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USE OF NARROW-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR MICROANALYSIS OF PROTEIN STRUCTURE*

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SUMMARY

We report here systematic approaches to microanalysis of protein structures using narrow-bore high-performance liquid chromatography of protein, peptide and derivatized [phenylthiocarbamyl (PTC-) and phenylthiohydantoinyl (PTH-)] amino acids. The utilization of columns with small diameters (2 mm or less) has improved resolution and sensitivity and enabled protein structure analysis at the low pmol level. Preparative isolation of proteins and peptides of pmol quantity is achieved and separation and identification of PTC- and PTH-amino acids can be routinized at the low pmol to subpmol level. The use of diode-array detection enables simultaneous multiple-wavelength monitoring and spectral retreat, which greatly enhances flexibility and usefulness of the present methods. In combination with protein microsequencing techniques, these narrow-bore high-performance liquid chromatographic procedures can facilitate structural analysis of minutely available proteins of biochemical and physiological significance.

INTRODUCTION

Recent progress in methodologies for protein structure determination has greatly improved the efficiencies of analyses of peptides and proteins. Among those methodologies, high-performance liquid chromatography (HPLC) has been often the method of choice for protein isolation, peptide separation, and analysis of amino acid derivatives[1–3]; and protein microsequencing has enabled highly sensitive protein sequence determination at the subnmol–low pmol level[4,5]. These technical advances have facilitated studies on multigene families such as plasma proteins including immunoglobulins and interferons, protein microheterogeneity caused by post-translational processing of gene-derived polypeptides such as incomplete glycosylation and proteolytic cleavage[6], and more strikingly, have facilitated generation of genes from proteins or proteins from genes via recombinant DNA technologies[7]. The former,

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generation of genes from proteins, very often faces the problem of dealing with proteins which are difficult to prepare or only available at trace quantity.

In the past several years, micro HPLC has emerged as a potential tool for micropreparation and microanalysis of polypeptides[8]. Commercially available reversed-phase columns with diameters of 1–2 mm are small columns which can be adapted for more sensitive separation of proteins, peptides and amino acids. However, there has been no report describing systematic approaches to microanalysis of protein structure, particularly primary and secondary structures, using these small columns. We report here a series of narrow-bore HPLC of proteins, peptides and amino acid derivatives for protein structural analysis; and illustrate such an analysis using protein and peptide samples prepared from natural sources or obtained by recombinant DNA technologies.

EXPERIMENTAL

HPLC apparatus

A Hewlett-Packard microbore LC system (HP1090) is used for micropreparation of proteins and peptides, and identification of phenylthiocarbamyl (PTC-) as well as phenylthiohydantoinyl (PTH-) amino acids. The system is equipped with a Hewlett-Packard autosampler and a diode array detector for automatic analysis and multiple-wavelength detection. A Nelson Analytical 4400 system using XTRA-CHROM software is connected to the chromatograph for data acquisition and processing.

Protein purification and peptide separation

Recombinant human interleukin-2 Ala analogue [rMetHuIL-2(ala125)] was produced in genetically modified *Escherichia coli* cells and the human urinary kallikrein was isolated from urine. Preparation procedures for isolation of these proteins will be published elsewhere. Human macrophage colony stimulating factor (M-CSF or CSF-1) was purified to homogeneity according to previous procedures[9] and kindly provided by Dr. M. C. Wu (Department of Biochemistry, North Texas State University, Denton, TX, U.S.A.).

Tryptic peptides of proteins were generated by incubating protein sample (10 μg), in 0.2 *M* ammonium bicarbonate, pH 8.0 buffer containing 1.0 m*M* calcium chloride, with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (substrate-to-enzyme ratio = 50:1, w/w) at 37°C for 6 h. Aliquots of tryptic digest were injected directly onto and separated in a narrow-bore C_4 column (Aquapore, 10 μm, 22 × 0.21 cm; Brownlee Labs.) using a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA). The flow-rate used for the C_4 narrow-bore column was kept at 150 μl/min. For multiple-wavelength detection, the diode array detector was set at 215, 254 and 280 nm to monitor chromophores arising from peptide bonds and aromatic amino acid side chains.

Hydrolysis of protein and peptide samples

Aliquots of protein and peptide samples (5–50 pmol) were transferred to 250-μl microvials (Hewlett-Packard) which had been pre-cleaned and then heated at 600°C. Samples in vials were carefully dried using a Speed Vac centrifuge (Savant), vials

were placed in Pico-Tag (Millipore) vacuum vials[10]. About 1.5–2.0 ml of 5.7 *M* hydrochloric acid (constant boiling) containing 0.2% phenol and 0.1% mercaptoethanol were placed in the Pico-Tag vacuum vial. The vacuum vials were flushed with a stream of nitrogen and then sealed *in vacuo*. Hydrolysis was carried out at 110°C for 24 h, at which time sample vials were removed from vacuum vials and dried before phenylisothiocyanate (PITC) derivatization. For analysis of cysteic acid after performic acid oxidation of the protein hydrolysate, mercaptoethanol is omitted from the hydrolysis acid.

Phenylisothiocyanate derivatization of amino acids

Coupling of protein or peptide hydrolysates with PITC was performed directly in the same hydrolysis vials as described above to avoid sample loss during transfer. Standard amino acids (1–5 nmol) and hydrolysates were derivatized with PITC using a modified coupling buffer [ethanol–trimethylamine–water (7:1:1, v/v)]. Trimethylamine (TMA) was specially purified and was free of any contaminating primary and secondary amines[5]. TMA (Eastman Kodak) which was added with isopropanol as a 25% solution was mixed with ninhydrin (3 g) and hydrindantin (0.3 g) and the mixture was stirred for 12 h at 25°C and stored at −20°C. TMA was then transferred from the mixture into ethanol–water (7:1) by bubbling the TMA solution with a small stream of nitrogen. Nitrogen bubbling was stopped when the desired TMA ratio was reached. The coupling buffer is stable at least for two weeks and is stored at −20°C after use.

For PITC derivatization, samples were pre-mixed with 50 μl coupling buffer and dried under vacuum. The same volume of coupling buffer containing 2% (v/v) PITC was then added and gently mixed with dried samples. The reaction was carried out at 25°C for 15–20 min. After completion, the sample solution was thoroughly dried using a Speed Vac centrifuge. The derivatized samples were stored in freezer or redissolved in solvent A (50 mM sodium acetate, adjusted to pH 6.5 with phosphoric acid) for immediate HPLC separation.

Separation of PTC-amino acids

Separation of PTC-amino acid derivatives was carried out by reversed-phase HPLC using an Altex narrow bore C_{18} column (25 × 0.2 cm) and a gradient of solvent B in solvent A. Solvent A was 50 m*M* sodium acetate (pH adjusted to 6.5 with phosphoric acid). Solvent B was 100 m*M* sodium acetate (pH adjusted to 6.5 with phosphoric acid)–acetonitrile–methanol (40:50:10). The column was equilibrated in A–B (95:5) and operated at 40°C using a flow-rate of 0.25 ml/min. Separation was achieved using a linear gradient of 5 to 50% B in 15 min, followed by an isocratic elution with 50% B for 5 min. The effluent was monitored at 254 nm.

