A simple modification converts the spinning cup protein sequencer into a vapour-phase sequencer

Wolf F. Brandt, Herman Alk, Madhu Chauhan and Claus von Holt*

CSIR Chromatin Research Unit, Department of Biochemistry, University of Cape Town, Private Bag Rondebosch 7700, Republic of South Africa

Received 8 May 1984; revised version received 19 June 1984

A spinning cup sequencer was converted into a vapour–liquid–solid phase instrument by replacing the chemical delivery valves with a simplified version of the Wittmann valve. The cup assembly was replaced by a Kel-F cartridge and the vacuum system slightly modified and fitted with a cold trap. The modification is inexpensive, easily performed and does not lead to the loss of the spinning cup capability of the instrument. The modified sequencer is easy to operate, reduces consumption of chemicals and exhibits a cycle time of 1 h. Small and larger peptides in amounts from 0.2–30 nmol were successfully degraded.

Spinning cup protein sequencer *Vapour-phase sequencer* *Protein structure*

1. INTRODUCTION

Micromethods for the elucidation of the primary structure of proteins have become increasingly important because the attention of investigators focusses more and more on peptides and proteins occurring in minute amounts in cells. Micro-sequencing is considerably more economic with respect to the consumption of chemicals. The coupling of the fast and highly sensitive analytical HPLC- and electrophoretic separation methods to micro-sequencing bypasses laborious and time-consuming macro-isolation procedures for the proteins and peptides.

In the most widely used macro method for the sequence determination of proteins [1], the spinning cup method, careful maintenance of the vacuum system, bearings, vacuum seals and scoop position are necessary to achieve constantly correct reagent levels in the cup as one of the prerequisites for high repetitive yields. The effects of sample loss during the extraction steps, contamination with aldehydes, problems to achieve an even protein film in the cup and to remove excess reagents and non-volatile reaction by-products were discussed previously proposing improvements in instrument design [2], buffers [3], film formation [4] and chemical modification of peptides to make the latter more suitable for sequential degradation [5].

A large number of these problems have been eliminated [6] by returning to the more simple principles of the sequential Edman degradation as in [7] and [8]. In the Hunkapiller automated methodology the polypeptide adsorbed to a fiber disk is sequentially degraded by alternating exposure to the reagents in the vapour and liquid phase as in the original manual micromethods previously cited. With the automated micromethodology as described by Hunkapiller 5 pmol of protein were successfully analysed [6].

A modification of the Beckman sequenator is reported here consisting of a cartridge similar to that by Hunkapiller but from Kel-F replacing the spinning cup and a modified valve similar to that in [2] to handle the stream of chemicals and products. These modifications allow the Beckman Sequenator to be used either in the 'vapour-phase' mode or the 'spinning cup' mode.

