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COMPUTER-ASSISTED HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF RADIO-LABELLED PHENYLTHIOHYDANTOIN AMINO ACIDS

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SUMMARY

A computer-controlled high-pressure liquid chromatographic (HPLC) system is described to identify *in vitro* phenyl [^{35}S]isothiocyanate-labelled phenylthiohydantoin (PTH) amino acids from a solid-phase sequencer. Each radio-labelled amino acid from the sequencer is added to a PTH amino acid standard and the mixture separated by HPLC using a computer, programmed to detect a slope change in the absorbance. Individual fractions corresponding to the PTH amino acids are collected and counted. The sensitivity of the system is demonstrated on 700 pmoles of lysozyme.

INTRODUCTION

Elucidation of the primary structure of cell surface proteins, tumor antigens, histocompatibility alloantigens and immune response gene products is of fundamental importance if we are to appreciate the role of these plasma membrane components in the regulation of the immune response. However, until recently the availability of only minute quantities of these proteins, poor yields obtained in their purification, and the limitations of efficient and sensitive microsequencing technology have seriously constrained the structural analyses of these macromolecules. Various approaches have advanced our microsequencing capabilities. These include the use of synthetic carrier[1], internal labelling of protein with ^{14}C-labelled amino acids[2], *in vitro* labelling with [^{14}C]phenyl isothiocyanate ([^{14}C]PITC)[3] and the use of quaternary ammonium compounds such as polybrene[4]. The involvement of this laboratory in the structural characterization of the mouse and human histocompatibility antigens H-2 and HLA has accelerated our efforts towards the development of microsequencing methods that would be sensitive at the picomole level of detection. A useful approach combines Edman degradation on a solid-phase sequencer with [^{35}S]PITC followed by a computer-assisted high-pressure liquid chromatographic (HPLC) separation and collection of the labelled phenylthiohydantoin (PTH) amino acids, and liquid scintillation counting. The details and design of such instrumentation are reported in this communication.

MATERIALS AND METHODS

Trifluoroacetic acid (TFA) and PITC were obtained from Pierce (Rockford, Ill., U.S.A.) and Quadrol from Beckman Instruments (Palo Alto, Calif.,U.S.A.). [^{35}S]-PITC (290 mCi/mmole) dissolved in acetonitrile was purchased from Amersham Searle (Arlington Heights, Ill., U.S.A.) in sealed vial each containing 2 mCi. Methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Porous glass beads (75 Å, 200–400 mesh) were supplied by Electro-Nucleonics (Fairfield, N.J., U.S.A.) and used without further purification. Non-porous glass beads for column packing were from Pierce and Liquifluor was from New England Nuclear (Boston, Mass., U.S.A.). Lysozyme was the product of Worthington (Freehold, N.J., U.S.A.). The scintillation fluid was prepared by mixing 21 ml of Liquifluor and 479 ml of toluene. An LKB 4020 solid-phase peptide sequencer with microsequencing capabilities was employed. The order of solvents and reagents placed in the sequencer was: methanol in position 1, followed by buffer (0.5 *M* Quadrol, pH 9.0) in position 2; positions 3 and 4 contained 5% PITC in acetonitrile and [^{35}S]PITC (2 mCi/4 ml of acetonitrile), respectively, and TFA was placed in position 5.

Protein attachment

In order to achieve maximum coupling a large excess of glass beads were added to protein solution. 10 nm of lysozyme (hen, egg-white) was dissolved in 250 μl of dimethylallylamine (DMAA; Beckman part No. 334702), containing guanidine hydrochloride to effect solubilization; 100 mg of phenyl diisothiocyanate-activated glass beads were added, a slow vacuum was applied for 1 min, and the mixture was incubated at 45°. After 1 h 1–2 drops of ethanolamine were added and incubation was continued for an additional 30 min. Throughout incubation the mixture was constantly stirred at a slow speed under nitrogen. Beads were then washed exhaustively with methanol and dried under vacuum. The extend of substitution was evaluated by hydrolysis of 25 mg of coupled glass beads with constant boiling hydrochloric acid for 24 h at 110° in a sealed evacuated ampule. The hydrolysate was then analysed on a Durrum 500 amino acid analyzer.