Sequence analysis

Automatic sequence analysis[4,5] was performed with a gas-phase sequencer using either a standard program or a program designated as MHNVAC supplied by Applied Biosystems. Protein or peptide samples were loaded onto a glass fiber disc containing precycled polybrene and sodium chloride[11]. The PTH-amino acid sample obtained from each sequencer cycle was carefully transferred from Wisp (Millipore) vials to HP microvials (Part No. 5080-8779).

In later sequencing runs, 300-μl microvials (Hewlett-Packard, Part No. 5180-0841, specific for HP1090 LC system), which correctly fit into the fraction collector in the sequencer, were used to replace Wisp vials for collection of S4 [methanol acetonitrile (9:1, v/v)] extract. This step has obviated tedious manual transfer and sample loss. After samples were completely dried, they were reconstituted in 12 m*M* sodium acetate (pH 4.1) containing 40% methanol. Usually an aliquot (5–15 μl) of PTH-amino acid sample was analyzed by reversed-phase microbore HPLC.

PTH-amino acids analysis

Standard PTH-amino acid derivatives and samples obtained from sequence analysis were separated by reversed-phase micro LC using an Altex narrow-bore C_{18} column (25 × 0.2 cm). PTH-amino acids were separated using a gradient of solvent D (acetonitrile) in solvent C (12 m*M* sodium acetate, pH adjusted to 4.2 with acetic acid). The column was initially equilibrated with C–D (90:10) and operated at 47°C and the flow-rate of the mobile phase was 0.2 ml/min. Typical chromatographic conditions were as follows: 10–12% D for 1 min; 12–40% D for 11 min; 40–55% D for 4.5 min; 55% D isocratic for 2 min and 55–60% D for 2 min. The column effluent was monitored at multiple wavelengths, *i.e.*, 254, 270 and 313 nm. The results obtained from the multiple-wavelength detection were stored on a HP hard disc. The detection signals at 270 and 313 nm were also stored in a Nelson Analytical 4400 data acquisition system. For immediate analysis of sequencing results, chromatograms monitored at 270 nm were also recorded by a Shimadzu CR3A recording integrator. Attenuation parameter setting for this recorder was 0.004 a.u.f.s. for detection of low pmol (1–10 pmol) levels of PTH-amino acids or 0.002 a.u.f.s. for subpmol (0.5–2 pmol) levels.

RESULTS AND DISCUSSION

Protein purification and peptide separation

The use of reversed-phase columns of small diameter has greatly improved the detection limit for the chromatographic separation of proteins. Fig. 1 shows the separation profile of 100 pmol (4 μg) of the purified preparation of human urinary kallikrein which is electrophoretically homogeneous, using an Aquapore C_4 column (22 × 0.21 cm). The material in this preparation was resolved into two components whose partial N-terminal amino acid sequences are identical. Since these two components can be individually re-chromatographed at their respective retention time, the result does not represent a chromatographic artifact. They may differ in the extent of post-translational processing such as glycosylation and proteolysis such that their overall protein hydrophobic properties are differentiable by the reversed-phase column. Observations of different iso-enzyme forms, glycosylation, and limited proteolytic processing have been reported on rat urinary and porcine pancreatic kallikreins[12,13].

The use of modified chromatographic conditions designed for columns of small diameters has effectively increased the concentration of the eluting proteins by minimizing sample dilution during the separation. Sample dilution resulting in reduced detectability often is observed with regular HPLC columns. Thus, higher sensitivity can be achieved with a sample load as small as several pmole of a protein, if columns with small diameters are used.

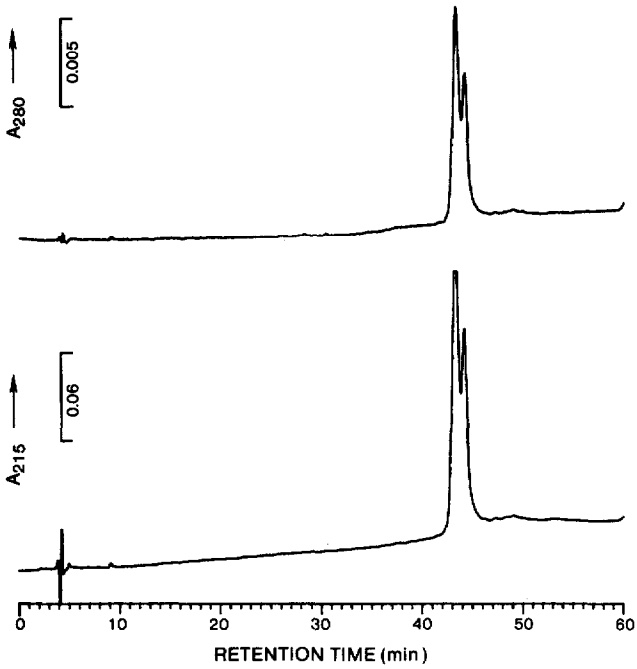

Fig. 1. HPLC separation of purified human urinary kallikrein (4 μg) in an Aquapore C_4 column (10-μm, 22 × 0.21 cm). The mobile phase components are 0.1% TFA (A) and 90% acetonitrile in 0.1% TFA (B). The column was initially equilibrated with A–B (85:15) at room temperature with a constant flow-rate of 150 μl/min. The separation was obtained by the following gradient elution conditions: isocratic at 15% for 3 min; 15–60% B for 57 min and 60–80% B for 10 min. The effluent was monitored at both 215 and 280 nm.

The same reversed-phase HPLC system can be used for the isolation of peptide fragments derived from enzymatic digestion of a narrow-bore column purified protein. This is illustrated by the HPLC peptide mapping of a tryptic digest of rMetHuIL-2(ala125). In Fig. 2A, a tryptic digest (1.0 μg) of rMetHuIL-2(ala125) was nicely separated using the same C_4 column. The improvement of the instrumentation and detection allows mapping being performed at the low pmol level. Peptides in fractions obtained from such chromatography were subsequently subjected to high-sensitivity amino acid analysis and sequencing.

The use of a diode-array detector has enabled the simultaneous designation of peptide fractions containing aromatic amino acids using detection at 280 nm. The chromatogram shown in Fig. 2A (top) is the separation profile of rMetHuIL-2(ala125) tryptic peptides detected at 280 nm. Spectral data of all peaks absorbing in the range of 210 nm to 400 nm were collected on a hard disc during the entire separation. An overlay of spectra taken at the peak maxima and the trailing edges is compared (Fig. 2B). The peptide containing a single tryptophanyl residue in rMetHuIL-2(ala125) is thus identified in the fraction with a retention time of 48.5 min (Fig. 2B, spectrum c). Peptides containing tyrosine are found in fractions with retention times of 25.1 min (Fig. 2B, spectrum b) and 50.6 min (Fig. 2B, spectrum d). These observations were confirmed by compositional and direct sequence analyses. Tryptophan and tyrosine contain only single and two degenerate codons, respective-

