USE OF PARVALBUMIN AS A PROTECTING PROTEIN IN THE SEQUENATOR: AN EASY AND EFFICIENT WAY FOR SEQUENCING SMALL AMOUNTS OF PEPTIDES

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1. Introduction

Since the description of the first automated apparatus for degradation of proteins by the phenylisothiocyanate method [1] it became obvious that the procedures worked out by Edman and Begg and later by Hermodson et al. [2] were not directly applicable to small and hydrophobic peptides. It has been shown that this was due to losses of the residual peptide during the extraction steps with organic solvents [1,3–6].

Several alternative approaches have been developed to overcome the problem. One of these is to render the peptide suitable for the usual procedures by coupling polar groups either to lysine [4] and *S*-(β-aminoethyl) cysteine residues [7] or to the C-terminal amino acid [5,8]. A second approach is to adapt the procedure to the peptide either by decreasing the concentration of quadrol [6,8,9] or by using a volatile buffer [8] which, in both cases, limits the number and the volumes of organic solvents used for extraction. A third possibility is to attach the peptide to an insoluble resin and perform the degradation in an heterogeneous phase [10] with the help of a specially designed apparatus [11].

We wish to report the results we obtained in sequencing unmodified peptides in very small amounts (down to 20 nmol) in an Edman and Begg type sequenator using dimethylbenzylamine as coupling buffer but in the presence of a naturally αNH_2-blocked protein (hake major parvalbumin) that acts as a protecting film. The technique proved also to be very efficient for the degradation of peptides attached to polystyrene resins: it prevents the loss of the insoluble matrix during extraction steps by organic solvents even in the usual sequenator cup.

2. Materials and methods

2.1. *Materials*

2.1.1. Parvalbumin

Parvalbumin from the white muscles of the hake *(Merluccius merluccius)* has been prepared according to a method previously published [12,13] with slight modifications (J. F. Pechère, personal communication) as under-mentioned. The extraction of the muscle was made with ice-cold water (2.5 litre/kg). The resulting suspension was centrifuged and the supernatant lyophilized. The lyophilizate was taken up in cold water (1/20 the volume of the first supernatant) and extensively dialyzed against cold water. The resulting precipitate was centrifuged off and the supernatant lyophilized. The lyophilizate, dissolved in a 0.02 M Tris-HCl buffer, pH 7 to 8, was then gel filtered on a Sephadex G-75 column (5 × 100 cm). The fraction containing the low molecular weight proteins was submitted to DEAE-cellulose chromatography following closely the prescriptions of Pechère et al. [13]. From 3.3 kg of muscle, 3.4 g of the major parvalbumin were obtained.

2.1.2. Peptides

The peptides were purified from enzymatic hydrolysates of scorpion and snake toxins whose primary sequences are under investigation in our laboratory. The peptides attached to the resin are those obtained after solid phase synthesis [14,15]

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and will be used for other projects. The resin employed is polystyrene cross-linked by 1% divinylbenzene and made reactive by a benzhydrylamine group.

2.1.3. Reagents and solvents

Benzene (solvent 1), 1-chlorobutane (solvent 3), *n*-propanol, phenylisothiocyanate, heptafluorobutyric acid (reagent 3) were purified according to Edman and Begg [1]. Heptane for u.v. spectroscopy (Fluka) was used without further purification for preparing 5% phenylisothiocyanate solutions (reagent 1). Dimethylbenzylamine (DMBA) (Merck) was purified according to Hermodson et al. [2] and the coupling buffer (reagent 2) prepared by mixing DMBA (6 ml), *n*-propanol (25 ml), water (27 ml) and acetic acid dropwise to pH 9.5 [16].

2.2. *Methods*

The sequenator used in these experiments was a Socosi PS-100 (94100 - St Maur, France) which is very similar in principle to the Edman and Begg apparatus [1]. This particular instrument was modified in order to obtain, when necessary during the vacuum steps, a regular flow of nitrogen, delivered through the single effluent line, into the spinning cup. The program of operation, which was derived from the program of Hermodson et al. [2], is presented in table 1. We also omitted the second cleavage step and the 1-chlorobutane extraction which follows it.

The operation was started by introducing in the spinning cup 0.4 ml of a 5 mg/ml water solution of parvalbumin*. The protein was dried. The peptide (20 to 400 nmol) was then deposited in solution in water** (0.2 to 0.4 ml) and dried. In the case of peptides attached to resin, they were introduced as a suspension in methanol and, after drying the resin, a second addition (0.4 ml) of the solution of parvalbumin was made. Then we started the program at step 22 (table 1) in order to first dry the protein and purge the reaction cell to eliminate any oxygen;

*Parvalbumin may not dissolve in water. The addition of dimethylallylamine (1 μl to 5 ml of solution) enables the solubilization of the protein.