Column packing

Glass beads equivalent to 700 pmoles of lysozyme were weighed and mixed with non-porous glass beads sufficient to pack half of the reaction column. Mixing of coupled and non-coupled beads was done to ensure efficient coupling with [^{35}S]-PITC. The sample mixture was then stacked in a 15 cm × 0.1 cm I.D. column in between two layers of non-porous glass beads.

Sequencing program

A pulse labelling ([^{35}S]PITC, then PITC), double cleavage program as shown in Table I was used for sequencing. The flow-rates of reagents and solvents for a column of 15 cm × 0.1 cm I.D. are shown in Table II. These parameters must be adjusted for a column of different dimensions.

PTH amino acid identification

Fractions from the sequencer run were converted to the PTH amino acids

TABLE I

PROGRAM FOR HIGH SENSITIVITY

Step No.	*Function**	*Pump/valve*	*Time*** *(min)*	*Effective time* *(min)*	
1	1	Methanol	2		
2	D	—	3	3	
3	2	Buffer	6		
4	D	—	7	7	
5	3	^{35}S-PITC	5		
6	D	—	1	1	
7	2	Buffer	4		
8	D	—	20	20	Coupling
9	2	Buffer	3		
10	D	—	4	4	
11	2	Buffer	11		
12	D	—	1	1	
13	5	PITC	10		
14	D	—	22	22	Coupling
15	1	Methanol	16		
16	D	—	17	17	Wash
17	10	Col. Sel. Valve	13		
18	9	Col. 1 coll.	17		
19	4	TFA	2		
20	D	—	7	7	Cleavage
21	4	TFA	5		
22	D	—	7	7	Cleavage
23	1	Methanol	1		
24	D	—	2	2	
25	13	Fraction collector step	No time		
26	D	—	1	1	
			Total	92	

* D = Delay; number denotes the programming codes for solvent and reagents pumps and valves.
** Only the delay times are additive.

TABLE II

FLOW-RATES OF REAGENTS AND SOLVENTS
Column: 15 cm × 0.1 cm I.D.

Solvent/reagent	*Flow-rate (µl/min)*
Methanol	1500
Buffer	40
PITC	40
[^{35}S]PITC	20
TFA	150

by a 10-min heating under nitrogen with 1 *M* hydrochloric acid at 80°. The derivatives were extracted with ethyl acetate, the organic layer dried under nitrogen, and the dried residue was redissolved in an appropriate volume of methanol. Up to 70% of the sample was added to 1 µl of a standard PTH amino acid mixture (0.6–0.9

nmole)[5] and the sample was injected into a Waters Assoc. Model 440 high-pressure liquid chromatograph. Separation of the PTH amino acids was achieved using the methanol program previously described[5]. The elution profile was recorded and each PTH amino acid collected directly into the scintillation vials in an LKB Redirac fraction collector. These steps were controlled by a PDP-81 computer. The details of this arrangement are described below and diagrammatically represented in Fig. 1.