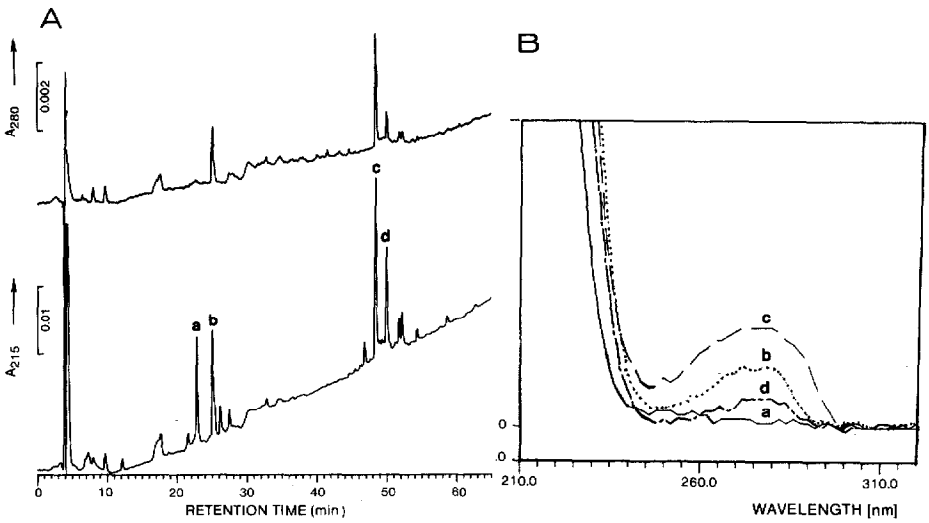

Fig. 2. (A) Peptide mapping of a tryptic digest of rMetHuIL-2(ala125) (1.0 μg) in an Aquapore C_4 column detected at 214 nm (bottom) and 280 nm (top) Mobile phases and conditions are the same as those described for Fig. 1. The column was equilibrated with A–B (97:3) and the following elution conditions were used: isocratic at 3% B for 3 min; 3–55% B for 57 min and 55–85% B for 30 min. (B) Overlay of spectra taken from (A) as designated. Full scale is set at 10 mAU.

ly, used for mRNA translation during protein synthesis. Peptides containing these amino acids are frequently used to make oligonucleotide probes with lower numbers of probe degeneration for molecular cloning.

Amino acid analysis of proteins and peptides

With the recent improvements in hardware design and column technologies, the ion-exchange chromatography of amino acids, as originally exploited by Moore *et al.*[14], is still being widely used as a routine procedure for compositional analysis of protein, hydrolysates and physiological fluids. However, the development of high-performance reversed-phase liquid chromatographic packing materials and the introduction of pre-column derivatization techniques have made possible rapid and sensitive HPLC analyses of the chemically labeled amino acid derivatives (for a review, see ref. 15). PITC pre-column derivatization is a later development[16,17], and it soon will become the method of choice for compositional analysis of proteins and peptides at much more sensitive levels[18–21]

HPLC separation of PITC derivatized amino acids is usually performed using regular reversed-phase columns (>3.9 mm I.D.). The sensitivity limit of a chromatographic separation is usually in the range of pmol levels. To improve sensitivity and to achieve better reproducibility and quantitation for routine analysis, we have used a micro LC system for the separation of PTC-amino acids. As shown in Fig. 3, PTC-amino acid standards (panel A) and standard with some oxidized amino acids as well as amino sugars (panel B) were separated on Altex C_{18} narrow-bore columns (25 × 0.2 cm). Samples of 10 pmol concentration were injected and a complete resolution profile was obtained using a detector sensitivity of 0.004–0.008 a.u.f.s. With columns ageing, all amino acid derivatives still remained well resolved except

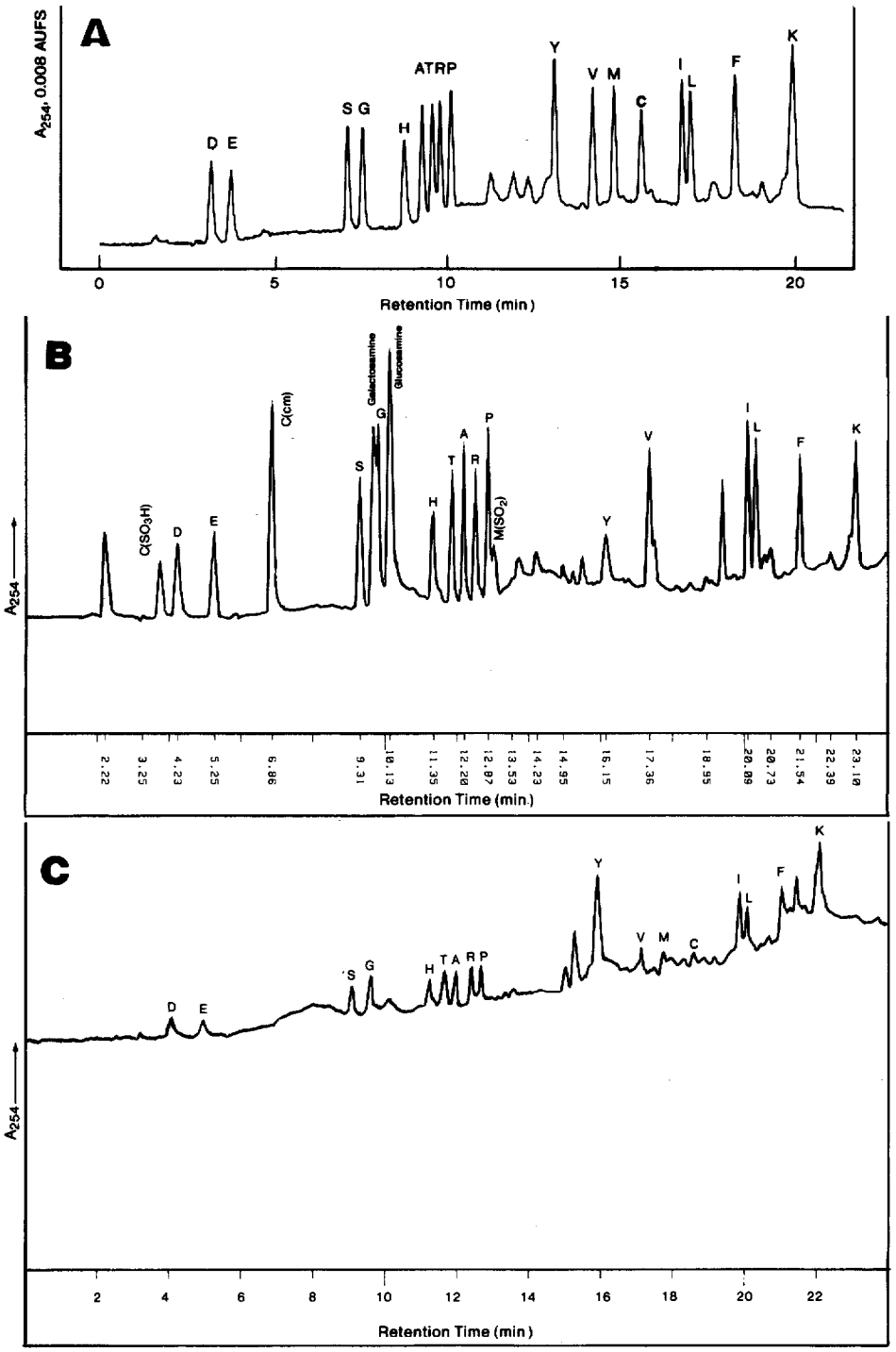

Fig. 3. HPLC separation of PTC-amino acids using an Altex C_{18} column (25 × 0.2 cm, 5 μm). (A) 10 pmol of Pierce amino acid standards; (B) 10 pmol of performic acid oxidized standards; (C) 800 fmol of standard. These chromatograms were obtained at different times, slightly shifted retention times were observed due to changes in initial equilibration (A, 5% mobile phase B; B and C; 3% mobile phase B) and gradient conditions.