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

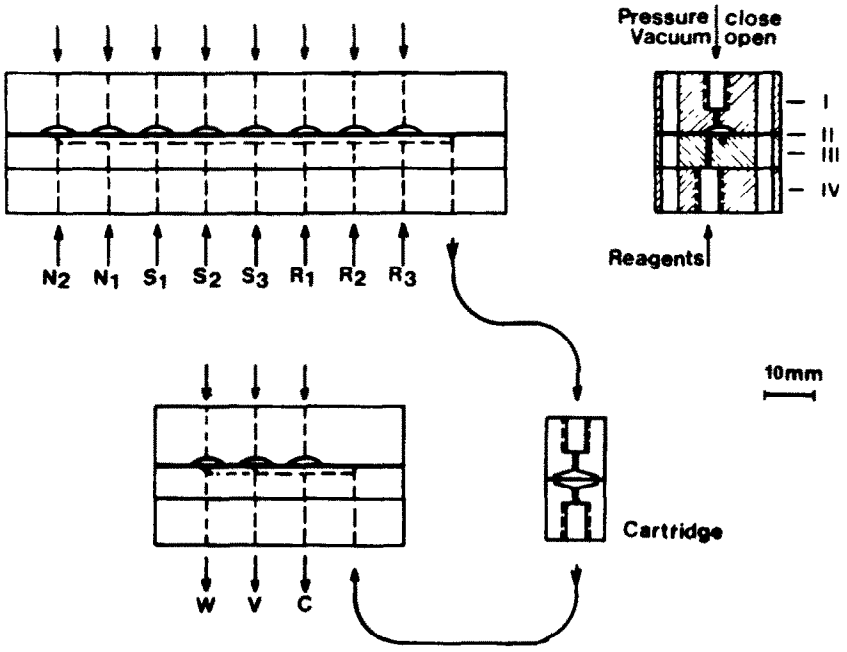


Fig.1. Schematic diagram of the delivery system and reaction cartridge of the modified vapour–liquid–solid phase sequencer. The delivery valves consist of a pneumatic connection block (I), a 0.3 mm thick PTFE membrane (II), a Kel-F valve block (III), and a reagent/solvent connection block (IV). The compression of the PTFE membrane (II) between the aluminium block (I) and the Kel-F valve block (III) effectively forms the seal between individual valve units as well as towards the outside. It is essential that the Kel-F block (III) is highly polished. The valves that control vacuum (V), waste (W) and collect (C) modes were designed in a similar fashion. The consumption of reagents and solvents per cycle are as follows: 30 μl 15% PITC in heptane (R_1), 8 ml vapour/min of 25% trimethylamine (R_2), 8 ml vapour/min of TFA (R_3), 2 ml benzene–10 ppm DTE (S_1), 3 ml ethyl acetate–10 ppm DTE (S_2), 2 ml *n*-chlorobutane (S_3) and N_1 and N_2 8 ml/min and 1 ml/min of nitrogen, respectively. In the case of R_2 and R_3 the vent valves were closed, the delivery lines raised above the liquid levels and the bottle pressure reduced to 60 mm Hg.

2. MATERIALS AND METHODS

2.1. *Sequenator*

A model 890 Beckman sequenator was modified as follows: The rough pump was solely used to control the pneumatic delivery–collect waste valves and the fraction collector. The fine pump was equipped with a liquid N_2 trap as in [2]. The pneumatic delivery valve was redesigned as in [2] (fig.1). The zig-zag delivery line to the sequenator cartridge was replaced by a single groove 0.2 × 0.15 mm on the surface of the Teflon or Kel-F block. The main advantages are the ease of manufacture and the absence of dead pockets and small dead volume. The sequencing programme is closely related to that in [6].

2.2. *Reaction cartridge*

The reaction chamber was made from Kel-F or Teflon (fig.1) and housed in a stainless steel cartridge fitted with a threaded lid accepting the cover to compress the two halves of the cartridge. Several reaction cartridges varying in diameter from 9–30 mm were made to accommodate various fiber disk sizes depending on the amount of protein or peptide to be sequenced. All experiments in this paper were performed on a 9 mm Kel-F cartridge.

Peptides or proteins were applied to Whatman GF/C glass filters which were punched out by a tool matching the diameter of a particular reaction chamber. A porous Teflon membrane was used to support the glass filters and simultaneously act as a seal. The glass filter discs were pretreated with a

solution of polybrene (6 mg/ml) at a coverage of about 1 mg/cm.

2.3. *Chemicals*

All the chemicals were purified as in [1]. Polybrene was purified on Dowex 1 and Al_2O_3 as in [8].

Trimethylamine was prepared in an all glass distillation apparatus from the hydrochloride by the slow addition of a concentrated NaOH solution. The condenser and the receiving bottle were cooled to −25°C. The collected trimethylamine was refluxed over phthalic anhydride at room temperature and then redistilled and diluted to 25% (v/v) with H_2O. This product is ninhydrin- and Tollens-test negative.

Melittin was purchased from Sigma. The pentapeptide Phe-Asp-Ala-Ser-Val was a kind gift from Professor F. Wunsch. The tryptic core of wheat H2A was donated by J. Rodrigues (unpublished results).

2.4. *PTH amino acid identification*

The amino acid anilinothiozolinones were collected in tubes containing between 1–10 nmol PTH nor-leucine and 0.1 mg DTE.

The anilinothiozolinones were converted to the PTH amino acids in 100 μl 20% TFA–0.1 mg DTE for 10 min in a heating block at 80°C.

