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Vapor-phase modification of sulfhydryl groups in proteins

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Proteins and peptides are readily and specifically modified at their sulfhydryl groups by the vapors of a mixture of 4-vinylpyridine and tributylphosphine. The phenylthiohydantoin derivative of *S*-β-(4-pyridylethyl)cysteine formed during sequence analysis is easily detectable in current identification systems.

Protein sequencing; Vapor-phase modification; Sulfhydryl group; *S*-pyridylethylation

1. INTRODUCTION

S-pyridylethylation is a very convenient method to block and determine free sulfhydryl groups and disulfide bonds in proteins and peptides [1–3]. Moreover, since the phenylthiohydantoin of *S*-pyridylethylcysteine is easily detectable with current HPLC procedures, positive identification of cysteine residues during protein or peptide sequencing is easily accomplished when the sample is *S*-pyridylethylated before. We have recently reported on such an *S*-pyridylethylation procedure in the cup of the liquid-phase sequencer prior to sequencing [4].

In the first instance we tried to use this principle in the gas-phase sequencer as well, simply by adding the reagents, 4-vinylpyridine and tributylphosphine onto the polybrene-coated glass-fiber filter disk containing the peptide or protein. The modification reaction was conducted by passing aqueous trimethylamine vapor (R2) through the filter disk for 1 h or so. Although *S*-pyridylethylation took place, it was found that excess of reagents and/or reaction products could only partially be removed by the solvents used in the gas-phase sequencer. Complete disappearance of spurious peaks was only accomplished by several cycles more.

In order to overcome this problem, *S*-pyridylethylation of proteins was performed in a small test tube, and it was tried to remove excess of reagents and reaction products by liquid-liquid extraction with various solvents including toluene, *n*-heptane, *n*-chlorobutane, ethyl acetate and *n*-butyl acetate. Unfortunately, stubborn impurities remained in the preparation and interfered with the subsequent purification of the peptide or protein by HPLC, as well as in the sequence determination.

It was, however, realized that 4-vinylpyridine and tributylphosphine are both rather volatile reagents, and that interfering impurities may be non-volatile. This, together with the fact that many chemical reactions occurring in the liquid phase also take place in the vapor phase – the gas-phase sequencer is a pertinent example! – prompted us to explore the modification of sulfhydryl groups and disulfide bonds in proteins in the vapor phase. We have developed a very simple and convenient procedure, requiring no special equipment.

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2. MATERIALS AND METHODS

2.1. *Reagents and solvents*

The following reagents and solvents were used.

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Trifluoroacetic acid (Merck, zur Synthese) was distilled from CrO_3, dried with $CaSO_4$ and redistilled. It was stored at room temperature.

4-Vinylpyridine (Aldrich) was distilled under reduced pressure and stored at −20°C.

S-β-(4-Pyridylethyl)cysteine was obtained from Pierce.

Tri-*n*-butylphosphine (Aldrich) was used as such; it was stored at −20°C.

n-Heptane and ethyl acetate, used for washing the glass-fiber filters, were 'protein sequencer grade' (Applied Biosystems) or of comparable quality.

Pyridine was distilled from phthalic anhydride and ninhydrin, respectively and stored in the dark at room temperature.

Polybrene (reinst) was from Serva, Heidelberg.

Glass-fiber filter disks (12 mm ∅) were cut from Whatman GF/C glass-fiber sheets by means of a cork borer, and subsequently washed twice with trifluoroacetic acid and air-dried in a cupboard. The filters were stored in a tightly closed bottle.

Amino acid sequences were determined with an Applied Biosystems model 470 A protein sequencer, on-line connected with a 120 A PTH analyzer. Program 3.0 was slightly modified (see also section 2.2).

