Journal of Chromatography, 297 (1984) 13–19
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 330

USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR PREPARING SAMPLES FOR MICROSEQUENCING

YU-CHING E. PAN*, JANUSZ WIDEMAN, RUSSELL BLACHER, MAY CHANG and STANLEY STEIN

Department of Molecular Genetics, Roche Research Center, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

SUMMARY

A method has been developed for the microsequencing of protein at subnanomole levels. The protein is carboxymethylated and freed of salts and reagents by reversed-phase chromatography prior to automated Edman degradation on a gas-phase sequencer. The carboxymethylated protein can also be fragmented chemically or enzymatically for further sequence analysis. The analytical techniques used to monitor the progress of the reactions all have picomole level sensitivity.

INTRODUCTION

Many of the biologically active peptides and proteins of current interest can be isolated in only small amounts and, therefore, microsequencing procedures are required for determination of their primary structure. One of the most significant advances in microsequencing has been the development of the gas-phase sequencer[1], which is now commercially available. However, the microsequencing instrument is only a tool, and appropriate methods for prearing protein samples are critical to obtaining meaningful data. Our laboratory has had extensive experience with protein microsequencing, and the following report illustrates our overall approach. An important step in our method is carboxymethylation of proteins followed by removal of salts and reagents on an inexpensive reversed-phase column.

EXPERIMENTAL

A model 470A sequencing instrument was purchased from Applied Biosystems (Foster City, CA, U.S.A.). A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatography (HPLC) system was used for phenylthiohydantoin (PTH)-amino acid analysis, according to the method of Hawke *et al.*[2]. Reagents for the sequencing instrument were obtained from Applied Biosystems, whereas all other solvents and chemicals were of the highest purity available from several sources. Protein solutions were always kept in polypropylene tubes.

For the carboxymethylation reaction, 300 μl of 6 *M* guanidine–HCl, 0.1 *M*

0021-9673/84/$03.00

Tris, pH 8.5 was added to the protein solution. The volume was reduced to 300 μl under a stream of nitrogen. The pH was checked and readjusted to 8.5, if necessary, by the addition of concentrated hydrochloric acid or sodium hydroxide. All reagents for carboxymethylation were dissolved in 6 *M* guanidine–HCl, pH 8.5. A 5-μl aliquot of dithrothreitol (50 mg/ml) was added. The tube was flushed with nitrogen, capped and kept a 37°C for 4 h. Then 200 μg of ^{14}C-labelled iodoacetic acid in 15 μl was added. The tube was again flushed with nitrogen and kept in the dark for 10 min. Two more portions of unlabelled iodoacetic acid (2.5 mg in 5 μl) were successively added as above to ensure completeness of reaction. The reaction could be terminated by the addition of excess dithiothreitol or the sample could be directly desalted on the reversed-phase column.

A 2 × 0.46 cm LC-308 cartridge column (Supelco, Bellefonte, PA, U.S.A.) was used for desalting. The carboxymethylated protein solution was adjusted to pH 2 with dilute trifluoroacetic acid (TFA) and loaded on the column, which had been preequilibrated with 0.1% TFA. The column was washed with 0.1% TFA at 24 ml/h until the baseline, monitored either with fluorescamine[3] or absorbance at 280 nm, returned to the pre-injection level. The eluent was then switched to 70% acetonitrile in 0.1% TFA and the protein was collected in a volume of 1–2 ml.

