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An alternative peptide sequencing procedure for low budget laboratories

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Abstract

We standardized the amino-terminal sequencing of peptides by using manual Edman chemistry followed by the separation of the derivatives obtained by high-performance liquid chromatography (HPLC). Initially, the 3-phenyl-2-thiohydantoins (PTH) of the twenty common amino acids were chemically synthesized and purified individually. With the exception of PTH-Met and PTH-Val, which coeluted when various gradient conditions were used, we were able to separate the remaining eighteen PTH-amino acids by reversed-phase HPLC on a C_{18} column. The pair PTH-Met/PTH-Val eluted from the column as a single peak at a different retention time than the other PTH derivatives. Then, the primary structure of a synthetic heptapeptide, kemptide, was verified using the standard Edman degradation procedure followed by HPLC separation of the PTH-amino acid liberated in each cycle. The sequence obtained, Leu-Arg-Arg-Ala-Ser-Leu-Gly, corresponds to the expected published sequence. Thus, the methodology reported here represents a simple, sensitive, reproducible, and inexpensive procedure that provides an alternative to peptide sequencing by automatic sequenators or mass spectrometers.

Key words: Edman degradation;high-performance liquid chromatography; peptide sequencing; 3-phenyl-2-thiohydantoin (PTH) derivatives of amino acids.

Un procedimiento alternativo de secuenciación de péptidos para laboratorios con bajo presupuesto

Resumen

Se estandarizó un procedimiento de secuenciación amino-terminal de péptidos, utilizando el método químico manual de Edman, seguido de la separación por cromatografía líquida de alta resolución (HPLC) de los derivados obtenidos. Inicialmente, los derivados 3-fenilo-2-tiohidantoínas (PTH) de los veinte aminoácidos comunes fueron químicamente sintetizados y purificados en forma individual. A excepción de los derivados PTH-Met y PTH-Val, los cuales coeluyeron bajo todas las condiciones de gradiente empleadas, los dieciocho PTH-aminoácidos restantes se separaron por HPLC de fase reversa usando una columna C_{18}. El par PTH-Met/PTH-Val eluyó como un

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único pico de la columna, a un tiempo de retención diferente que los otros derivados PTH. Posteriormente, la estructura primaria de un heptapéptido sintético, el kémptido, fue verificada usando el procedimiento estándar de degradación de Edman, seguido de la separación por HPLC de los PTH-aminoácidos liberados en cada ciclo. La secuencia obtenida, Leu-Arg-Arg-Ala-Ser-Leu-Gly, correspondió a la secuencia publicada en la literatura. La metodología reportada aquí representa un procedimiento simple, sensible, reproducible, y barato, que provee una alternativa a la secuenciación de péptidos mediante secuenciadores automáticos o espectrómetros de masa.

Palabras clave: Cromatografía líquida de alta resolución; degradación de Edman; derivados 3-fenilo-2-tiohidantoínas (PTH) de los aminoácidos; secuenciación de péptidos.

Introduction

The Edman degradation sequentially removes one residue at a time from the amino end of an unblocked peptide (1). Phenyl isothiocyanate reacts with the uncharged terminal amino group of the peptide to form a phenylthiocarbamoyl derivative, which in acidic solution is cleaved to form a 2-anilino-5-thiazolinone derivative of the terminal amino acid, and the peptide chain shortened by one residue. The 2-anilino-5-thiazolinone compounds hydrolyze to give the phenylthiocarbamoyl amino acids (PTC-amino acid), which are converted by ring formation into the 3-phenyl-2-thiohydantoin (PTH)-amino acids (2). Various procedures, which differ in the composition of the coupling buffer solution, the extraction of by-products and 2-anilino-5-thiazolinones, or other details, have been used for the direct manual phenylisothiocyanate degradation (3-6).

The PTH-amino acids can be identified by several chromatographic procedures: thin layer chromatography (7, 8), high-performance liquid chromatography (HPLC) (9-19), or gas chromatography/mass spectrometry (20). The sensitivity of each of these analytical techniques ranges from nanomoles to fentomoles.

HPLC has been widely used in manual and automatic peptide sequencing, due to its high sensitivity and speed. A variety of reversed-phase resins containing octylsilane, octadecylsilane, phenylalkyl and cyanopropylsilane, have been employed for the analysis, by HPLC, of the PTH-derivatives with different elution buffers and numerous gradient systems (9-19).