2.2. *Procedure*

An amount of 1–100 μg of protein, dissolved in water or in another appropriate volatile solvent, is spotted in small aliquots on a glass-fiber paper disk, previously treated with trifluoroacetic acid (see section 2.1). The solvent is allowed to evaporate completely in a current of air at 40°C. *S*-pyridylethylation is performed in an ampoule, which has a second constriction in its centre (see fig.1). The lower part of the ampoule is filled with a freshly prepared mixture of 100 μl H_2O, 100 μl pyridine, 20 μl 4-vinylpyridine and 20 μl tributylphosphine (the relative composition of this mixture being not very critical). The protein- (or peptide-) coated filter disk is placed into the upper part of the ampoule above the central constriction (see fig.1). This constriction still allows a free diffusion of reagent vapors, but prevents the filter disk from being in direct contact with the liquid reagent mixture. The ampoule is then evacuated, and sealed by means of an oxygen flame. Evacua-

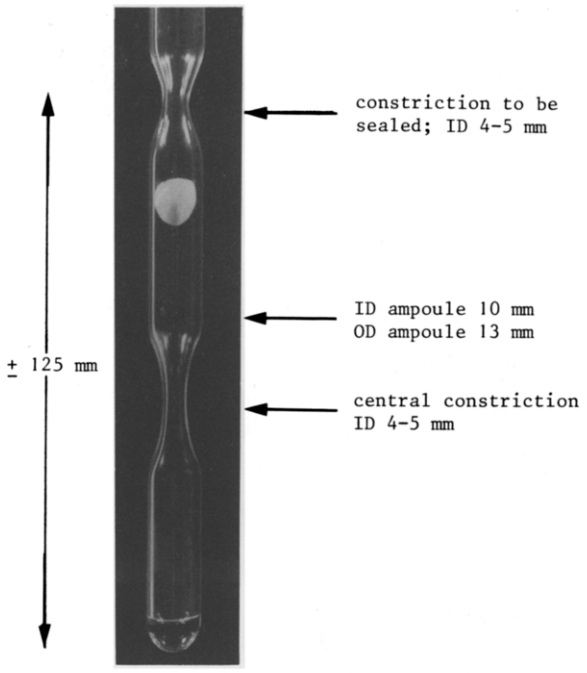

Fig.1. The ampoule, used for vapor-phase *S*-pyridylethylation. See the text for further details.

Table 1

Precycling program, prior to sequencing, for proteins or peptides *S*-pyridylethylated according to the procedure described

Step	Cartridge function	Flask function	Time (s)
1	argon dry	pause	300
2	deliver R3	pause	300
3	argon dry	pause	120
4	prep S3	pause	6
5	deliver S3	pause	5
6	pause	pause	120
7	deliver S3	pause	100
8	argon dry	pause	120
9	deliver R2	pause	300
10	argon dry	pause	120
11	prep S1	pause	6
12	deliver S1	pause	120
13	prep S2	pause	6
14	deliver S2	pause	300
15	argon dry	pause	120

R2, vapor of trimethylamine; R3, vapor of trifluoroacetic acid; S1, *n*-heptane; S2, ethyl acetate; S3, 1-chlorobutane

tion removes oxygen and accelerates vapor diffusion. The vapor-phase reaction is conducted for 2 h at 60°C. Subsequently, the filter disk is removed from the ampoule, and washed three times in each of the following solvents: *n*-heptane; *n*-heptane:ethyl acetate (2:1, v/v); ethyl acetate, and dried. The filter disk is then wetted with 30 μl of a solution of polybrene (50 mg/ml H_2O), dried and loaded onto the gas-phase sequencer. To remove virtually all traces of reagent, it is recommended to precycle the sample with the program depicted in table 1.

Alternatively, the filter disk containing the *S*-pyridylethylated protein can be stored in a box at −20°C until use.

3. RESULTS AND DISCUSSION

Fig.2 depicts the sequence analysis of the first steps of about 1 nmol *S*-pyridylethylated basic protease inhibitor, fig.3 that of about 1 nmol human insulin. The method for on-line PTH-amino acid analysis is according to Hunkapiller [5]. As one can see, the first 2–3 steps still contain some impurities, which however do not interfere with the PTH analysis. The PTH of *S*-pyridylethylcysteine is clearly visible. It elutes with or near the peak of diphenylthiourea, and can be separated from the latter by a slight change in the ionic strength of the first buffer, used for the gradient separation of the PTH-amino acids: a