Prior to tryptic digestion, the TFA and acetonitrile were removed under a

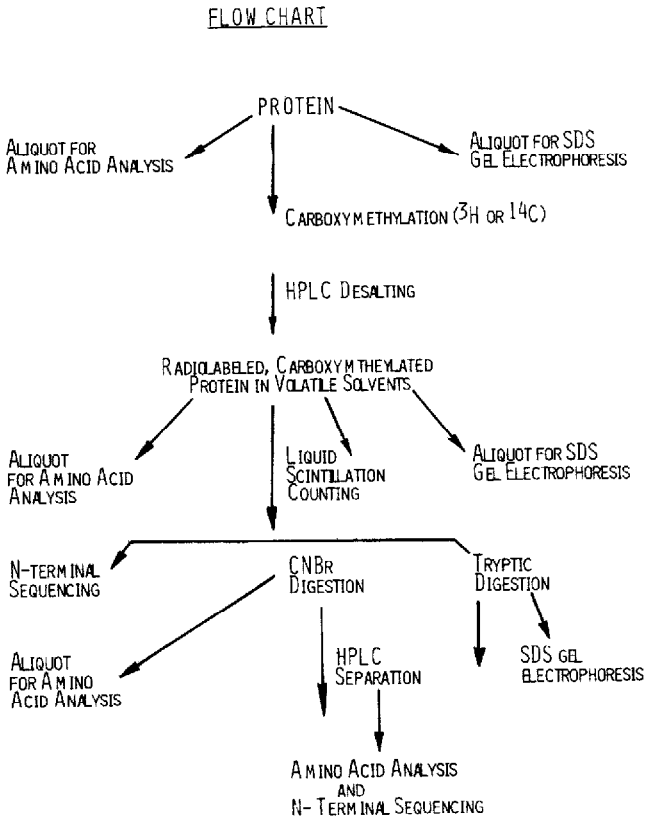

Fig. 1. Flow chart summarizing the procedures used in our sequencing laboratory.

stream of nitrogen with repeated additions of water. The solution was taken to a final volume of 10–20 μl, without ever being allowed to dry completely. A 100-μl aliquot of 0.2 *M* ammonium bicarbonate (pH 8) was added. Then trypsin was added at a trypsin:protein ratio of 1:30. After incubation at 37°C for 10–20 h, the reaction was stopped by adjusting to pH 2 with 0.1% TFA. The peptides were resolved on a 15 × 0.46 cm I.D. LC-318 column using a gradient of increasing acetonitrile in 0.1% TFA.

For cyanogen bromide digestion, the acetonitrile and TFA were removed under nitrogen with repeated additions of water to a final volume of 10–20 μl. Then 100 μl of 88% formic acid and a 100-fold excess (over methionine) of cyanogen bromide were added. The tube was flushed with nitrogen, capped and kept at room temperature for 24 h. An aliquot was taken for amino acid analysis and the reaction was allowed to proceed for another 24 h. After the 48-h digestion, and if amino acid analysis indicated complete destruction of methionine, the reaction was stopped by adding 10 volumes of cold water. Excess reagent was removed under nitrogen with repeated additions of water.

Amino acid analysis was performed on an instrument using post-column reaction with fluorescamine[4]. Proteins were hydrolyzed in 6 *M* hydrochloric acid containing 4% thioglycollic acid at 110°C for 20–24 h *in vacuo*.