Mass spectrometric sequencing methods are gaining increasing importance due to the suitability of novel ionization techniques like matrix-assisted laser desorption (MALDI) (21, 22), and electrospray ionization (ESI) (23, 24) for peptide and protein analysis. Tandem mass spectrometry, which requires two mass analyzers (MS-MS), also is used for obtaining sequence information for peptides and proteins (25). The molecular ion formed by ionization is selected in the first mass analyzer and reaches a collision cell, where it is fragmented by collision with argon atoms (collision-induced dissociation or CID). The fragment ions are separated according to their m/z ratio in the second mass analyzer and are registered in the corresponding mass spectrum. Depending on the peptide sequence, this spectrum contains a series of N-terminal, C-terminal, or internal fragment ions, formed by cleavage of bonds along the peptide backbone. In favorable cases it is possible to determine the entire sequence from the observed mass differences between corresponding mass peaks in the spectrum. The novel method of sequencing peptides by means of metastable ions in a time-of-flight mass spectrometer is quite promising (26). This technique is character-

ized by high sensitivity, allowing sequencing in the fentomole range.

In other approaches Edman degradation and mass spectrometry are combined. In order to determine the sequence of a peptide, either the mass of the cleaved PTH-amino acid (27) or of the remaining peptide (28) can be determined after each degradation step. Recently, Chait *et al.* (29) introduced the interesting method of ladder sequencing which consists of two steps: i) the chemical degradation of the protein into peptide fragments, each of which differ by one amino acid (generation of the "peptide ladder"), and ii) the readout of the sequence from the MALDI mass spectrum, which results from the mass differences between adjacent mass peaks. To generate the ladder, the stepwise Edman degradation with phenylisothiocyanate is performed in the presence of phenylisocyanate as the terminating reagent (ratio of phenylisothiocyanate to phenylisocyanate is ~ 95:5). The phenylcarbamoyl peptide (PC-peptide) is stable in acidic solution, whereas the phenylthiocarbamoyl peptide is degraded. Thus, in each cycle a small amount of peptide is blocked N-terminally by phenylisocyanate and protected from further Edman degradation. A defined number of degradation cycles are performed without isolating and analyzing the reaction products formed with phenylisocyanate. Only at the end is a MALDI spectrum of the whole mixture recorded. The spectrum contains the mass peaks of the individual phenylcarbamoyl peptides. The mass difference of adjacent mass peaks corresponds to one particular amino acid residue, and the order of their occurrence defines the sequence of the original peptide chain (30).

With the development of the sophisticated sequencing procedures described above, there is a pervasive feeling that the manual sequencing degradation method is becoming old-fashioned and superannuated. However, commercial automatic sequenators, as well as mass spectrometers, are very expensive instruments. In this work, we report the optimal separation of the PTH derivatives from eighteen of the twenty common amino acids, using HPLC on a C_{18} column. Although three pairs of PTH-amino acids (PTH-Glu/PTH-Gly, PTH-Met/PTH-Val, and PTH-Ile/PTH-Phe) coeluted in our initial chromatogram of the mixture, the use of different gradient conditions permitted the resolution of PTH-Gly from PTH-Glu, and the resolution of PTH-Phe from PTH-Ile. The pair PTH-Met/PTH-Val was not separated under any of the chromatographic conditions assayed, but it coeluted from the column at a different retention time than any of the other eighteen PTH-amino acids. The standard Edman degradation procedure followed by the HPLC method described here was subsequently applied to determine the total primary structure of a commercially available synthetic peptide, kemptide. The sequencing procedure employed in this work represents a valid and valuable alternative for low budget laboratories that do not own an automatic sequenator or a mass spectrometer.

Methods

Materials

Reagents were purchased from the following sources: the 20 common L-amino acids, kemptide, PTH-carboxymethyl cysteine, triethylamine, trifluoroacetic acid (TFA, sequanal grade), Sigma; acetonitrile (HPLC grade), Mallinckrodt; pyridine (HPLC grade), J. T. Baker; phenyl isothiocyanate (PITC), Pierce. All other reagents were analytical grade. Triethylamine was distilled three consecutive times before use.

