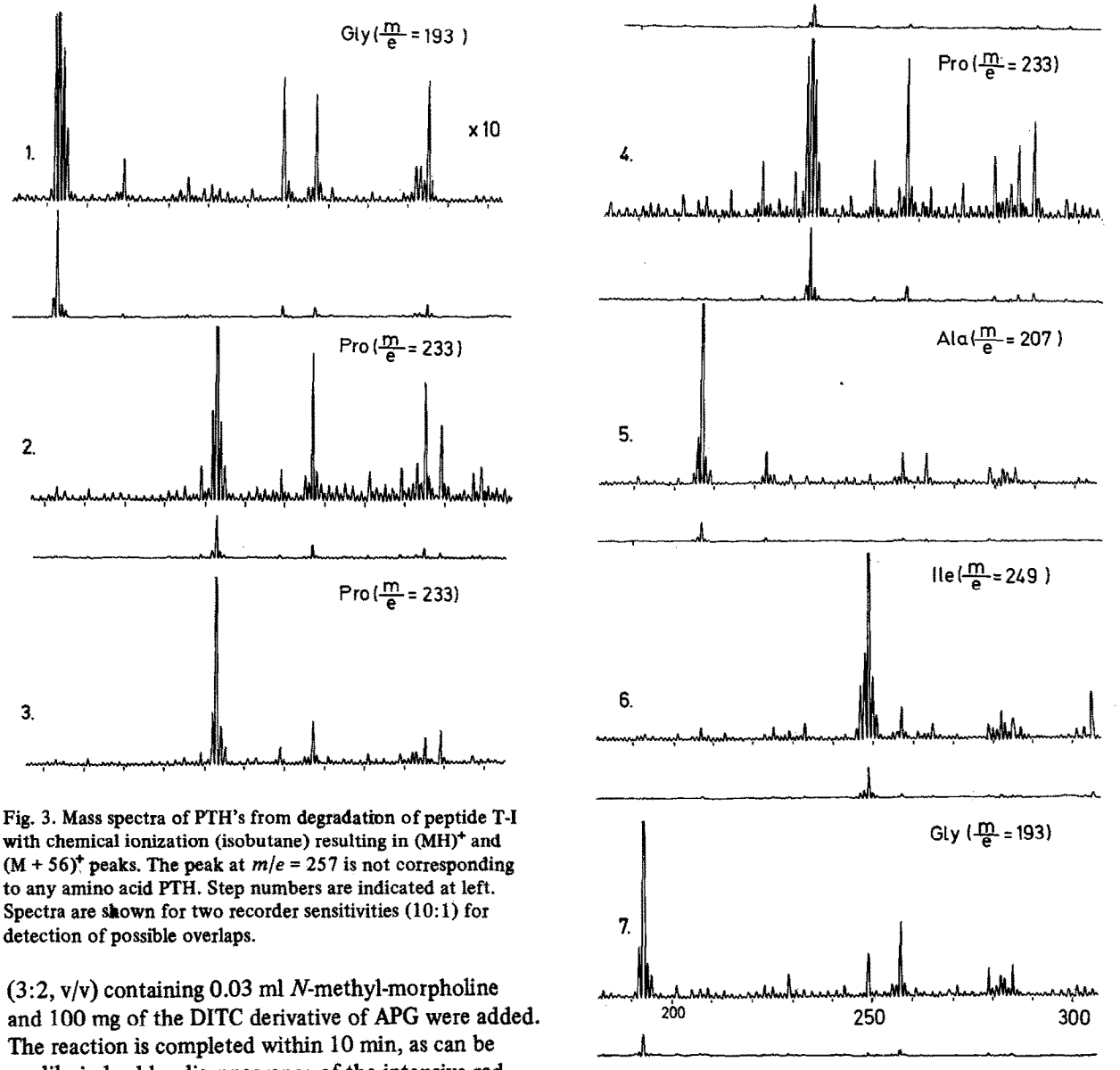

Fig. 3. Mass spectra of PTH's from degradation of peptide T-I with chemical ionization (isobutane) resulting in $(MH)^+$ and $(M + 56)^+$ peaks. The peak at $m/e = 257$ is not corresponding to any amino acid PTH. Step numbers are indicated at left. Spectra are shown for two recorder sensitivities (10:1) for detection of possible overlaps.