that PTC-Pro and PTC-Arg tended to lose some resolution. This can be corrected by decreasing mobile phase B to 40–45% for the first 15 min gradient as seen in Fig. 3B. However, a higher salt concentration in mobile phase A for example, up to 80 m*M*, is also able to effectively separate PTC-Pro from Arg in older columns. PTC-Ala and PTC-Thr were sensitive to changes in buffer concentration and pH. They tended to co-elute and then a reversed elution order could occur depending on the adjusted pH and concentration of mobile phase A. The high efficiency of narrow-bore columns has allowed separation of all amino acid derivatives including cysteic acid, proline and methionine sulfone (Fig. 3B). Carboxymethylcysteine (6.8 min), galactosamine (between Ser and Gly), glucosamine (after Gly) and Trp (after Phe) can also be quantified using identical chromatographic conditions (data for Trp not shown). With the exception of lysine, all derivatives have similar peak areas and thus should differ little in response factors. The peak area for lysine is twice of that for other amino acids because of its side chain derivatization. The use of a lower flow-rate (0.25 ml/min) in micro LC system gives a rather stable baseline at higher sensitivity (<0.004 a.u.f.s.). The increased resolution and improved signal-to-noise ratio allow highly sensitive analysis at subpmol levels (as shown in Fig. 3C). In comparison to the separation using regular HPLC, the present method shows at least a four-fold increase in sensitivity for the analysis of PTC-amino acid derivatives.

Fig. 4A illustrates the separation of PITC-derivatized hydrolysates of human urinary kallikrein. In this run, with 0.2-μg sample analyzed, an excellent separation profile was achieved and a much stabler baseline was obtained at 10–15 mV output signal. At a higher sensitivity level (<0.004 a.u.f.s.), a comparable chromatogram could also be generated from the analysis of a subpmol protein hydrolysate (chromatogram not shown; see Table I). The amino acid composition of this protein was determined using the response factors from the amino acid standards (Table I). The data calculated from different sensitivity levels match reasonably well. The only exception is the low yield of Met at higher sensitivity analysis, possibly due to the instability of this particular PTC-amino acid derivative. The compositional data obtained from these analyses agree well with those deduced from the cDNA sequence[22] and also with our protein sequencing results[23]. The sensitivity and reproducibility obtained with the column was established after a long period of usage. For example, Fig. 4B illustrates the analysis of human CSF-1 hydrolysate oxidized by performic acid prior to PITC derivatization using a 6 month-old column after hundreds of injections. All amino acids were completely resolved, except that PTC-Thr and PTC-Ala were poorly separated and eluted in a reverse order due to a slight increase in buffer concentration and changes in gradient conditions. However, the computer data processing capability allows good quantitation of amino acids for human CSF-1 including Cys(SO_3H), Thr and Ala (Table I). The composition of human CSF-1 determined by this method is comparable to that derived from the cDNA sequence in which the amino terminal signal peptide and possible C-terminal unprocessed region were not estimated[24].

PTC-amino acid analysis can be used as a procedure to quickly identify disulfide-containing peptides in a protein digest[21]. For example, rMetHuIL-2(ala125) contains a single disulfide pair (Cys_{59}–Cys_{129}), tryptic digestion would generate one disulfide-containing peptide, which was found in the fraction with a retention time of 50.6 min shown in Fig. 2. Determination of cystine content of the identified di-

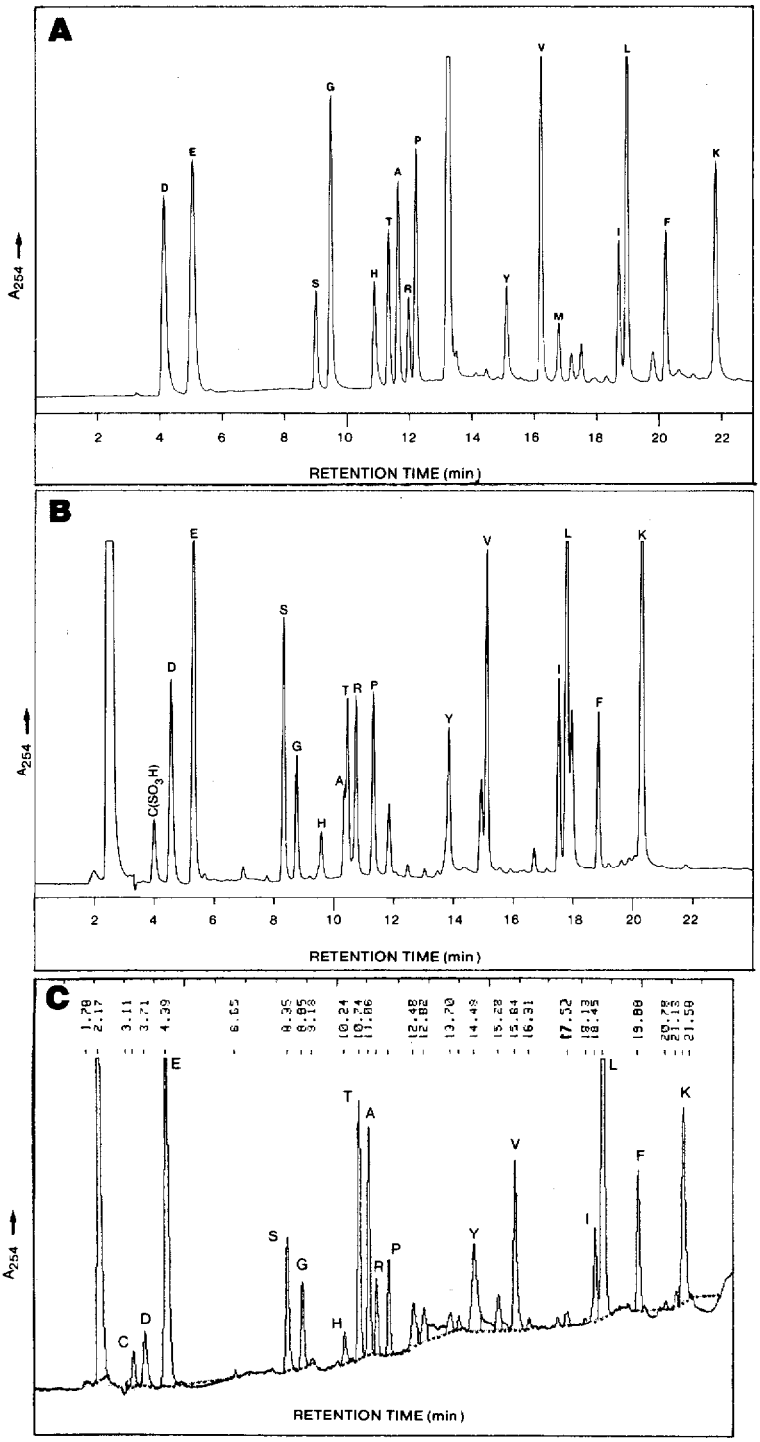

Fig. 4. Chromatograms of PTC-amino acid analysis of protein and peptide hydrolysates. Differences in retention time and elution order of Thr and Ala as well as Pro and Arg are due to analyses performed at different times and slight changes in initial buffer concentration, pH, and gradient conditions. (A) Analysis of human urinary kallikrein hydrolysate (0.2 μg injected); (B) analysis of human CSF-1 hydrolysate oxidized by performic acid (0.21 μg injected); (C) analysis of cystine-containing tryptic peptide obtained from HPLC mapping of rMetHuIL-2(ala125) trypotic digest (11 ng injected).