Synthesis of PTH-amino acids

PTH-amino acids were synthesized using 5×10^{-5} mol of each amino acid, and a slight excess (6.5×10^{-5} mol) of PITC. The mixture contained ethanol:triethylamine:water:PITC in the proportions 7:1:1:1 (V/V). All reactions were carried out in 1 mL vials under inert atmosphere. Initially, the

reagents were incubated at room temperature for 20 min. After incubation at 50°C for 30 min, the products were dried at 50°C, under vacuum. TFA (200 µL) was added and the mixtures were further incubated for 5 min at 50°C. Then, TFA was removed at 60°C for 10 min, under vacuum. The resulting compounds were finally incubated with 200 µL of aqueous TFA (20%), for 10 min at 60°C. The products, which corresponded to the crude PTH-amino acid derivatives, were dried under high vacuum for 5 min.

Purification by HPLC of crude PTH-amino acids

Crude PTH-amino acids were purified by reversed-phase HPLC on an LKB Bromma system with a Super Pac Spherisorb ODS C_{18} column (25 µm, 4 x 250 mm, Pharmacia), or a LiChrospher 100 RP-8 (5 µm, Merck) C_8 column. The following buffers were employed: in reservoir A, 10 mM sodium acetate, pH 4.0 : acetonitrile (9:1 V/V); and in reservoir B, 10 mM sodium acetate, pH 4,0 : acetonitrile (1:9 V/V). Absorbance was monitored at 269 nm on a Bio-Rad spectrophotometer equipped with a flow-through cell. In all cases, the flow rate was 1 mL/min and the columns were equilibrated in 6% B prior to the isolation procedure. The PTH derivatives of Ile, Met, Pro, Phe, Trp, Tyr, Lys, Asp, Gln, Leu and Val were purified using the C_8 column with a 15-min linear gradient from 6 to 80% B, followed by a 5-min linear gradient from 80 to 100% B. PTH-Glu and PTH-Asn were purified using the C_{18} column with a 30-min linear gradient from 6 to 80% B, followed by a 5-min linear gradient from 80 to 100% B. The PTH derivatives of Arg, His, Ala, Gly, Ser, Thr and Cys were purified using the C_8 column with a 15-min linear gradient from 6 to 80% B, and a 10-min linear gradient from 80 to 100% B.

PTH-Ser, PTH-Thr and PTH-Cys, obtained by the synthesis procedure described above, were partially decomposed. Therefore, the three compounds were resynthesized according to the method described by Ingram (31). PTH-Ser and PTH-Thr were recrystallised twice and three times in ethanol, respectively. PTH-Cys was recrystallised twice in a mixture of ethanol:isopropanol:petroleum ether in the proportions 6:1:4 V/V. The identity of the PTH derivatives of Ser, Thr and Cys was verified by Fourier transformed infrared (FTIR) spectroscopy, ^1H nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography/mass spectrometry (GC-MS).

HPLC separation of PTH-amino acids

A mixture containing the PTH derivatives of the twenty common amino acids and of carboxymethyl Cys (~3 nmol of each) was separated by HPLC on a Super Pac Spherisorb ODS C_{18} column (25 µm, 4 x 250 mm, Pharmacia), using a modification of the procedure described by Downing and Mann (32). The solutions used were the following: in reservoir A, 10 mM sodium acetate pH 4.0:acetonitrile (9:1 V/V); and in reservoir B, 10 mM sodium acetate pH 4,0:acetonitrile (1:9 V/V). Optical density was monitored at 269 nm. The column was equilibrated in 6% B before starting the separation procedure, and the flow rate was 1 mL/min during the whole chromatogram. The PTH-amino acids were eluted with a 9-min linear gradient from 6 to 30% B, a 5-min linear gradient from 30 to 35% B, and a 16-min linear gradient from 35 to 43% B.

HPLC runs, under similar conditions, were performed with each PTH-amino acid in order to relate their individual elution times with the ones obtained in the chromatogram of the mixture. Under this condition, three pairs of PTH-amino acids coeluted: 1) PTH-Glu/PTH-Gly, 2) PTH-Met/PTH-Val, and 3) PTH-Ile/PTH-Phe. With the exception of the pair PTH-Met/PTH-Val, the other two pairs of PTH derivatives were successfully separated by a 15-min linear gradient from 6 to 80% B, using the same C_{18} column.