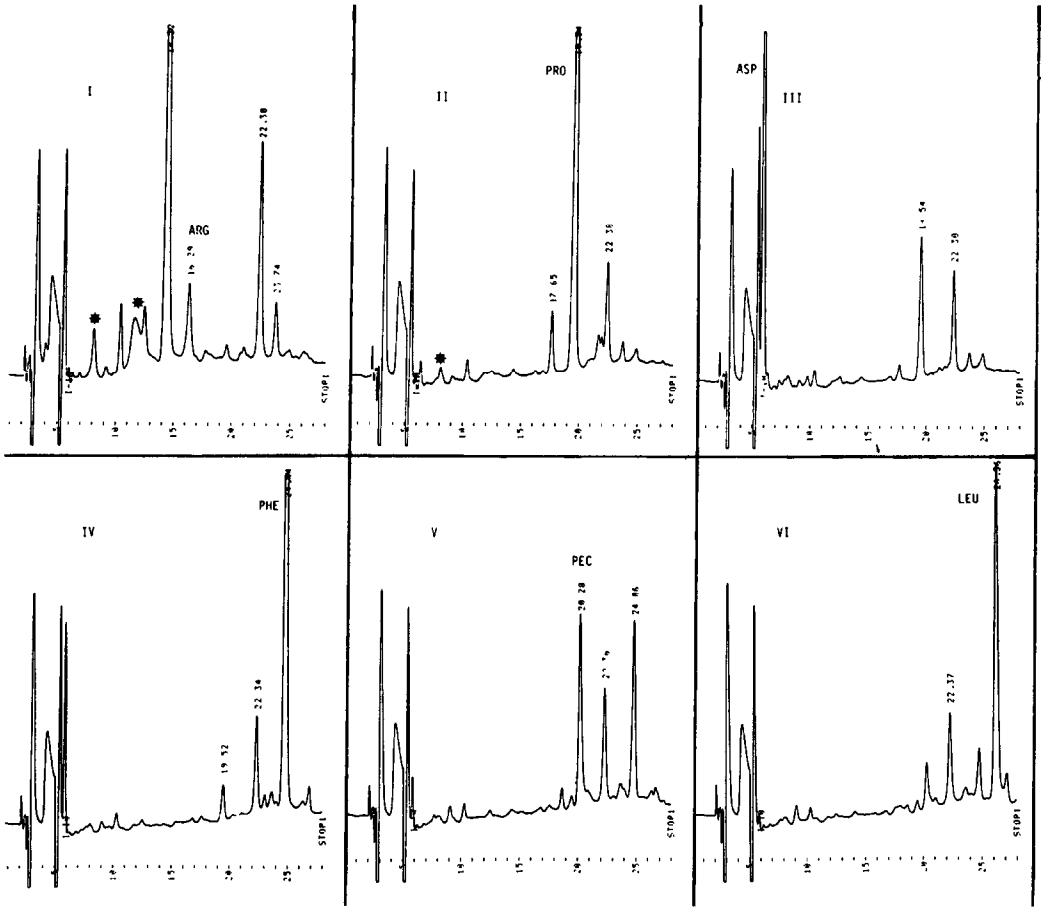

Fig.2. 1 nmol basic protease inhibitor (bovine) was *S*-pyridylethylated as described. The first six steps obtained by automated Edman degradation are shown. The peaks eluting at about 22.30 and 23.74 min are diphenylthiourea and diphenylurea, respectively. The peak appearing at 14.32 min in the first panel is not due to the modification reaction, but arises from the preparation of the inhibitor. (*) Impurities due to modification reaction. PEC, *S*-pyridylethylcysteine.