TABLE I

AMINO ACID COMPOSITION OF HUMAN URINARY KALLIKREIN, HUMAN COLONY STIMULATING FACTOR-1 AND rMetHuIL-2(ala125) DISULFIDE PEPTIDE OBTAINED FROM PITC DERIVATIZATION AND NARROW-BORE HPLC

Amino acids	*Human urinary kallikrein**					*Human CSF-1***				*Disulfide peptide*** [rMetHuIL-2(ala125)]*		
	pmol	*No. of res.*	*pmol*	*No. of res.*	*Theoretical*	*pmol*	*No. of res.*	*pmol*	*No. of res.*	*pmol*	*No. of res.*	*Theoretical*
$Cys(SO_3H)$	–	–	–	–	–	–	–	59	5.0	4.1	1.8	2
Asp	193	22.5	16.5	21.6	26	155	12.0	138	11.7	5.6	2.4	3
Glu	254	29.7	21.1	27.4	29	272	21.0	240	20.3	25.2	10.8	11
Ser	97	12.0	8.2	10.7	16	132	10.2	114	9.7	3.6	1.6	2
Gly	166	19.4	15.4	20.1	19	66	5.1	57	4.8	2.4	1.1	1
His	77	9.6	8.0	10.5	11	36	2.8	30	2.5	1.7	0.7	1
Thr	95	11.7	8.9	11.6	14	29	2.3	25	2.1	10.1	3.2	4
Ala	101	12.5	10.1	13.1	12	76	5.8	76	6.4	8.4	3.6	3
Arg	48	5.7	5.3	7.2	5	83	6.4	79	6.7	2.9	1.2	1
Pro	115	13.4	10.9	14.3	13	70	5.4	74	6.3	2.9	1.2	1
Tyr	44	5.2	3.9	5.1	5	81	6.1	47	4.0	5.5	2.3	2
Val	168	19.9	15.9	20.8	21	153	11.8	136	11.5	2.6	1.1	1
Met	28	3.3	2.4	3.2	4	32	2.5	–	–	–	–	1
1/2 Cys	–§	–	–	–	10	61	4.7	–	–	–	–	(2)
Ile	62	7.5	6.3	8.2	8	82	6.3	78	6.6	3.1	1.3	1
Leu	184	21.5	17.0	22.2	22	217	16.7	203	17.1	18.7	7.6	7
Phe	81	9.4	7.2	9.6	9	72	5.6	68	5.8	5.8	2.4	2
Lys	69	8.1	6.2	8.1	7	148	11.4	135	11.4	5.5	2.3	2
Trp§§	–	–	–	–	7	–	–	–	–	–	–	0
Amount injected	0.2 μg		18 ng			0.21 μg		0.19 μg		11 ng		

* Sample (1 μg) was hydrolysed in 6N HCl containing 0.05% mercaptoethanol and 0.1% phenol. After derivatization, aliquots of sample were injected for HPLC analysis. Theoretical numbers are obtained from cDNA sequence (Biochemistry, 24, 8037-43, 1985).

** Human CSF-1 (2 μg) was hydrolysed in 6N HCl in the absence of mercaptoethanol. The hydrolysate was split in half for direct analysis and performic acid oxidation.

*** Peptide (approximately 0.2 μg) was hydrolysed and prepared according to text. Theoretical numbers are obtained from *Proc. Natl. Acad. Sci., U.S.A.* 80 (1983) 5990-5994.

§ Half cysteine being destroyed by mercaptoethanol.

§§ Not determined.