(3:2, v/v) containing 0.03 ml *N*-methyl-morpholine and 100 mg of the DITC derivative of APG were added. The reaction is completed within 10 min, as can be readily judged by disappearance of the intensive red colour of cytochrome *c* from the supernatant.

The peptide-loaded glasses were washed thoroughly with DMF and finally with methanol on a small fritted glass filter column and dried *in vacuo.*

2.3. *Automatic Edman degradation*

The automatic sequencer used for the degradations will be described in detail elsewhere. It was constructed following the basic ideas of Laursen [1] with some technical modifications. As in the Laursen machine, Chromatronix valves are used. They are activated by nitrogen, which is controlled by solenoid valves (Lucifer). The connections are made by teflon tubing with Chromatronix fittings and adapters. All the pumps are of the syringe type with driving units based on perfusion pumps developed in this laboratory earlier [9]. The reservoirs for reagents and solvents are sealed by teflon plugs pressed upon the grinded bottle-necks. The bottles are connected alternatively to prepurified nitrogen (0.2 atmos.) at intervals of 2 min. The reac-

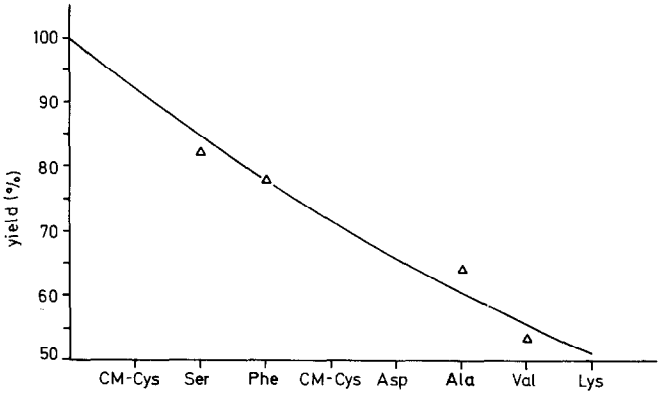

Fig. 4. PTH yields obtained during degradation of peptide T-VIII as estimated by gas chromatography. The solid line is a theoretical curve for an average yield of 92% per cycle.

tion columns (0.3 X 5 or 0.3 X10 cm) are embedded in a thermostated block containing a coil which allows pre-heating of reagents. The head pressure of the column is recorded. Typical pressure profiles and the sequencer program are shown in fig. 2.

2.4. *Identification of phenylthiohydantoins*

The conversion procedure was adapted from Laursen [1]. The resulting amino acid phenylthiohydantoins (PTH's) were identified either by gas chromatography [10] or mass spectrometry [11] or both. Gas chromatography was done with a Beckman GC 45 following the procedure of Bober [12], mass spectrometry was performed on a Finnigan 3100 with chemical ionization (isobutane).

3. Results and discussion

A solid-phase support for Edman degradation has to be resistant against trifluoroacetic acid. Until now, this condition has been fulfilled only by a few supports, such as polystyrene or glass matrices. Since controlled-pore glass (CPG) beads [13] with a wide range of well-defined pore diameters are commercially available, such support has become promising for attaching proteins and peptides [14]. In our experiments, the introduction of amino groups to porous glass with 3-aminopropyltriethoxysilane [4] proved to be simple and led to constant binding capacities of at least 150–170 nmol/mg CPG, as estimated by titration with trinitrobenzene sulphonic acid for a CPG with a mean pore diameter of 75 Å. The product showed no significant loss of activity over a period of two months.

