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Separation of phenylthiohydantoin–amino acids by temperature-controlled reversed-phase high-performance liquid chromatography

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Abstract

A unique, but general, separation method of phenylthiohydantoin (PTH)–amino acids has been devised by using a temperature-controlled gradient-elution mode of reversed-phase high-performance liquid chromatography. The gradient was established from two solvents (A* and B*) which consisted of an aqueous acidic phosphate buffer solution (pH 2.1, 0.1 *M*) and alcoholic organic modifiers, which afforded stable analysis. The column used was Develosil ODS UG-5. The addition of acetonitrile to solvent B* and running the column at a higher temperature was essential for separating PTH–valine and 1,3-diphenyl-2-thiourea. It was found that separation of the early-eluting solvophilic amino acids was more efficient at lower column temperatures, but that a higher temperature was required for separating PTH–methionine from PTH–valine, using a 60 Å pore diameter ODS silica as model gel. Therefore, temperature control from 35 to 60°C was introduced. Since this separation method is reproducible, convenient, and quantitative, it was applied to the yield analysis of bovine β-lactoglobulin and several peptides after covalent bonding to glass fiber disks.

Keywords: Gradient elution; Amino acids, PTH derivatives; Amino acids

1. Introduction

Liberation from the influences of the silanol groups of the silica gel support [1] and the ionization of acidic solutes [2] is important in high-recovery and stable chromatographic analysis with reversed-phase high-performance liquid chromatography (RP-HPLC) column. Therefore, stable analytical methods for peptides [3] and proteins [4,5] using gradient-mode RP-HPLC were developed using acidic phosphate buffer and alcohols; i.e., sufficient ionic strength of more than 0.05 *M* combined with suppression of ionization at high proton concentration of pH 2.1.

In protein sequencing it is essential to precisely determine the phenylthiohydantoin (PTH)–amino acids after Edman degradation [6]. RP-HPLC has been used as an important separation method of PTH–amino acids [7]. However, the influence of the silanol groups on separation of PTH–amino acids with RP-HPLC remains, although reports on separation of it [8–15] exist. In order to minimize this effect, we previously proposed an isocratic separation of PTH–amino acids using aqueous acidic trifluoroacetic acid (TFA) solution (pH 1.9) [16]. Although this TFA method is stable and reproducible, we recently discovered that improved recovery of sulphur containing compounds, such as thioctic acid (lipoic acid), from the reversed-phase ODS column is achievable only when the eluent is an

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acidic phosphate buffer (pH 2.1) and a short 30 mm column is used instead of aqueous TFA eluent [17]. Thus, TFA method with a 25 cm ODS column seemed not to be suitable for sulphur containing amino acids. Furthermore, recent advances in the computer-aided HPLC gradient system affords reliabilty and convenience. Therefore, we decided to develop a PTH–amino acid separation method using the gradient system with acidic phosphate buffer.

The effect of column temperature on the separation of PTH–amino acids with acidic phosphate buffer condition was first tested using a 60 Å pore diameter ODS column, since we had previously observed that when using Nucleosil 5 C_{18}, higher temperatures were necessary for separating PTH–valine from PTH–methionine [16]. It was found that separation of solvophilic early-eluting amino acids was better at low temperatures, while higher temperatures were required for separating glutamic acid and alanine, and methionine and valine. Thus, we developed a convenient, stable, and reproducible system for determining twenty-one PTH–amino acids using a reversed-phase support of Develosil ODS UG-5 and a temperature-controlled programme.

2. Experimental

2.1. Chemicals and reagents

PTH–amino acids in a mixed standard set containing 20 PTH–amino acids [Table 2, except *S*-(4-pyridyl-ethyl)cysteine (PeC)], the powder-form of each 20 PTH–amino acids, *n*-heptane (amino acid sequencing grade), pyridine (amino acid sequencing grade), phenyl isothiocyanate (PITC; amino acid sequencing grade), acetonitrile (HPLC grade), tetrahydrofuran (HPLC grade), and other highly pure substances such as ethyl acetate, 2-propanol (99.5%), methanol (99.8%), orthophosphoric acid (85%, w/w), sodium dihydrogenphosphate dihydrate, trimethyl amine, triethylamine, *p*-phenylene diisothiocyanate (DITC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), dimethylformamide (DMF), acetone, 2-aminoethanol, 1,3-diphenyl-2-thiourea (DPTU), tri-*n*-butylphosphine, chloroform, and glass fiber disk (TFA treated; 12 mm diameter) were obtained from Wako (Osaka, Japan). The Develosil ODS UG-5 (250×4.6 mm I.D.; Mfg.No. 0907743; carbon content 17.6%; monomeric-type; pore diameter 140 Å) column, Develosil ODS HG-5 (250×4.6 mm I.D.; carbon content 18%; polymeric-type) column, and Develosil ODS UG-3 (3 μm particle diameter; 30×4.6 mm I.D.) were obtained from Nomura (Aichi, Japan). 3-Aminopropyltriethoxy-silane was from Shin-Etsu Chemical, Tokyo, Japan. ODS-silica-gel (60 Å mean pore diameter, carbon content 20.4%; SP-60-5-ODS-AP; Lot. 50954) was kindly donated from Daiso, Hyogo, Japan. PeC, β-casein and dephosphorylated β-casein (from bovine milk: relative molecular mass M_r=24 000), Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg (an active fragment of myelin basic protein), and Gly–Arg–Gly–Leu–Ser–Leu–Ser–Arg (a substrate for cAMP dependent protein kinase) were purchased from Sigma (St.Louis, MO, USA). PTH–pyridylethylcysteine (PTH–PeC) was synthesized from PeC using an automated protein sequencer. Bovine β-lactoglobulin (M_r=18 400), which was a gift from Dr. Kunio Yamauchi, Honorary Professor of the University of Tokyo, was purified from cows' milk according to the procedure described previously [18].