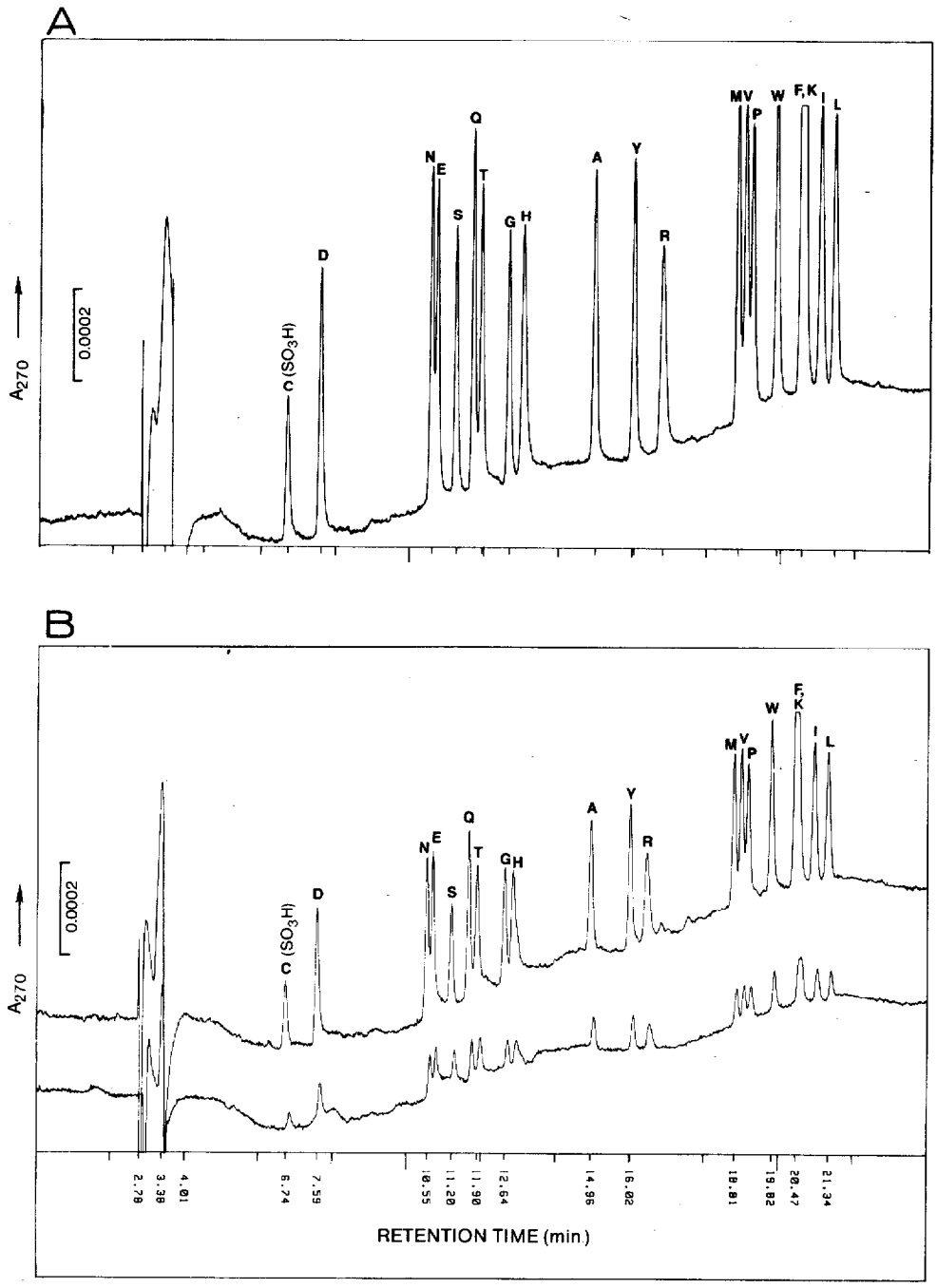

Fig. 5. HPLC separation of PTH-amino acids. (A) Separation of a sample (10 pmol) on Altex C_{18} columns (22 × 0.2 cm, 5 μm). (B) Higher sensitivity analyses of 3 pmol and 750 fmol of standard mixture.

sulfide peptides was performed by PTC-amino acid analysis of the performic acid oxidized sample. Cystine was detected as cysteic acids in a PTC form. Fig. 4C illustrates the analysis of an oxidized sample of disulfide-containing peptide obtained from a tryptic digest of rMetHuIL-2(ala125) using 1–2 pmol of the derivatized material. PTC-cysteic acid was recovered in a molar ratio of 1.8 per peptide molecule, while the rest of the amino acids were recovered at a level close to the expected theoretical values (Table I). Routinely, we were able to hydrolyze 0.5–1 μg protein sample with an average molecular weight of 20–40 kilodalton, and to inject 5–20% derivatized hydrolysate. Therefore, a number of analytical runs can be performed for comparison and estimation of reliable composition data. These analyses are particularly useful in comparison with the data deduced from the cDNA sequence of an unknown protein sample only available with limited partial N-terminal sequence[25].

PTH-amino acid analysis and microsequencing

Fig. 5 shows the elution profile of a mixture of 20 PTH-amino acids at the 10 pmol level (panel A). PTH-Asp elutes at 7.6 min, but is partially obscured by the reduced and oxidized forms of dithiothreitol (DTT) usually obtained from S4 (methanol–acetonitrile, 9:1) extraction during sequencing. To avoid interference of DTT, the salt concentration in mobile phase C can be slightly increased (see Experimental) to cause later elution of PTH-Asp. Practicallly, the methyl esters of PTH-Asp (between PTH-Ala and PTH-Tyr) and PTH-Glu (between PTH-Tyr and PTH-Arg) are more suitable for analysis than the corresponding free acids. The esters are formed during Edman degradation if methanolic hydrochloric acid is used as conversion acid. PTH-Phe and PTH-Lys were barely separated with the gradient elution used, but they were resolvable by changing the final concentration of mobile phase D at the last step of elution. The Altex narrow-bore C_{18} column exhibited a characteristic property in the separation of PTH-His and PTH-Arg. At conditions using mobile phase C of 12 m*M* sodium acetate, pH 4.2, PTH-His eluted behind PTH-Gly and PTH-Arg behind PTH-Tyr with response factors comparable to other PTH-amino acids. After prolonged use of the column, PTH-His and PTH-Arg tended to be retained longer in the column, while other PTH-amino acids still eluted unchanged. However, the retention times of both amino acids could be adjusted easily by changing the pH of mobile phase C.

The sensitivity of the PTH-amino acid separation using a narrow-bore column increased approximately four-fold, as also observed in the PTC-amino acid separation described above. This increase has enabled the routine PTH-amino acid analysis at the 1–10 pmol level. Panel B in Fig. 5 shows original chromatograms of approx. 3 pmol (top) and 700–800 fmol (bottom) amounts of PTH-amino acids.

Fig. 6 shows analyses of PTH-amino acids derived from the sequencing of 100 pmol (≈4 μg) human urinary kallikrein at cycles 1–4 and 26–27. The sample was loaded onto a polybrene-precycled glass fiber disc and yielded 69 pmol of PTH-Ile at the first cycle. Strong PTH-amino acid signals were obtained at the first ten cycles by injecting 48% of sample. Background peaks and carry-over occurred after extended analysis as can be seen on the chromatograms obtained for cycles 26–27. At cycle 27, however, the recovered PTH-amino acid (PTH-Gly) still remained detectable at *ca.* 7 pmol as opposed to 58 pmol at cycle 4. The sequencing of this protein has carried out further and thus 35 identifiable residues were obtained with a repetitive yield of 91–93%.