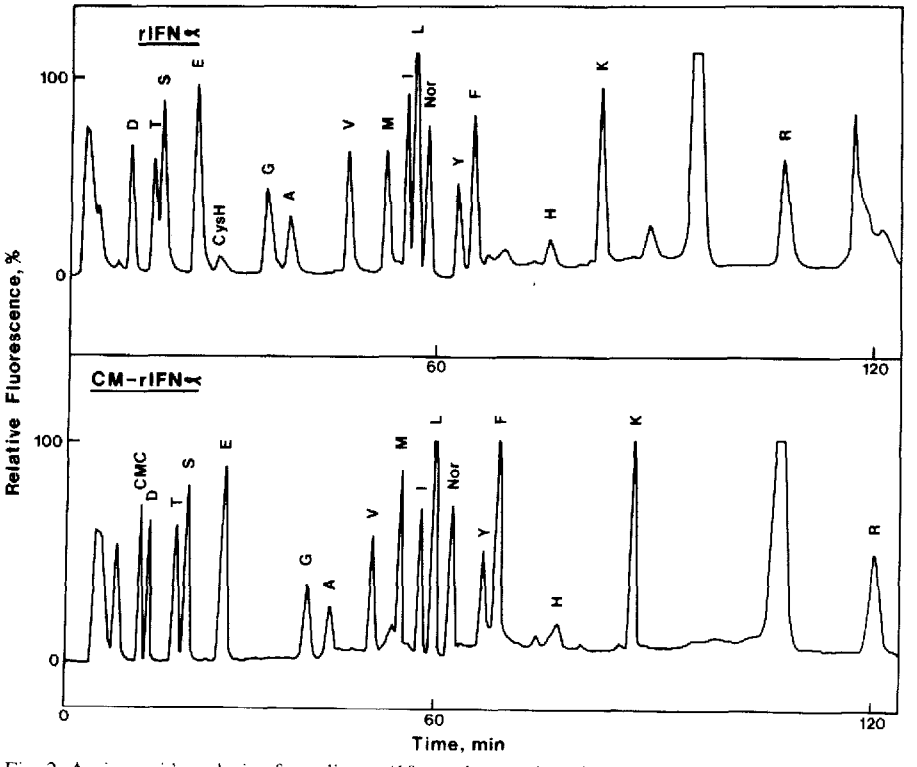

Fig. 2. Amino acid analysis of an aliquot (10 pmole protein) of recombinant, human leukocyte interferon (top) and an equivalent aliquot taken after carboxymethylation and desalting (bottom). Single-letter amino acid codes are used to designate the peaks, except CMC (carboxymethyl cysteine) and Nor (norleucine). Detection is with fluorescamine[4].

RESULTS AND DISCUSSION

A flow chart summarizing the general approach is given in Fig. 1. All proteins, except those known not to contain cysteine, are carboxymethylated. After this treatment, the protein is fully denatured and, as our experience indicates, more amenable to reversed-phase chromatography and sequencing. The use of a radiolabel is convenient for determining recovery of protein at various steps. Carboxymethyl cysteine is easier to quantitate as either the free or PTH-amino acid than is cysteine itself.

It should be noted from Fig. 1 that amino acid analysis and gel electrophoresis are used to monitor the progress of the reactions. Fig. 2 verifies the conversion of cysteine to carboxymethyl cysteine. Likewise, the disappearance of methionine (not shown) is used to monitor cyanogen bromide cleavage. Gel electrophoresis is a good indicator of sample purity, as well as a monitor for successful tryptic digestion. Recovery of protein is also determined by amino acid analysis. Fluorometric amino acid analysis and silver-staining of gels both have picomole level sensitivity.

The desalting procedure (Fig. 3) has been used successfully on a variety of proteins. Recovery is generally over 90%, even with only a fraction of a nanomole of protein (Fig. 4). The protein is recovered in volatile solvents and can be readily dried on the filter of the sequence. Alternatively, the sample may be concentrated *in vacuo* before drying on the filter. Proteins processed in this manner usually give an initial yield of 30–40% and a repetetive yield of about 90%, as exemplified in Table I.

The proteins prepared in the above manner can also be successfully fragmented with trypsin, cyanogen bromide or any other known method. Reversed-phase chromatography using a gradient of acetonitrile in 0.1% TFA for separating the peptide

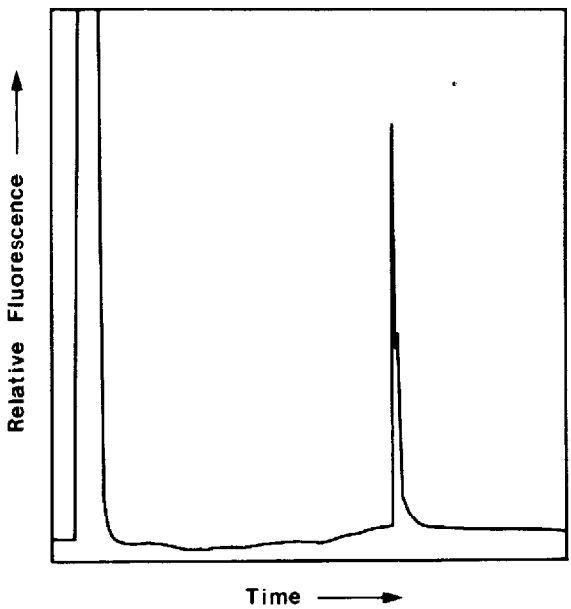

Fig. 3. Desalting of carboxymethylated, recombinant, human leukocyte interferon. See Experimental section for details.