The TFA was removed under a stream of N_2, the sample was then redissolved in 150–500 μl of methanol of which 70 μl were subjected to identification. PTH amino acids were identified in a Hewlett Packard HPLC instrument on a LICHROSORB RP-8 column 4 × 250 mm (Merck) using a 0.05 M sodium acetate buffer, pH 3.85, and a methanol gradient from 8–50% at a flow rate of 1.3 ml/min. Small adjustments of the pH and the molarity of the buffer had to be made for each column and as a given column aged to optimize the separation of the pairs Glu/Asp and His/Arg (fig.2). The PTH derivatives of Lys, Ile and Phe were resolved in a subsequent isocratic run (25% of methanol in the same buffer on the same column) [9].

3. RESULTS AND DISCUSSION

In order to test the performance of the modified sequencer, various peptides and proteins were subjected to 'vapour-phase' sequence analysis. Fig.2 and table 1 shows that pentapeptide, Phe-Asp-Ala-Ser-Val can be sequenced successfully in the 5000 to 200 pmol range.

All of the 26 amino acids of melittin could be identified using either 20 or 2 nmol of the peptide (table 2). The repetitive yield based on PTH–Leu was 94% in both cases. An example of a large number of different tryptic peptides isolated from histone digests have been subjected to gas-phase sequence analysis in amounts varying between 30–3 nmol is given in table 3. In every instance,

Table 1

Degradation of the pentapeptide Phe-Asp-Ala-Ser-Val

Step no.	PTH amino acid	Yield in nanomoles		
		5 nmol (2.5 μg) peptide	1 nmol (0.5 μg) peptide	0.2 nmol (0.1 μg) peptide
1	Phe	5.1	0.99	0.21
2	Asp	4.8	0.77	0.23
3	Ala	4.3	1.10	0.24
4	Ser[a]	2.4	0.50	0.15
5	Val[b]	1.9	0.02	0.01

[a] Quantitated as dehydroalanine
[b] Carboxyl terminus (yields are variable)

Table 2

Yields of some PTH amino acids obtained after vapour-phase isothiocyanate degradation of melittin

PTH amino acid	Position	Yield in nanomoles (melittin applied)	
		100 μg (20 nmol)[a]	20 μg (4 nmol)[a]
Gly	1	15	2.4
Leu	6	17	1.6
Leu	9	16	1.8
Leu	13	13	1.1
Leu	16	9	0.9
Gln	25	1	0.1
Gln	26[b]	1	0.2

[a] Corrected for moisture and salt content based on amino acid composition (57% melittin of the dry weight)
[b] Carboxyl terminus

Table 3

Yields of some PTH amino acids obtained by vapour and spinning cup sequencing of the tryptic core of wheat histone H2A

PTH amino acid	Position	Yield in nanomoles	
		Vapour phase 0.1 mg (27 nmol)[a]	Spinning cup 1.0 mg (270 nmol)[a]
Val	1	20	ND[b]
Gly	2	20	94
Leu	9	10	59
Leu	13	19	32
Leu	16	8	19
Leu	21	5	8
Lys	33	1.5	ND[b]
Repetitive yield (Leu)		94%	89%

[a] Uncorrected for moisture and salt content

[b] This hydrophobic peptide was treated with sulphonated PITC [5] in order to retain it in the cup. The core was obtained by digesting maleylated H2A with trypsin yielding a core 35 amino acids long.

results obtained from 'vapour-phase' mode were better on less material when compared to results obtained in the spinning cup mode (table 3).

Proteins have been studied to a limited extent only but with similar repetitive yields.

These results show that a spinning cup sequencer can easily be converted into a vapour–liquid solid phase instrument by a few relatively minor modifications. This allows the sequence of proteins and peptides on a Beckman sequenator in the vapour–liquid solid phase sequencing mode as described by Hunkapiller with most of the advantages inherent in that method. Simple reorientation of the exit line from the new valve to the cup