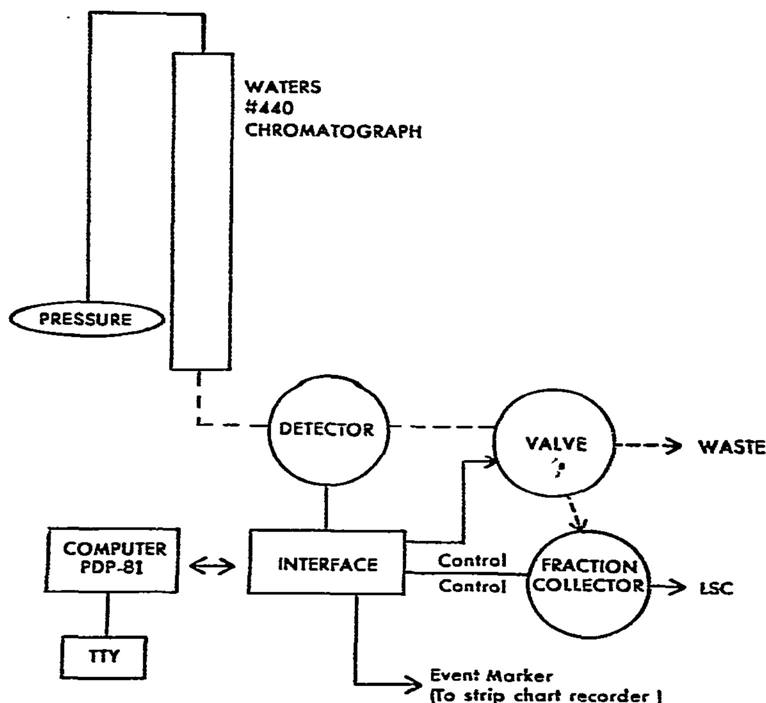

Fig. 1. Schematic representation of computer-controlled HPLC system.

Computer control

To achieve precise peak collection a PDP-81 computer with specially designed interface circuitry and software has been employed. The output of the HPLC detector is connected to the computer via an interface that converts the analog signal into digital levels. The computer in turn activates an LKB Redirac fraction collector and a Hamilton rotary valve (part No. 86430) which directs the eluate stream to waste or to collect. All communication with the computer are via a remote teleprinter. The computer is programmed to monitor signal levels and changes in slope with the onset of each peak and to index the valve function and fraction collector stepping. The data are accumulated in memory for computation of peak areas. Suitable delay times are programmed to compensate for residence time of fluids within the tubing, valve and fittings, thereby insuring discrete collection. In addition, the system has the advantages of interpeak volume collection and of the fact that when the peak interval time exceeds 2 min (approximately 5 ml of eluate) the fraction collector is

indexed and the event recorded. The computer is programmed at the operators option to terminate the run if no peak is detected for any five continuous minutes. At termination of a run, the eluate is directed to waste, and the retention time and peak area are reported. The software is returned to standby, awaiting another start command.

Counting

The peaks are collected directly into counting vials and 10 ml of scintillation cocktail are added and samples counted in an LKB liquid scintillation counter (1210 UltraBeta).

Quantitations

Yields at each cycle are calculated from the activities of the peak relative to that in the injected sample. Counts are plotted against cycle number and the repetitive yield is calculated using the slope of the regression line.

RESULTS

A 70% sample injection with 1 μl of a standard PTH amino acid mixture produces a profile as shown in Fig. 2. The vertical lines at the bottom indicates fraction collector indexing and peak collection. No spurious peaks are seen, thus eliminating collection of peaks other than those proven to contain PTH amino acids. Baseline drift has been minimal.

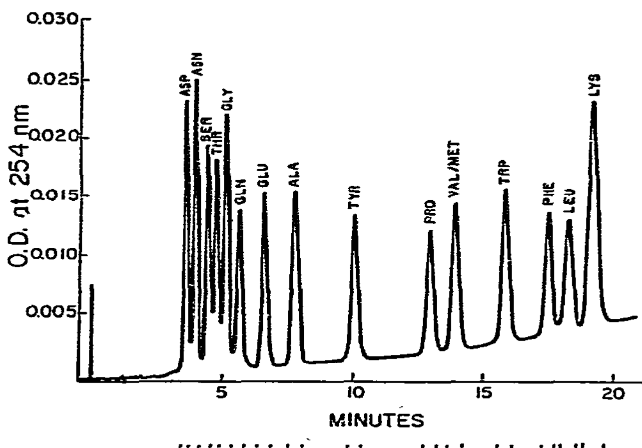

Fig. 2. HPLC of the PTH amino acids[5]. The vertical lines shown below the figure denote fractions collected and analyzed by liquid scintillation.

Fig. 3 presents the counts per minute obtained in various peaks when twelve cycles of lysozyme (700 pmoles) were analysed. Although low counts were obtained for glycine and glutamic acid at residues four and seven, a high repetitive yield (98%, Fig. 4) and low background (<10%) have been realized.