Peptide sequencing

Kemptide (30 nmol) was reacted with 10 µl of PITC, in a mixture containing ethanol:water:triethylamine:PITC in a ratio 7:1:1:1 (V/V). All the steps were performed under helium gas in 1 mL vials. The reagents were incubated at 50°C with occasional agitation. After 30 min, the vial was placed in a –20°C freezer for 5 min, and 1 mL of cold acetone was added. The reaction mixture was vortexed vigorously and centrifuged at 12,000 g. The pellet contained the phenylthiocarbamoyl derivative of the peptide, and the supernatant was discarded. This washing procedure was repeated twice. The resultant product was dried at 50°C, under vacuum, for 5 min. Immediately, 150 µL of TFA were added and the mixtures were further incubated for 3 min at 50°C. TFA was removed at 50°C for 5 min, under vacuum. Liquid partition was then applied to separate the 2-anilino-5-thiazolinone derivative of the subtracted amino acid (ATZ-amino acid) from the remaining peptide. The reaction mixture vial was placed on ice and cold 5% pyridine (100 µL) was added. The ATZ-amino acid was extracted three consecutive times with 200 µL of cold butyl acetate (saturated with water). After centrifugation at 12,000 g for 0.5 min, two phases were separated: the organic phase containing the ATZ-amino acid, and the aqueous phase holding the shortened peptide (n-1 peptide). The ATZ-amino acid was treated with 75 µL of aqueous TFA (20%), and incubated for 10 min at 60°C. The PTH-amino acid derivative formed was dried under high vacuum for 5 min. The n-1 peptide was then used for the next cycle of the Edman degradation analysis.

The PTH-amino acids obtained in each manual sequencing step were separated by HPLC as described in the previous section, and their identity was determined by comparison with the elution times observed for the mixture of standard PTH-amino acids. The last Edman degradation cycle of kemptide eluted at the corresponding retention time for the pair PTH-Glu/PTH-Gly. We were able to, unequivocally, identify the PTH-amino acid in this step cycle of kemptide, by reversed-phase HPLC, using the same C_{18} column with a 15-min linear gradient from 6 to 80% B (See previous section).

Results and Discussion

Synthesis and purification of PTH-amino acids

The PTH derivatives of the twenty common amino acids were individually synthesized using the Edman chemistry. Each crude PTH-amino acid was then purified chromatographically by reversed-phase HPLC. Examples of the chromatograms obtained during the purifications of these compounds are shown in Figure 1 for PTH-Trp, PTH-Lys and PTH-Arg. Common contaminant peaks were detected among the HPLC profiles when the column and gradient conditions used were the same.

As can be seen in Figure 2 (Panel A), the chemical reaction to produce crude PTH-Ser, PTH-Thr and PTH-Cys using the Edman methodology, resulted in a partial decomposition of the compounds of interest and the formation of sub-products, especially in the case of PTH-Cys. Furthermore, our attempts to isolate the PTH derivatives of Ser, Thr and Cys from the rest of the reagents present in the reaction mixture resulted in an almost intolerable increase in the HPLC column pressure, probably due to the polymerization of some of the decomposed compounds that were produced. Therefore, the PTH derivatives of Ser, Thr and Cys were resynthesized using a different method (31). Figure 2 (Panel B) illustrates the HPLC purification profiles for the PTH-Ser, PTH-Thr and PTH-Cys that were produced using the Ingram chemistry followed by recrystallisation. The identity of PTH-Ser, PTH-Thr and PTH-Cys was confirmed by FTIR spectroscopy, ^{1}H NMR spectroscopy, and GC-MS (Data not shown).