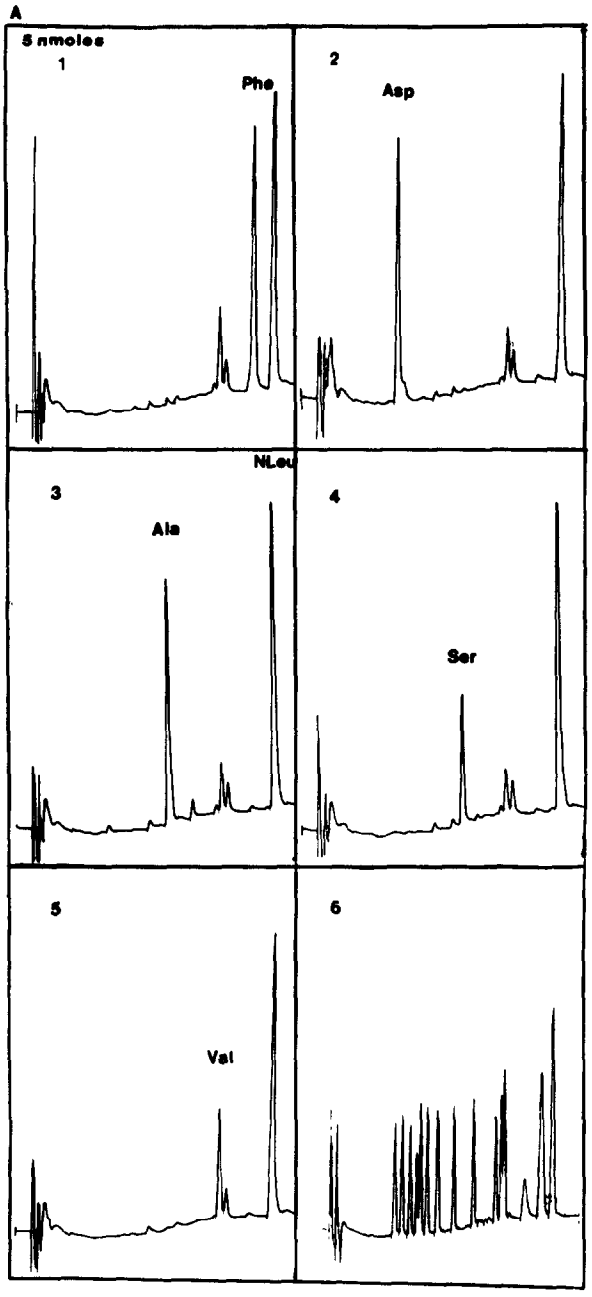
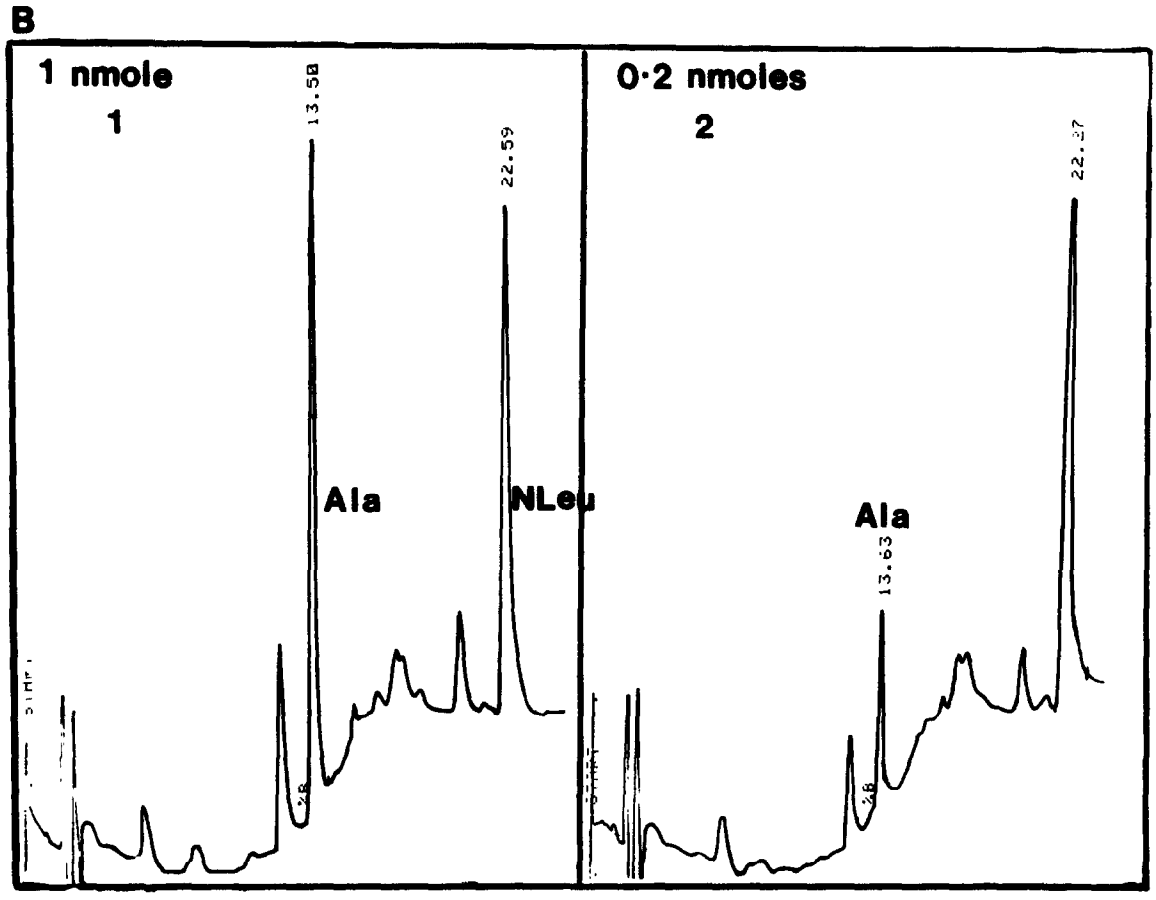