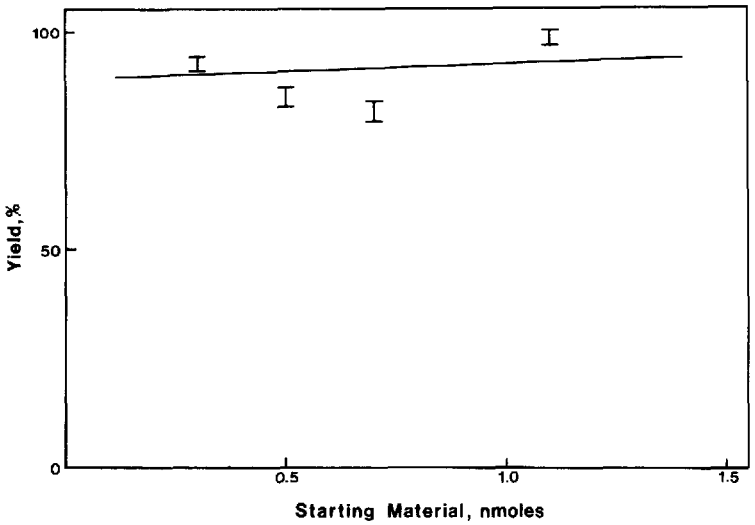

Fig. 4. Recovery of protein through the carboxymethylation and desalting steps. Varying amounts of recombinant, human leukocyte interferon were separately carboxymethylated and desalted. Quantitation was by amino acid analysis. The 300-pmole sample was successfully sequenced.

fragments is shown in Fig. 5 for cyanogen bromide cleavage and in Fig. 6 for tryptic cleavage. The cyanogen bromide derived fragments of human interleukin-2 (Il-2) (Fig. 5), were determined to correspond to the structure predicted from the cDNA sequence by amino acid analysis and peptide sequencing[5]. In a similar fashion, sequence analysis of the tryptic peptides of L-factor[6] (Fig. 6) corresponded to the structure predicted from the cDNA (unpublished results).

In conclusion, carboxymethylation of a protein, followed by desalting on a reversed-phase column produces material suitable for all aspects of microsequencing. The desalting procedure has also been found to be useful in other situations. It has

TABLE I

SEQUENCE ANALYSIS OF 0.5 nmole OF RECOMBINANT PROTEIN

Cycle	*PTH-amino acid*	*Yield (pmole)*
1	Met	216
2	Phe	198
3	Thr	–
4	Ile	184
5	Pro	76
6	Leu	161
7	Gly	94
8	Lys	127
9	Phe	127
10	Met	125

Initial yield = 43%.
Repetitive yield = 94%.