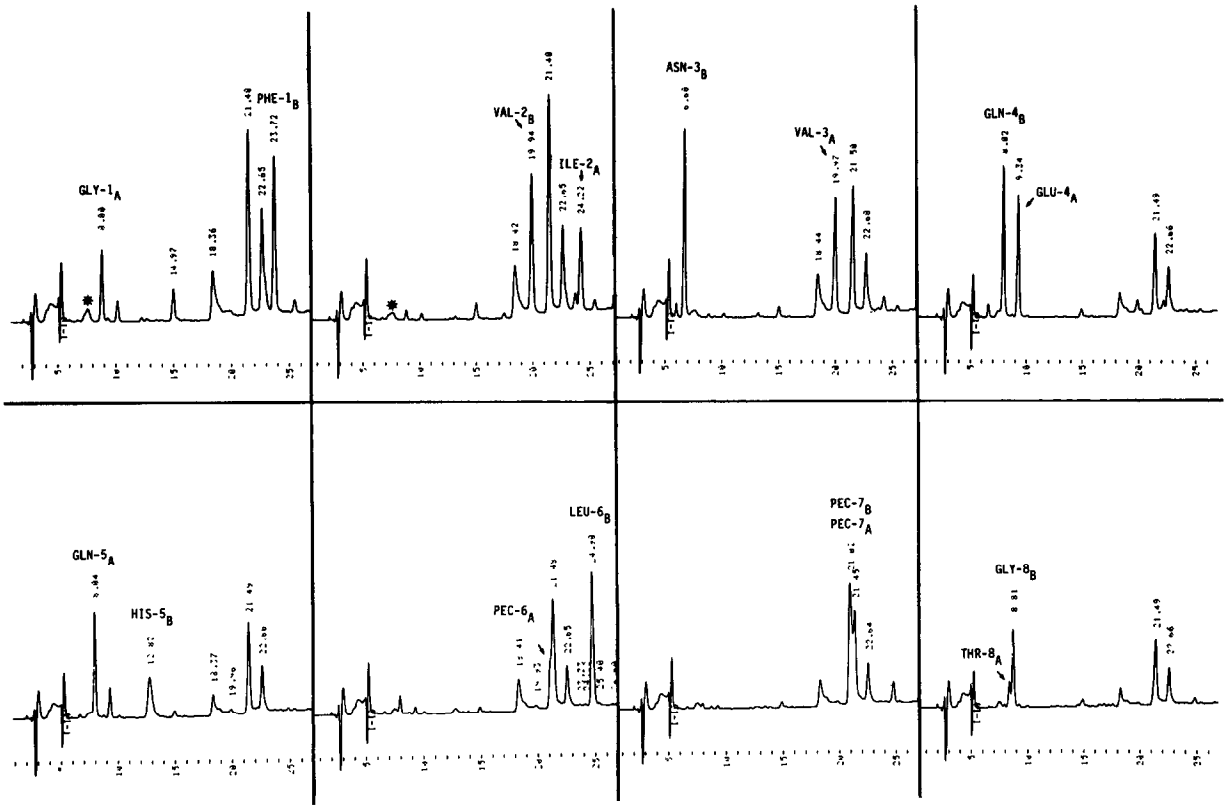

Fig.3. 1 nmol human insulin was *S*-pyridylethylated as described. The first eight sequencing steps are shown. The peak eluting at about 18.36 min originates from the presence of dithioerythritol in the sequencing solvents S_1, S_2 and S_3; the peaks originating at about 21.48 and 22.65 min are diphenylthiourea and diphenylurea, respectively. (*) Impurities due to modification reaction. PEC, *S*-pyridylethylcysteine. The subscripts A and B denote to the A- and B-chains of insulin, respectively.

decrease of ionic strength shifts the peak away from injection, an increase of ionic strength has the opposite effect. The position at which the PTH of *S*-pyridylethylcysteine is eluted can be easily determined before by sequencing an amount of 1–2 nmol *S*-β-(4-pyridylethyl)cysteine, absorbed on a polybrene-coated glass-filter disk to reduce extraction losses.

With respect to the efficiency of the modification reaction, it was found in initial experiments that *S*-pyridylethylation was already virtually complete after 30 min incubation time; no increase in peak height was detectable with reaction times longer than 2 h. Until now, we have no indications that certain disulfide bridges are refractory to vapor-phase modification.

Even peptides with an extremely high cysteine (or cystine) content pose no difficulties: all six cysteine residues (at positions 2, 6, 12, 16, 19 and 26) of a 27-residue peptide, isolated from a plant-fungus interaction, could be clearly identified (Toma, I. and De Wit, P., to be published).

Considering the specificity of the modification reaction, we have no indications that amino acids other than cysteine react under the conditions described.

The peak height of the PTH of *S*-pyridylethylcysteine is always lower than that expected from the amount of material under consideration (cf. figs 2 and 3). This is probably due to the diminished extraction efficiency of PTH-derivatives of amino acids bearing a positive charge, like Arg and His. In addition, the quality of polybrene seems to be of utmost importance in

this respect. We generally obtained best results with polybrene from Serva (unpublished). The method has now been routinely in use for several months and a large number of proteins and peptides have been modified and sequenced successfully.

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