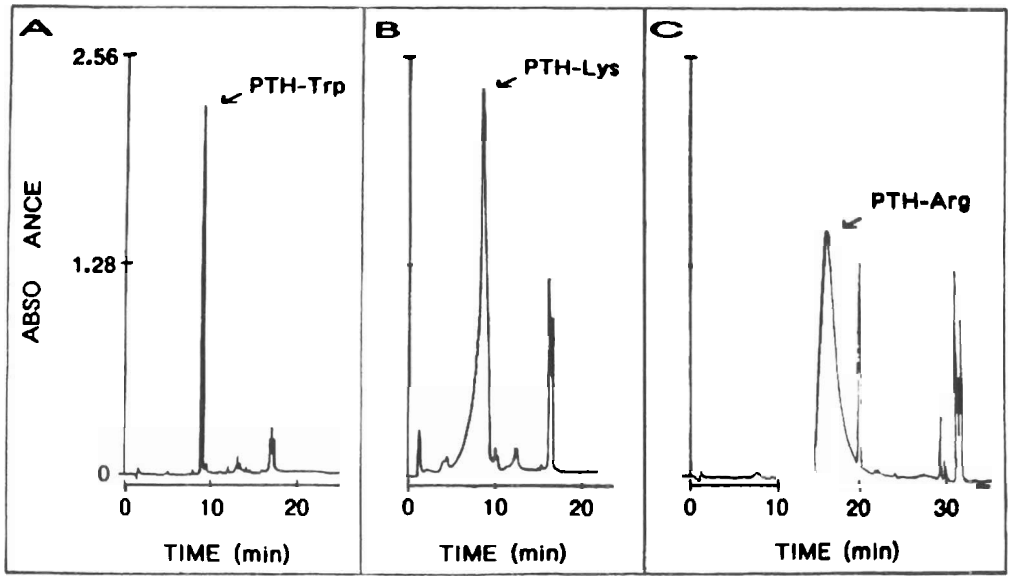

Figure 1. **Purification of crude PTH-Trp, PTH-Lys and PTH-Arg.** The synthesized PTH-amino acids were isolated by reversed-phase HPLC using a C_8 column. The buffers employed were in A, 10 mM sodium acetate, pH 4.0:CH_3CN (9:1 V/V), and in B, 10 mM sodium acetate, pH 4.0:CH_3CN (1:9 V/V). PTH-Trp (Panel A) and PTH-Lys (Panel B) were purified with a 15-min linear gradient from 6 to 80% B, followed by a 5-min linear gradient from 80 to 100%. PTH-Arg (Panel C) was purified with a 30-min linear gradient from 6 to 80%, followed by a 5-min linear gradient from 80 to 100%. The optical density was monitored at 269 nm.

All the synthesized and purified PTH-amino acids presented an absorbance maximum peak at an average wavelength of 269-270 nm. (Data not shown).

HPLC separation of PTH-amino acids

Reversed-phase HPLC runs, on a C_{18} column, were performed with each synthesized PTH-amino acid, in order to determine their individual elution times using the conditions described in **Methods** (Data not shown). A concentration dependence study on the limit of sensitivity of this method indicated that ~10 pmol of each PTH-amino acid was the minimum amount that could be detected at 269 nm (Data not shown). A mixture containing PTH-carboxymethyl Cys and the PTH derivatives of all twenty common amino acids was then separated under these same conditions (Figure 3). The identity of each peak was determined by comparing their elution times with the ones obtained in the individual runs.

As can be seen in Figure 3, three pairs of PTH-amino acids coeluted in the chromatogram of the mixture: 1) PTH-Glu/PTH-Gly, 2) PTH-Met/PTH-Val, and 3) PTH-Ile/PTH-Phe. We were able to separate the PTH derivatives of Gly and Glu, and those of Phe and Ile, by using a 15-min linear gradient from 6 to 80% B (Figure 4). No further improvement of the resolution of the pair PTH-Met/PTH-Val was obtained by this procedure (Data not shown). Various gradient conditions, using the same buffer system, were assayed in order to resolve the Met and Val derivatives, but no positive results were obtained (Data not shown). However, the pair PTH-Met and PTH-Val was found to elute at a different retention time than the eighteen remaining PTH derivatives (Figure 3). Thus, the method described here al-