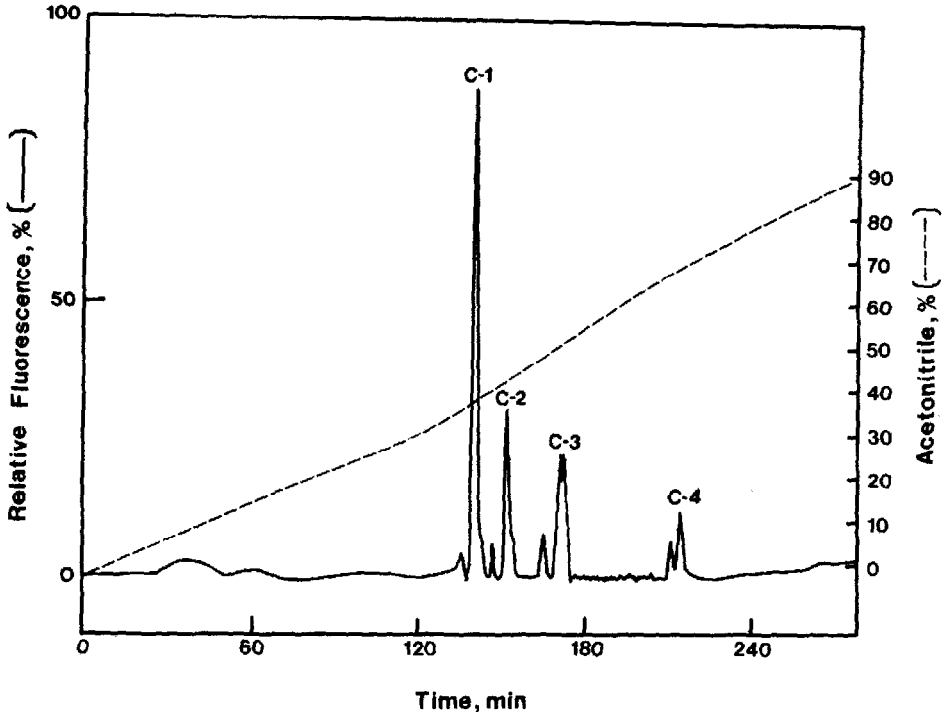

Fig. 5. Separation of the cyanogen bromide cleavage peptides of human IL-2 by reversed-phase chromatography. A 15 × 0.46 cm I.D. LC-18-DB column (Supelco) was eluted at 20 ml/h using a linear gradient of acetonitrile in 0.1% TFA[5].

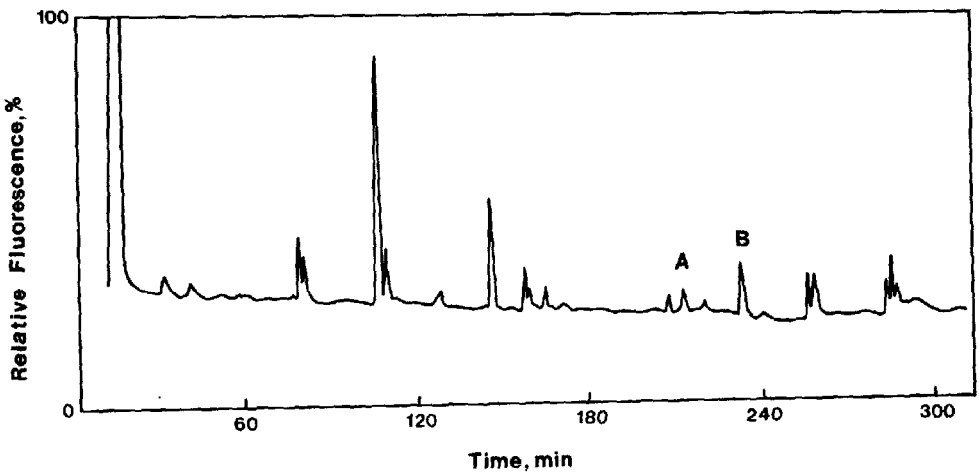

Fig. 6. Separation of the tryptic peptides of L-Factor by reversed-phase chromatography. A 15 × 0.46 cm I.D. LC-318 column (Supelco) was eluted at 24 ml/h using a linear gradient of acetonitrile in 0.1% TFA[6].

been possible to extract proteins from polyacrylamide gels with 6 *M* guanidine–HCl and then desalt them. Although, in this case, an increased level of contamination was observed in the first cycle, successful sequencing was possible. Other samples, which were found to be heterogenous by gel electrophoresis, could be resolved into their components by using a shallow gradient on the reversed-phase column subsequent to the carboxymethylation step. The care taken in the preparation of protein samples allows the gas-phase sequencer to be used to its fullest potential.

REFERENCES

1 R. M. Hewick, M. W. Hunkapiller, L. E. Hood and W. J. Dryer, *J. Biol. Chem.*, 256 (1981) 7990.
2 D. Hawke, P.-M. Yuan and J. E. Shively, *Anal. Biochem.*, 120 (1982) 302.
3 S. Stein and J. Moschera, in S. Pestka (Editor), *Methods Enzymol.*, 79 (1981) 7.
4 S. Stein and L. Brink, in S. Pestka (Editor), *Methods Enzymol.*, 79 (1981) 20.
5 A. S. Stern, Y.-C. E. Pan, D. L. Urdal, D. Y. Mochizuki, S. DeChiara, R. Blacher, J. Wideman and S. Gillis, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 871.
6 H.-F. Kung, in D. Schlessinger (Editor), *Microbiology*, Amer. Assoc. of Microbiol., NY, 1983, p. 53.