**The peptide can be introduced in basic or acid solutions provided that they are volatile.

Table 1
Peptide program[a]

Step	Duration (sec)
1 Reagent 1 (0.4 ml)	2
2 Restricted vacuum	30
3 Reagent 2 (0.4 ml)	14
4 Reaction	600
5 Reagent 2 (0.2 ml)	4
6 Reaction	600
7 Restricted vacuum	200
8 Vacuum	60
9 Vacuum + N_2	300
10 Solvent 1 (20 ml)	280
11 Delay	60
12 Restricted vacuum	300
13 Vacuum	200
14 Vacuum + N_2	600
15 Reagent 3 (0.12 ml)	11
16 Reaction	180
17 Restricted vacuum	50
18 Vacuum	10
19 Vacuum + N_2	50
20 Solvent 3 (4 ml)	50
21 Delay	60
22 Restricted vacuum	300
23 Vacuum	20
24 Vacuum + N_2	800
25 Advance collector tube	4

[a]Pressuring steps are not shown. The total cycle time is 81 min. The operating temperature was kept at 50°C. The differential pressure between the reagent and solvent bottles and the reaction cell was 160 mbar.

step 24 could be shortened by up to 200 sec for this preliminary cycle.

Characterization of the phenylthiohydantoin (PTH) derivatives of amino acids was generaly obtained by gas chromatography [17] using a Beckman CG-65 apparatus and thin-layer chromatography except in the case of PTH-His and PTH-Arg which were detected on paper strips by Pauly's and Sakaguchi's reactions [18]

4. Results and discussion

Table 2 shows the results obtained when 14 peptides different in length (4 to 28 residues) were submitted to this technique. With the exceptions of Thr-15 (peptide 5) and of the three last residues of the longest peptide that was sequenced (peptide 6),

Table 2
Sequences of peptides determined using parvalbumin as protecting protein

Amount (nmoles)	Nb	Peptide[a]
400	1	H-MeC[b]–MeC–Ser–Thr–Asp–Asn–MeC–Asn–Pro–Phe–Pro–Val–Trp–Asn–Pro–Arg-OH
400	2	H-Leu–Glu–MeC–His–Asn–Gln–Gln–Ser–Ser–Glu–Pro–Pro–Thr–Thr–Thr–Arg-OH
400	3	H-Val–Glu–Leu–Gly–MeC–Ala–Ala–Thr–MeC–Pro–Lys-OH
300	4	H-Val–His–Tyr–Ala–Asp–Lys-OH
250	5	H-Ala–Pro–Glu–Cys(CM)[c]–Leu–Leu–Ser–Asn–Tyr–Cys(CM)–Asn–Asn–Gln–Cys(CM)–[Thr]–Lys-OH
230	6	H-Ser–Ser–Gly–Gly–Tyr–Cys(CM)–Tyr–Ala–Phe–Ala–Cys(CM)–Trp–Cys(CM)–Thr–His–Leu–Tyr–Glu–Gln–Ala–Val–Val–Trp–Pro–Leu–[Pro,Asx,Lys]-OH
200	7	H-Gly–Ile–Gln–Leu–His-OH
150	8	H-Lys–Thr–Cys(CM)–Asn-OH
100	9	H-Ala–Gly–Val–Gly–Ile–Lys-OH
100	10	H-Trp–Asn–Pro–Arg–Gly-OH
100	11	H-Asn–Pro–Arg–Gly-OH
70	12	H-Gly–Leu–Asn–Asp–Asp–Lys–Lys–Val–Leu-OH
60	13	H-Val–Leu–Glu–Ile–Ser–Asp–Thr–Arg-OH
40	14	H-Cys(CM)–Asp–Thr–Thr–Ile–Ile–Asn-OH
20	9	H-Ala–Gly–Val–Gly–Ile–Lys-OH

[a] The residues into square brackets were not identified.
[b] MeC: S-methylcysteine.
[c] Cys(CM): S-carboxymethylcysteine.