Fig.2. HPLC tracings from the amino acid sequence analysis of the pentapeptide Phe-Asp-Ala-Ser-Val. (A) Tracings 1–5 correspond to vapour-phase degradation cycle 1–5 of 2.5 μg (5 nmol) pentapeptide. Each sample represents 28% of the total and contained 2.8 nmol PTH–nLeu. Analysis performed on a Hewlett Packard HPLC (1084 A) at an attenuation of 2^5 (0.054 AU/FS). Elution of a standard mixture of PTH amino acid (0.4 nmol each) is given in trace 6 [9]. (B) Tracings 1 and 2 represent Edman degradation cycle 3 (Ala) of 1 and 0.2 nmol of the pentapeptide by vapour-phase degradation. Yields of all the cycles have been recorded in Table 1. 60% of the samples were injected containing 0.6 nmol nLeu at an attenuation of 2^3 (0.014 AU/FS).



assembly allows the instrument to be used in either the vapour phase or spinning cup mode.

With the modified sequencer and the programme described the sensitivity limit is reached between 100–200 pmol level (fig.2). This may well be the result of not using more rigidly purified chemicals and the application of Teflon and Kel-F in the construction of parts. Optimization of the relative positions of valves, cartridge and fraction collector may yield smaller dead volumes, together with an isocratic PTH–amino acid system may extend the sensitivity to well below the 100 pmol level.

REFERENCES

[1] Edman, P. and Henschen, A. (1975) in: Protein Sequence Determination (Needleman, S.B. ed.) 2nd edn, pp.259–262, Springer-Verlag, Berlin.

[2] Wittmann-Liebold, B. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1415.

[3] Niall, H.D., Jacobs, J.W., Van Rietschoten, J. and Tregear, G.W. (1974) FEBS Lett. 41, 62.

[4] Klapper, D.A., Wilde, C.E. and Capra, J.D. (1978) Anal. Biochem. 85, 126.

[5] Braunitzer, C. and Schrank, B. (1979) Hoppe-Seyler's Z. Physiol. Chem. 351, 417.

[6] Hewick, R.M., Hunkapiller, W., Hood, L.E. and Dreyer, W. (1981) J. Biol. Chem. 256, 7990–7997.

[7] Fraenkel-Conrat, H., Harris, S.I. and Levy, A.L. (1955) Methods Biochem. Anal. 2, 393.

[8] Wittmann-Liebold, B. (1981) Chemical Synthesis and Sequencing of Peptides and Proteins (Liu, Schechter, Heinrikson and Condliffe eds) pp.75–110, Elsevier/North-Holland, Amsterdam, New York.

[9] Strickland, M., Strickland, N.W., Brandt, W.F., Von Holt, C., Wittmann-Liebold, B. and Lehmann, A. (1978) Eur. J. Biochem. 89, 443–452.