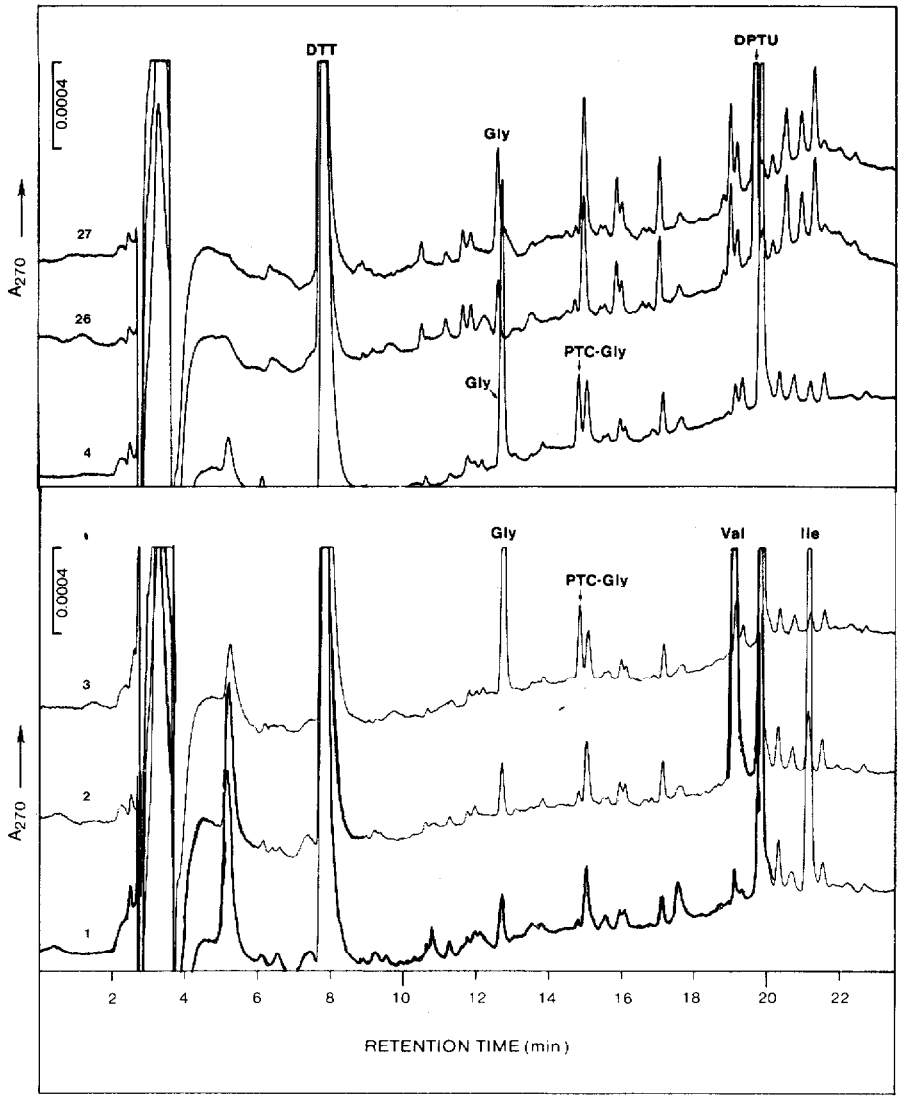

Fig. 6. Identification of PTH-amino acids derived from the sequence analysis of 100 pmol human urinary kallikrein at cycles 1–4 and 26–27. The samples obtained from sequencing runs were dried and reconstituted into 25 μl of methanol–sodium acetate (40:60) buffers and 48% aliquots were injected for analysis. Cycle 26 is not identified, Cys is assigned when carboxymethylated kallikrein is sequenced.

The detection of PTH-amino acids using the diode-array detector has the advantage of simultaneously monitoring dehydration products of PTH-Thr and PTH-Ser at 313–320 nm without peak broadening as usually observed in a regular dual-wavelength detector. Fig. 7 shows the chromatograms derived from the sequence analysis of urinary protease at cycle 11 and of a tryptic peptide at cycle 5. Smaller peaks corresponding to PTH-Thr and PTH-Ser and their respective dehydrated products are seen at 270 nm. On the contrary, the dehydrated products show

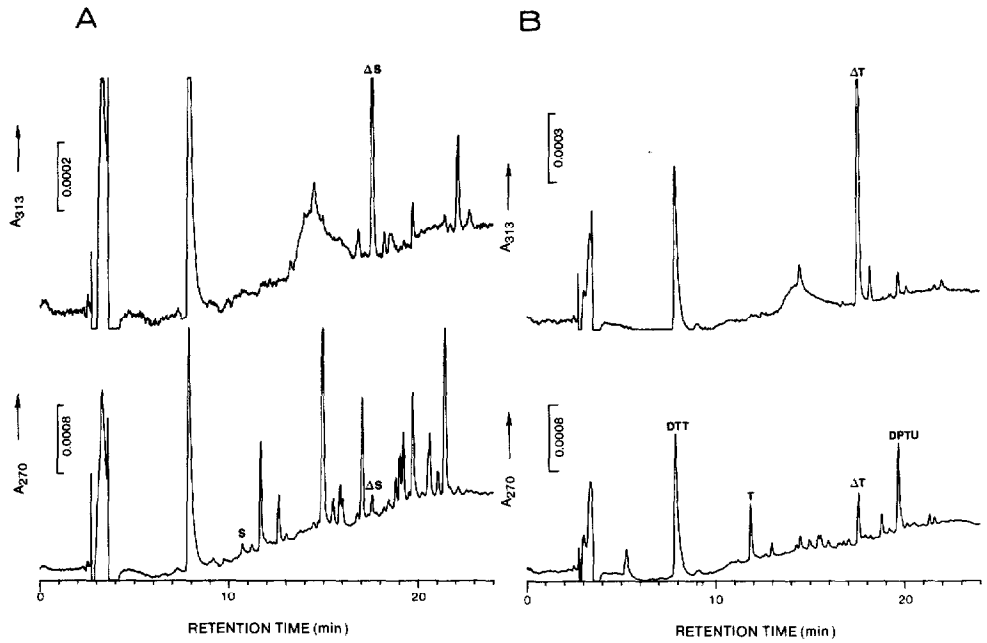

Fig. 7. Detection of PTH-Thr and PTH-Ser (270 nm) and their respective dehydro products (313 nm) by diode-array detection. (A) Analysis of cycle 13 from the sequence determination of human urinary kallikrein; (B) cycle 5 from the sequence analysis of a tryptic peptide derived from human urinary kallikrein.

strong absorption at 313 nm and can be unequivocally differentiated from the other PTH-derivatives.

The sequence analysis of a tryptophan-containing peptide derived from [rMet-HuIL-2(ala125)] (Fig. 2, retention time = 48.6 min) is shown in Fig. 8. The sample obtained from HPLC was dried and redissolved and 60% of the material (60 pmol if 100% recovery was obtained) was sequenced. Only 2.5 pmol of PTH-Trp and 3.5 pmol of PTH-Ile were obtained at cycles 1 and 2, respectively. This result accounts for approx. 9% initial yield according to concentration determination using PTC-amino acid analysis described above. However, the repetitive yield is 94.3% and the peptide could be sequenced through 12 cycles. As summarized in Table II (sample A), the identification of PTH-amino acids was actually performed at the subpmol level. The low initial yield could be caused by improper sample handling after the peptide is collected from the HPLC eluate. Improved absolute yield was observed when the sample was directly collected during the HPLC separation (Table II, sample B). A much stronger signal (33% initial yield) of PTH-amino acids detected in HPLC indicates that sample loss due to improper drying and handling has been reduced significantly.

In conclusion, we have demonstrated the systematic use of narrow-bore HPLC for microanalysis of protein structure. Recently, a highly sensitive on-line PTH-amino acid analysis[26] and a more versatile sequencing data processing method[27] have been reported. The methods described in our paper, when used in conjunction with these techniques, can be especially useful for further improvement in the recov-