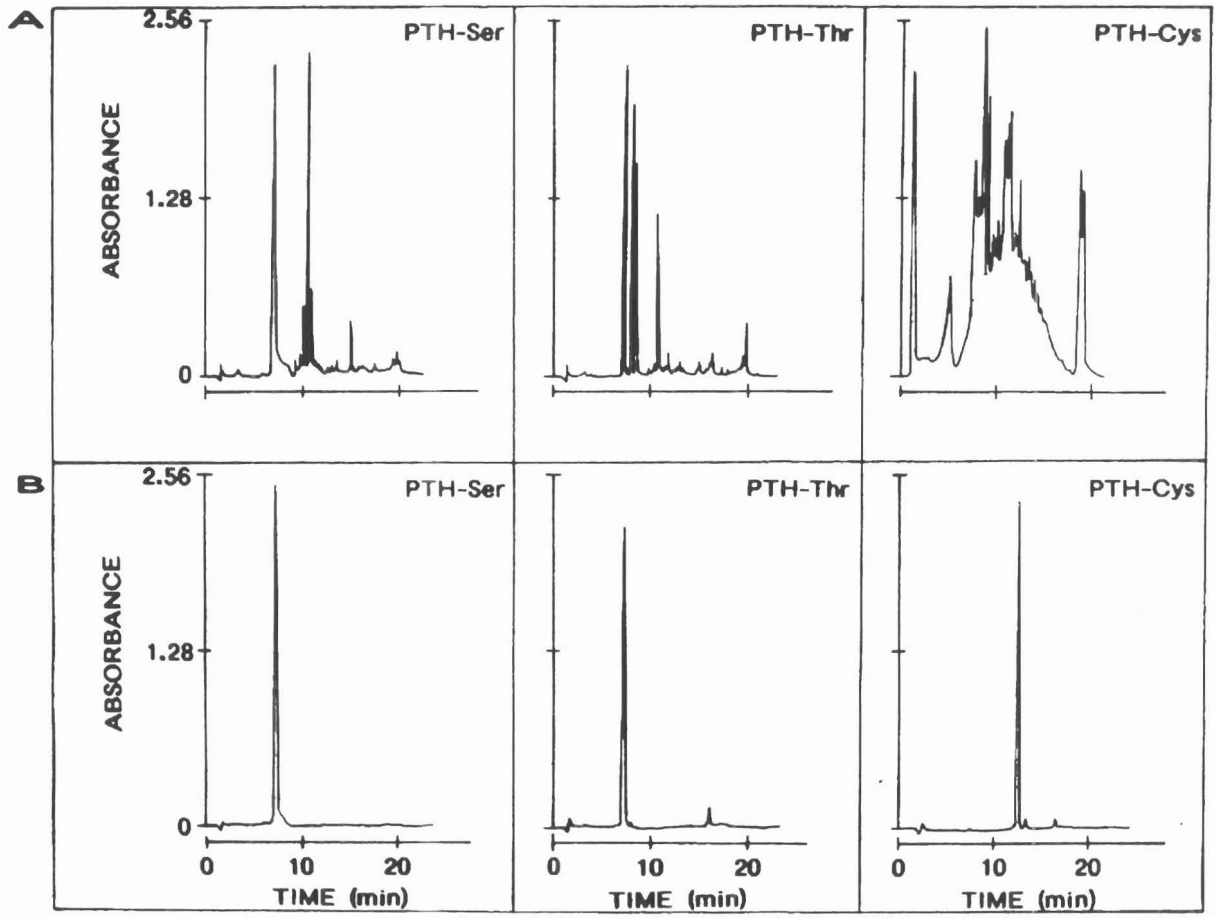

Figure 2. **Purification of synthesized PTH-Ser, PTH-Thr and PTH-Cys.** *Panel A*, HPLC purification of crude PTH-Ser, PTH-Thr and PTH-Cys prepared using Edman chemistry. *Panel B*, HPLC isolation of recrystallised PTH-Ser, PTH-Thr and PTH-Cys that were synthesized according to Ingram (1953). PTH-amino acids were chromatographed on HPLC using the procedure described for PTH-Arg in Figure. 1.

lowed the successful separation of eighteen of the twenty PTH-amino acids.

It is evident, from the published data, that the separation of the PTH derivatives of Met and Val is difficult (13, 15, 32). However, it has been reported that the use of other types of HPLC columns, like cyanopropylsilane (17), or phenylalkyl (16); as well as other solvents in the HPLC reservoirs (10, 14, 18) allowed the resolution of the pair PTH-Met/PTH-Val. In this work, all the chromatographic separations of the compounds tested were performed with a reversed-phase C_{18} column, at room temperature, using the same solvent composition in the HPLC reservoirs (See **Methods**). The elution pattern of the PTH-amino acids also may be influenced by changes in pH and temperature, variations in the ionic strength of the buffer and in the flow rate. The effect of some of these factors on the resolution of the pair PTH-Met/PTH-Val should be investigated and requires further studies.