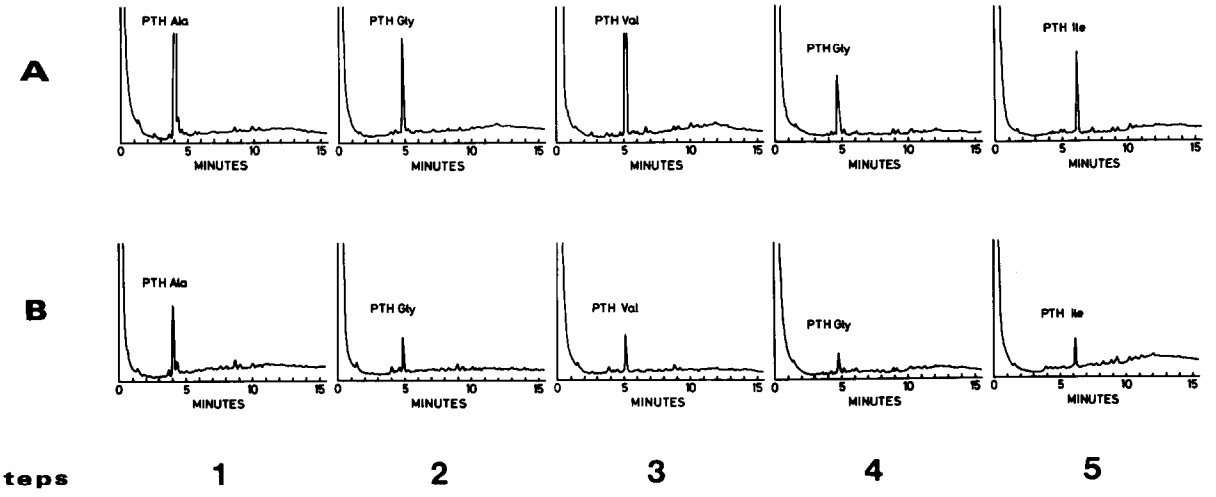

Fig.1. Gas chromatography analyses of PTH-amino acid derivatives obtained after degradation of peptide H-Ala–Gly–Val–Gly–Ile–Lys-OH. (A) 100 nmol of peptides; (B) 20 nmol of peptides. 15% of the sequenator products were injected in the column without silylation (PTH-Ile was confirmed with silylation). Ordinate is in arbitrary units; attenuation of the gas chromatograph was set at 1600.

unambiguous identifications were made. As an example fig.1 gives gas liquid chromatographic analyses of the first 5 steps concerning the degradation of peptide 9 in two different amounts (100 and 20 nmol). This peptide can be considered, after the first coupling step, as quite a hydrophobic one. Nevertheless, in both cases, 15% of the sequenator product was enough to identify all PTH derivatives. These results were confirmed by thin layer chromatography which gave also PTH-Lys at step number 6 even when only 20 nmol of peptide were used.

From these results one can expect every peptide, however low its polarity may be, to be successfully sequenced to the last amino acid residue even with a very small amount of starting material. This is certainly related to the property of parvalbumin, a very hydrophilic protein, to precipitate in the form of a gel in the DMBA buffer and thus protect the peptide from extraction by organic solvents. Crewther and Inglis were probably looking for this effect when working out what they called a 'hydrated phase in the sequenator cup' [6]: they adjusted the conditions of extraction so that one leaves enough quadrol in the cup after coupling reaction to obtain this hydrophilic phase but there should remain no excess as it could interfere with the following steps, particularly during the acid cleavage. We feel that these adjustments may not prove to be easily reproducible. Due to its insolubility in the DMBA buffer parvalbumin was chosen rather than succinylated sheep apomyoglobin which was first used in our laboratory giving also good results with greater amounts of peptides [19] but the film of protein had a tendency to come unstuck from the wall of the cup. Parvalbumin was also found to be very resistant to acid treatment: when 30 cycles were performed using the quadrol* program [1], which means 60 heptafluorobutyric acid treatments, no significant rise of background was noticed. This is of interest for it is possible to sequence several peptides using the same aliquot of parvalbumin. To avoid the possibility of overlapping contamination, the apparatus is programmed to perform one or two steps more than the number of residues contained in the first peptide (as determined by amino acid analysis) before the second peptide is introduced into the cup. Niall et al. [20] also showed the protecting effect of a polypeptide in a sequenator but they used it for the degradation of very small amounts of protein.

The technique that we recommend is different

*Hake parvalbumin is perfectly soluble in the quadrol buffer.

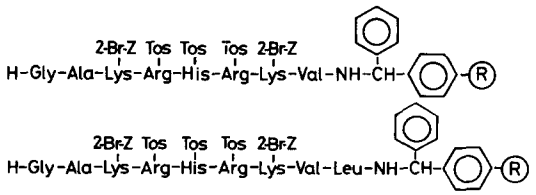

Fig.2. Structure of the two protected peptides obtained after solid phase synthesis that were submitted to Edman degradation. 2-Br-Z = 2-bromobenzyloxycarbonyl; Tos = tosyl; ® = polystyrene 1% divinylbenzene.

from the first two approaches quoted in the introduction. The presence of parvalbumin, as a protecting protein, allows the use of the DMBA procedure for analysis by Edman degradation of free peptides without any special chemical preparation. Moreover, due to its insolubility in the DMBA buffer, parvalbumin acts as a trap for resins. The degradation of peptides attached to resin is then possible (the cup need not be of a special design) which is very useful for detection deletion peptides during solid phase peptides synthesis [21]. Two syntheses have been controlled with the peptides described in fig.2. In both cases even the last amino acid (attached to the benzhydrylamine resin) was recovered with the expected yield.

In conclusion, we can now say that, using a single apparatus, it has become possible to sequence automatically as well proteins as peptides (free or attached to a solid phase).

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