3.1. *Peptides attached to aminopropyl glass*

APG was applicable for both commonly used coupling methods, the carboxyl-activating method [1], as demonstrated with peptide T-I (fig. 3), as well as the DITC method, as demonstrated with peptide T-VIII (fig. 4) and melittin (fig. 5). Compared with aminopolystyrene of similar particle size, APG exerts a considerably lower flow resistance in the reaction column as indicated by the pressure profiles in fig. 2. Therefore APG can be used without dilution by glass beads and we were able to employ shorter columns (5–10 cm). A further decrease of column length seems possible and should afford an advantage in the optimization of the degradation cycle.

The result from the automatic degradation of peptide T-I is shown in fig. 3. The mass spectral identification of the amino acid PTH's reveals a very low overlap. This may be due to the good accessibility of the peptide attached to APG.

The PTH's from peptide T-VIII, coupled by the DITC method, were clearly identified by gas chromatography, with the exceptions of the glass-bound N-terminal carboxymethyl cysteine (CMCys) and C-terminal lysine, and of the aspartic acid PTH which was identified by mass spectrometry. The repetitive yields in this degradation are plotted in fig. 4.

As an example for a longer peptide attached by the

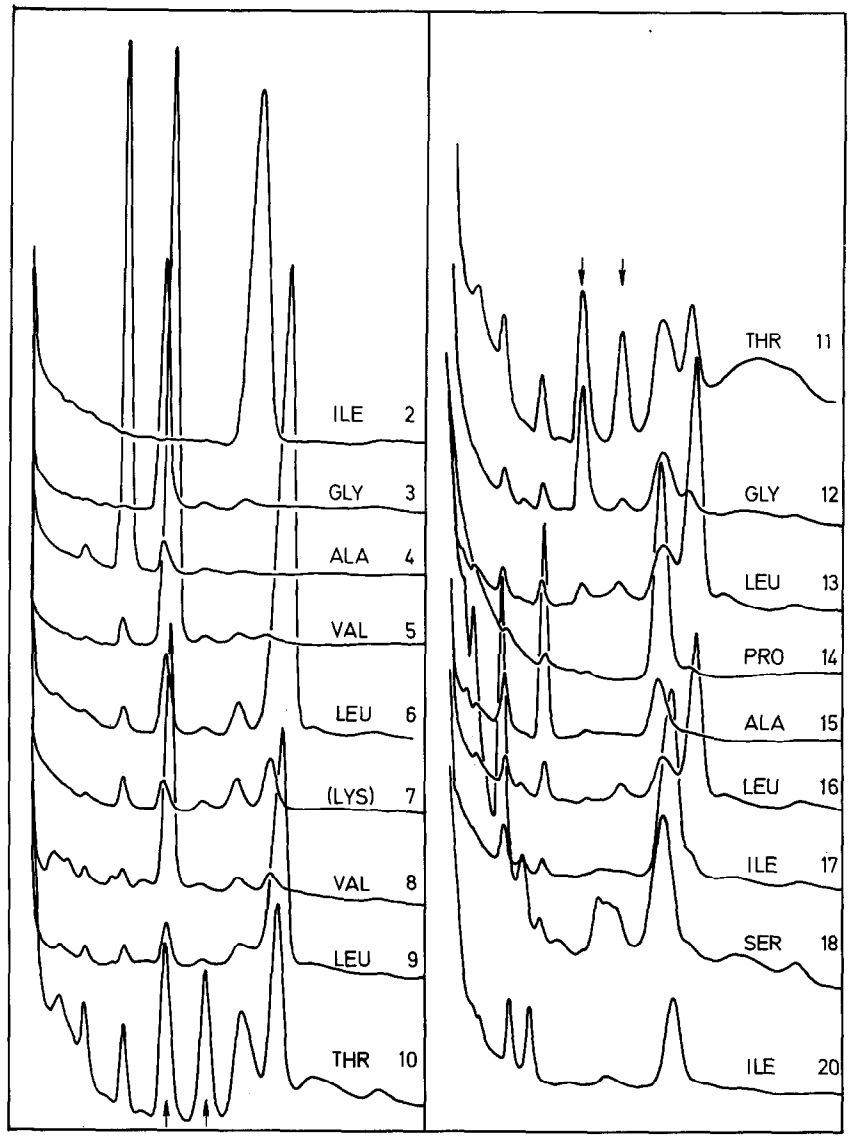

Fig. 5. Gas chromatograms of PTH's from degradation of melittin. Sensitivities are increased 4-fold for Thr^{10} and Thr^{11}, 2-fold for steps 12–17 and 20 when compared to steps 1–9, and 8-fold for Ser^{18}.

DITC method we have degraded melittin which contains 26 amino acids, among them 3 lysines in positions 7, 21, and 23. The PTH's from positions 2 to 20 have been identified by gas chromatography (fig. 5), with the exceptions of the anchor points, i.e. Gly^1 and Lys^7, and of Trp^{19}. The latter amino acid leads to red reaction products. The decrease of PTH yield after step 7 may be due to the loss of this part of the peptide which was anchored only at positions 1 and 7. The average yield per cycle was 89% as calculated from Ile^2 and Ile^{20}.