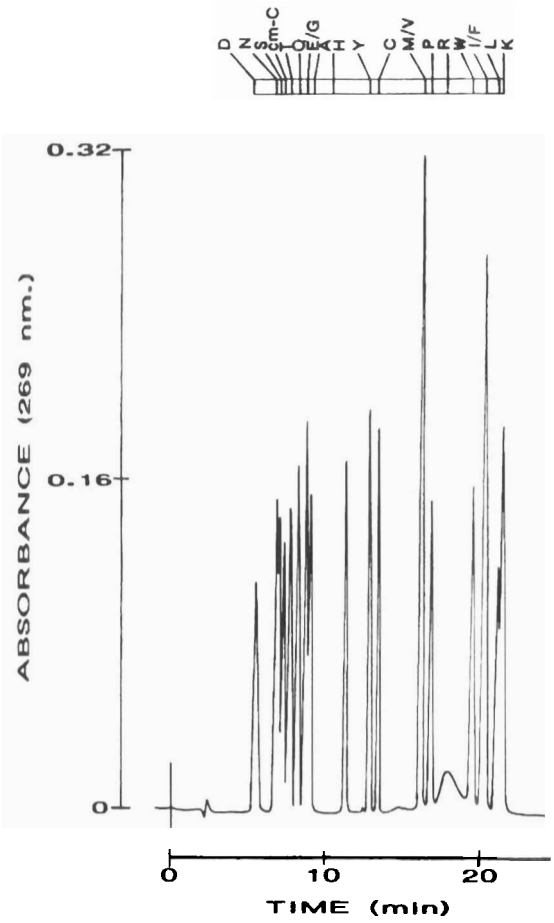

Figure 3. **HPLC separation of PTH-amino acids.** A mixture containing PTH-carboxymethyl Cys and the PTH derivatives for the twenty common amino acids (~3 nmol of each) was separated by HPLC on a C_{18} column. The buffers employed were in A, 10 mM sodium acetate, pH 4.0:CH_3CN (9:1 V/V), and in B, 10 mM sodium acetate, pH 4.0:CH_3CN (1:9 V/V). The compounds were eluted with a 9-min linear gradient from 6 to 30% B, a 5-min linear gradient from 30 to 35% B, and a 16-min linear gradient from 35 to 43% B. The peak for each PTH derivative is labeled with the one-letter abbreviation for that amino acid. PTH-carboxymethyl Cys = cm-C.

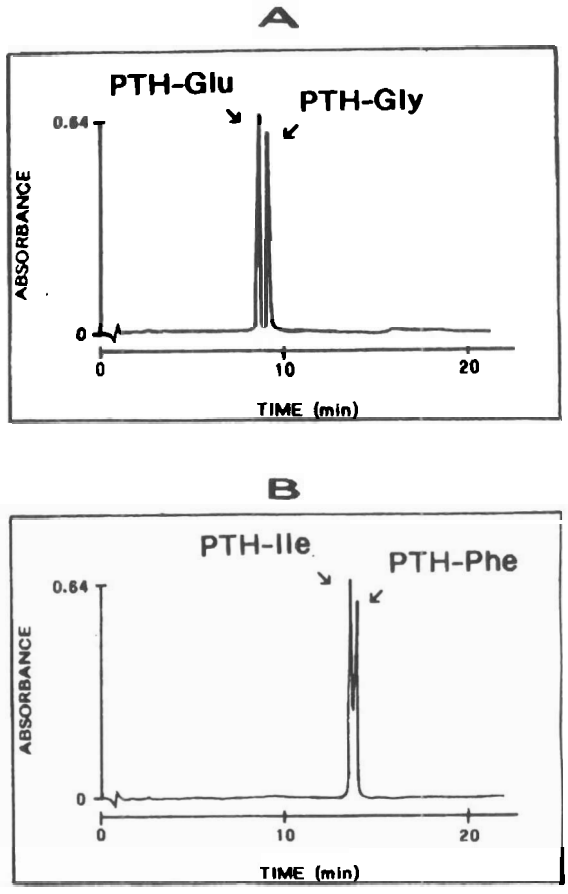

Figure 4. **Separation by HPLC of the pairs PTH-Glu/PTH-Gly and PTH-Ile/PTH-Phe.** The PTH derivatives of Glu and Gly, as well as those for Ile and Phe coeluted under the conditions shown in Figure. 3. Both pairs were clearly separated by rechromatography on HPLC, using a 15-min linear gradient from 6 to 80% B with a C_{18} column. *Panel A,* pair PTH-Glu/PTH-Gly. *Panel B,* pair PTH-Ile/PTH-Phe.