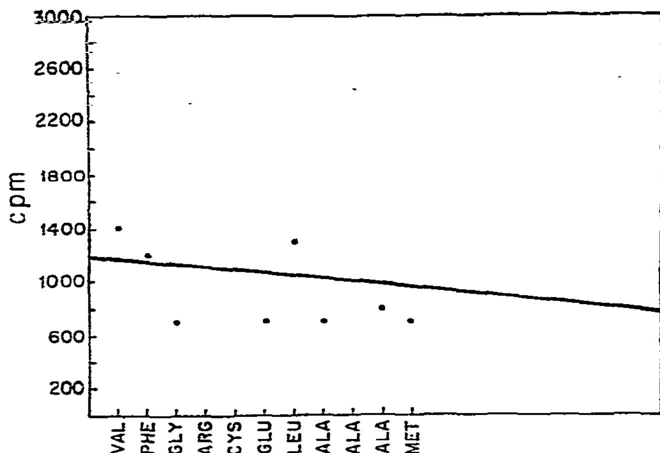

Fig. 3. Plot of the cpm recovered as PTH amino acids during the analysis of 700 pmoles of lysozyme. The sequence indicated below the graph was determined by HPLC.

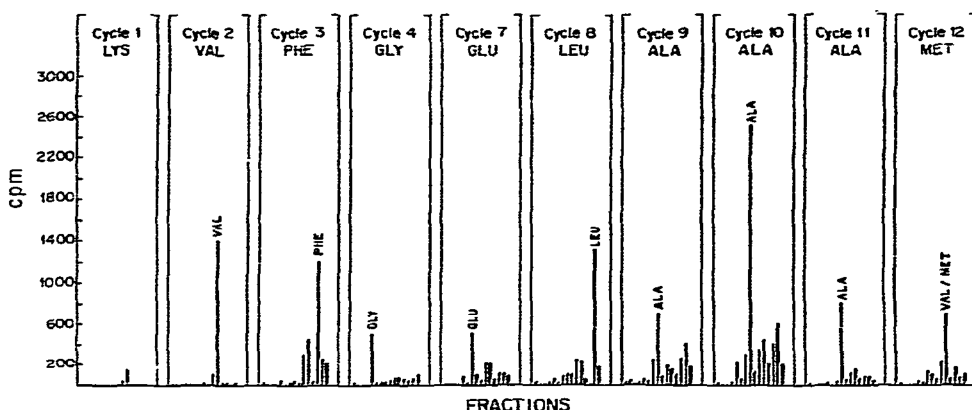

Fig. 4. Plot of the cpm for individual fractions obtained by HPLC at each cycle of the degradation of 700 pmoles of lysozyme.

DISCUSSION

Over the past few years several methods[1–4] have been successfully applied to the sequencing of nanomole quantities of proteins and peptides. Unfortunately these techniques suffer from a common technical drawback of sample extraction when less than 10 nmoles of protein are sequenced. The introduction of polybrene by Tarr[4] has greatly reduced sample losses from the spinning cup of the liquid-phase sequencer and may be used with either DMAA or Quadrol as the coupling buffer (unpublished observation). Alternatively, Edman degradation of proteins or peptides at the picomole level using [^{35}S]PITC as an initial pulse coupling reagent has been made feasible by the development of automatic solid-phase sequencer by Laursen[6]. However, identification of labelled PTH amino acids after each cycle of the degradation has been routinely achieved only by thin-layer chromatography (TLC) followed by auto-

radiography or liquid scintillation counting of each spot subsequent to punching it out from the TLC plate. Although these methods are reliable, they are less sensitive and certainly more time consuming than direct scintillation counting. Furthermore, the methods have an additional disadvantage in that they are not very quantitative. In the present system we have established that with computer-assisted HPLC the labelled PTH amino acid from the sequencer run could be effectively separated and the individual peak collected directly into scintillation vials for counting. The system is extremely reliable as evident by low background and no spillover of counts in the adjacent peak of the chromatogram. The system is also explicitly reproducible, and more economical than autoradiography. It should be feasible to enhance the sensitivity further by increasing the specific activity or concentration of [^{35}S]PITC such that one may reasonably expect to achieve sequence data on 20–50 pmoles of sample.

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