3.2. *Cytochrome c attached to the p-phenylene diisothiocyanate derivative of 3-aminopropyl glass*

It appeared promising to attach the isothiocyanate groups to the support in a separate reaction before the

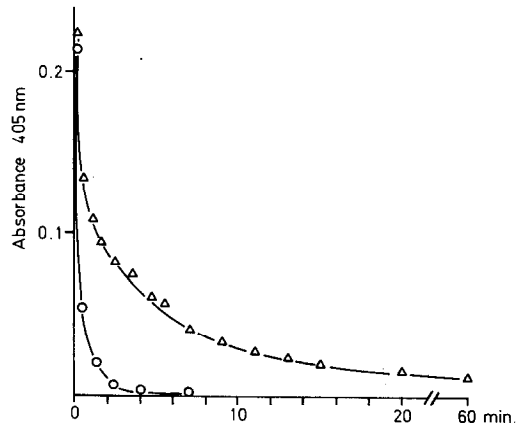

Fig. 6. Kinetics of attachment of cytochrome *c* to the *p*-phenylene diisothiocyanate derivative of 3-aminopropyl glass, as followed by absorbance of residual cytochrome *c* at 405 nm. In pyridine–water–*N*-methyl-morpholine (30:20:3, v/v/v; o—o—o) the reaction is faster than in absence of *N*-methyl-morpholine (△—△—△).

coupling of the peptide. In this case maximal loading of the support with reactive groups should be attainable. Furthermore, the crosslinking reaction of the support caused by excess DITC should not compete with the attachment of the peptide at the favourable sites of the glass.

Cytochrome *c* from horse heart was used as a model to substantiate this hypothesis, because it is rich in lysines exposed at the surface of the molecule [15]. As estimated from the disappearance of the absorbance at 405 nm, cytochrome *c* was coupled rapidly and completely (fig. 6). The red-coloured support was washed extensively with water, pyridine, and NH_4HCO_3-buffer in order to exclude an unspecific adsorption. Cytochrome *c* from horse heart could not be degraded directly, because it is acetylated [15]. For confirmation of the attachment of the protein a sequencer run was performed after tryptic digestion of the glass-fixed cytochrome *c*. The resulting PTH's, though very complex, clearly indicated that the attached fragments are degradable.

Degradations of N-terminal free cytochromes *c*, e.g. from *Candida krusei* and *Neurospora crassa*, may provide further evidence for the applicability of the DITC derivative of APG for sequencing proteins by the solid-phase method. In this respect, recent experiments with large polypeptides, like the protease inhibitors of pig pancreas [16] and *Anemonia sulcata* [17] are encouraging.

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