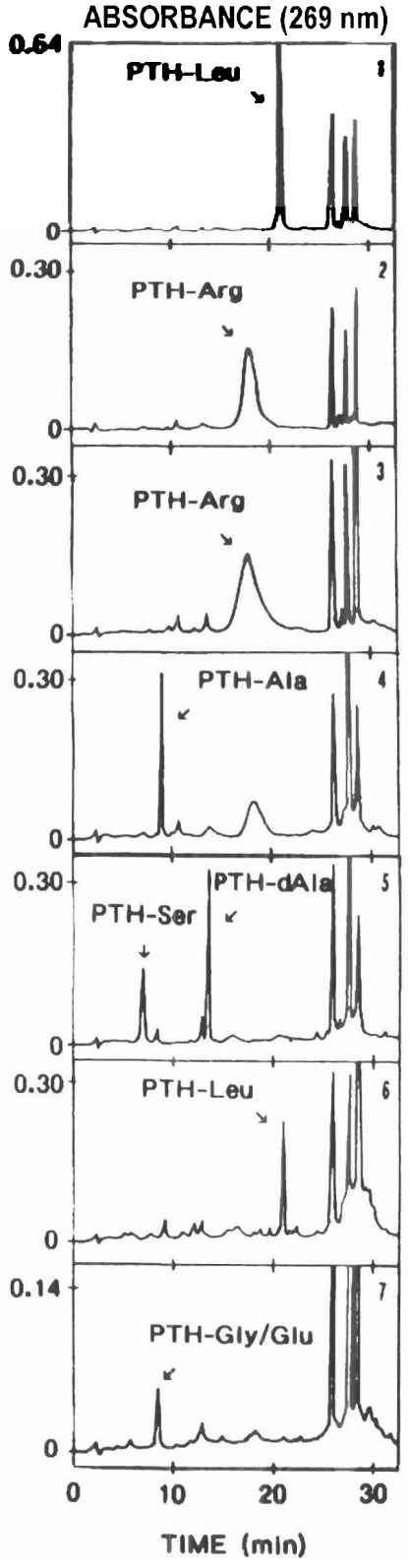

Figure 5. **Sequencing of kemptide.** Kemptide (30 nmol) was manually sequenced and the PTH-amino acid obtained at every step was chromatoghaphed by HPLC, as described in Figure. 3. At the top right corner of each panel is shown the number of the corresponding degradation cycle. The elution time for every peak was compared to a standard chromatogram similar to the one shown in Figure 3. PTH-dALA = PTH-dehydro Ala.

Peptide sequencing

Kemptide was subjected to manual sequencing using the standard Edman degradation procedure. The PTH-amino acid obtained after each reaction cycle was then isolated by HPLC, under our optimal separation conditions described in **Methods**. Figure 5 shows the chromatographs for each degradation step of the phosphate acceptor heptapeptide. The amino acid sequence obtained for kemptide was: Leu-Arg-Arg-Ala-Ser-Leu-Gly/Glu. In step 5, we observed the appearance of PTH-dehydroalanine (PTH-DAla), a common decomposition by-product of PTH-Ser. In order to discriminate between PTH-Gly and PTH-Glu in step 7, the PTH derivative obtained in this cycle was further separated by HPLC using a 15-min linear gradient from 6 to 80% B as described in **Methods**. This procedure, unequivocally, identified step 7 of kemptide as Gly (Data not shown). As expected, the sequence obtained for kemptide corresponded to its published primary structure.

The peptide sequencing procedure reported in this manuscript illustrates an alternative methodology that is simple, rapid, sensitive, very reproducible, and less costly than automated analysis. Even though, automation confers a higher cycle-to-cycle consistency and circumvents the problems of sample loss during repeated cycles of chemistry, commercial sequenator instruments are extremely expensive. Mass spectrometric sequencing methods also are

gaining increasing significance. However, mass spectrometers are very costly and luxurious devices. The manual approach presented here has the advantage of requiring a lower investment and operating costs than any commercial automatic sequenator or mass spectrometer available. The other virtue of this method is the personal involvement of the investigator with the material to be sequenced. Thus, this procedure represents an alternative for low budget laboratories without an automatic sequenator or a mass spectrometer.

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