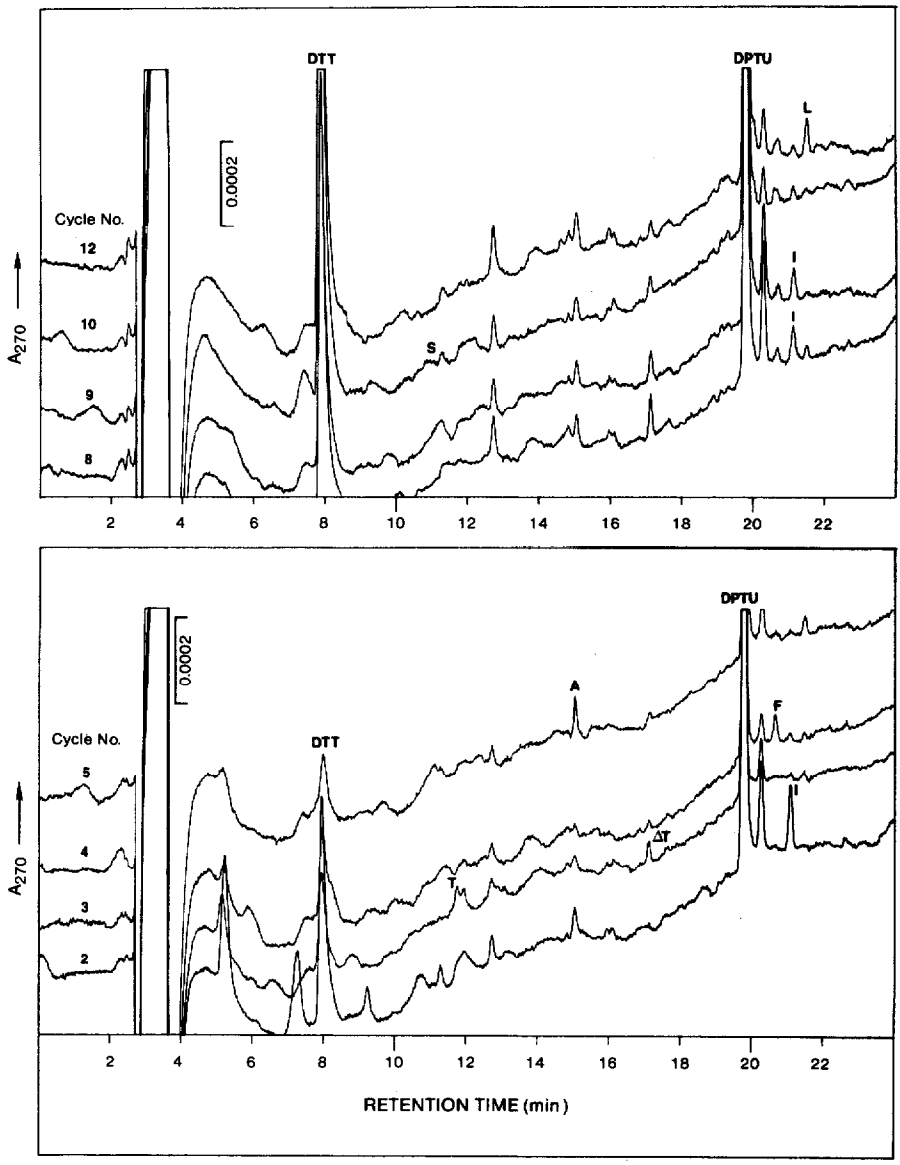

Fig. 8. Identification of PTH-amino acids derived from the sequence analysis of a rMetHuIL-2(ala125) tryptic peptide. Cycles 2–5 and cycles 8–10 and 12 are shown. 48% sample was analyzed at each cycle. For quantitation data, see Table II.

ery of sequencing cycles and for optimizing the signal-to-noise ratio in PTH-amino acid analysis. More potentially, the use of these procedures in conjunction with high-resolution mass spectrometric methodologies can substantially complement the automated Edman degradation in the strategy for protein sequencing.

TABLE II

RECOVERY OF PTH-AMINO ACIDS OBTAINED FROM SEQUENCING OF A rMetHuIL-2(ala125) TRYPTIC PEPTIDE

Cycle	*Amino acid*	*Recovery (pmol)*	
		*A**	*B***
1	Trp	2.5	9.9
2	Ile	3.5	21.0
3	Thr	0.8	8.3
4	Phe	1.2	13.3
5	Ala	2.2	13.9
6	Gln	1.0	9.2
7	Ser	+***	1.7
8	Ile	1.5	6.8
9	Ile	1.4	11.3
10	Ser	+	+
11	Thr	–§	+
12	Leu	1.2	2.1
13	Thr	–	–

* The isolated peptide is collected in a tube and the dried sample is redissolved in 50% formic acid before loading onto the sequencer.

** The isolated peptide is collected in a polypropylene tube (0.5 ml volume) and an aliquot of the sample is directly applied onto the sequencer.

*** Quantitative data not determined.

§ Undetected.

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REFERENCES

1 F. E. Regnier, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High-Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1983, p. 1.
2 M. Elzinga (Editor), *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1982, p. 1.
3 K. A. Walsh, L. H. Ericsson, D. C. Parmelee and K. Titani, *Ann. Rev. Biochem.*, 50 (1981) 261.
4 R. M. Hewick, M. W. Hunkapiller, L. E. Hood and W. J. Dreyer, *J. Biol. Chem.*, 256 (1981) 7990.
5 P.-H. Lai, *Anal. Chim. Acta*, 163 (1984) 243.
6 N. L. Anderson, R. P. Tracy and N. G. Anderson, in F. W. Putnam (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, Orlando, FL, 2nd ed., 1984, p. 221.
7 M. Hunkapiller, S. Kent, M. Caruthers, W. Dreyer, J. Firca, C. Giffin, S. Horvath, T. Hunkapiller, P. Tempst and L. Hood, *Nature (London)*, 310 (1984) 105.
8 G. Guiochon and H. Colin, in P. Kucera (Editor), *Microcolumn High-Performance Liquid Chromatography*, Elsevier, Amsterdam, 1984, p. 1.
9 M. C. Wu, J. K. Cini and A. A. Yunis, *J. Biol. Chem.*, 254 (1979) 6229.
10 S. A. Cohen, T. L. Tarvin and A. Bidlingmeyer, *Am. Lab.*, August (1984) 48–59.
11 *Applied Biosystems User Bulletin No. 12, Protein Sequencer*, Foster City, CA, 1985.
12 J. Chao and H. S. Margolius, *Biochem. Pharmacol.*, 28 (1979) 2071–2079.
13 F. Fiedler, E. Finke, H. Tschesche and H. Fritz, *Methods Enzymol.*, 80 (1981) 493–532.

14 S. Moore, D. H. Spackman and W. H. Stein, *Anal Chem.*, 30 (1958) 1185.
15 M. W. Hunkapiller, J. E. Strickler and K. J. Wilson, *Science (Washington, D.C.)*, 226 (1985) 304–311.
16 D. R. Koop, E. T. Morgan, G. E. Tarr and M. J. Coon, *J. Biol. Chem.*, 257 (1982) 8472–8480.
17 R. Heinrickson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65–74.
18 B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, 336 (1984) 93–104.
19 T. H. Maugh, *Science (Washington, D.C.)*, 225 (1984) 42.
20 R. R. Granberg, *LC, Liq. Chromatogr. HPLC Mag.*, 2 (1985) 778–789.
21 H. S. Lu, R. R. Everett, M. L. Klein and P. H. Lai, in J. L'Italien (Editor), *Modern Methods in Protein Chemistry*, Plenum Press, New York, in press.
22 D. Fukushima, N. Katamura and S. Nakanishi, *Biochemistry*, 24 (1985) 8037–8043.
23 H. S. Lu and P. H. Lai, manuscript in preparation.
24 E. S. Kawasaki, M. B. Ladner, A. M. Wang, J. V. Arsdell, M. K. Warren, M. Y. Coyne, V. L. Schweikart, M. T. Lee, K. A. Wilson, A. Boosman, E. R. Stanley, P. Ralph and D. F. Mark, *Science (Washington, D.C.)*, 230 (1985) 291–296.
25 L. M. Souza, T. C. Boone, J. Gabrilove, P. H. Lai, K. M. Zesbo, D. C. Murdock, V. R. Chazin, J. Bruszewski, H. S. Lu, K. K. Chen, J. Barendt, E. Platzer, M. A. S. Moore, R. Mertelsmann and K. Welte, *Science (Washington, D.C.)*, 232 (1986) 61.
26 *Applied Biosystems Users Bulletin No. 11*, Foster City, CA, 1985.
27 S. Kent, L. Hood, R. Aebersold, D. Teplow, L. Smith, V. Farnsworth, P. Cartier, W. Hindes, P. Hughes and C. Dodd, in J. L'Italien (Editor), *Modern Methods in Protein Chemistry*, Plenum Press, New York, in press.