2.2. Instruments for Edman reaction

Direct Edman degradation of protein was performed using two protein sequencer-models 471A and 473A (Perkin-Elmer, Foster City, CA, USA). PTH–amino acids produced were fractioned by fraction collectors, and stored at −20°C until analysis. Volumes of each fraction were 0.125 ml for model 473A and 0.25 ml for model 471A, respectively, using tentative degradation programmes.

Since yields of Thr and Ser were considered to be low when using TFA as a cutting reagent as compared to HCl [19], we also used HCl as a cutting reagent.

2.3. Instruments for PTH–amino acid analysis

The chromatograph assembly (Shimadzu, Kyoto, Japan) consisted of two pumps (model LC-10AD; with auto-washing kit), a system controller (model SCL-10A), an injector (model U6K, Waters, Milford,

MA, USA), and a column oven/cooler (air-stirring type; model CTO-10AC, Shimadzu). For detection, an Hitachi Model L-4000 UV Detector was used at 269 nm; the data processor was Shimadzu Model C-R6A. The sample loop of U6K was 2 ml, and the volume of the mixing chamber SUS (Shimadzu) was 0.5 ml. Although, the combination of a sample loop of 0.05 ml together with a mixing chamber of 2.6 ml also worked well, we used former combination in this work. A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the separation column of Develosil ODS UG-5. The column oven/cooler used (CTO-10AC; Serial No. C20383406701KL; with 550 VA or W) showed the ability of temperature control to within 0.2°C at adjusted temperature of 50°C tested at a room temperature of 18°C.

Standard PTH–amino acids were dissolved in methanol at a concentration of 1 mg/ml. PTH–amino acid mixed standard (20 nmol each) was dissolved in 2 ml of methanol (10 nmol/ml). These standards were stored at −20°C. Immediately before use, they were diluted with distilled water (methanol concentration less than 10%, v/v), or by aqueous acetonitrile solution (18%, v/v solution).

2.4. Gradient separation of PTH–amino acids

Gradient separation of the 21 amino acids was performed according to the gradient programme detailed in Table 1.

2.4.1. Stock solutions

First, 0.1 *M* sodium phosphate buffer (pH 2.1) was made as follows; 78.0 g of sodium dihydrogenphosphate dihydrate and 35 ml of 85% orthophosphoric acid (w/w) were added and mixed to a volume of 5 l in distilled water, and pH adjusted to 2.1. Solvent A consisted of 0.1 *M* sodium phosphate buffer (pH 2.1)–methanol (90:10, v/v). Solvent B was made as follows; one liter of 0.1 *M* sodium phosphate buffer (pH 2.1)–methanol (20:80, v/v) was prepared, and to this mixture 3 ml of 2-propanol and 20 ml of 85% orthophosphoric acid was added (finally apparent pH of 2.7 was observed with a usual glass-electrode). These two solutions were stored at room temperature.

Table 1
The elution programme for PTH–amino acid analysis[a]

Step	Time	Function	Value
0	0.01	B.conc	0.5%
1	0.01	B.curv	0
2	14.00	B.conc	7.0%
3	14.00	B.curv	1
4	14.01	B.conc	17.0%
5	14.01	B.curv	−5
6	14.02	Oven T.	60 C
7	14.50	B.conc	29.0%
8	14.50	B.curv	−2
9	14.60	B.conc	34.5%
10	14.60	B.curv	−1
11	26.00	Oven T.	35 C
12	28.90	B.conc	35.0%
13	28.90	B.curv	−2
14	29.00	B.conc	44.0%
15	29.00	B.curv	1
16	40.00	B.conc	50.0%
17	40.01	B.conc	100.0%
18	45.00	B.conc	100.0%
19	45.01	B.conc	0.5%
20	45.02	Stop	

[a] Initial condition: T.flow (total flow), 1.000 ml/min. B.conc (concentration % of Running solvent B*), 0.5%. Oven T.(oven temperature), 35°C. Column was Develosil ODS UG-5. Curve types by number (B.curv; shape of gradient curve) were according to the description of the manufacturer; i.e., 0 was a linear gradient, from −10 to 10. Eluent: Eluents of Running solvent A* and Running solvent B* were as described in the Experimental (section 2.4.2). Time; min. Stop; stop of time-programme or return to the initial condition.

2.4.2. Running solutions

Running solvent A* was a mixture of solvent A and solvent B; solvent A:solvent B=83:17 (v/v). Running solvent B* was a mixture of solvent B–acetonitrile–tetrahydrofuran–orthophosphric acid (1000:100:10:15, v/v). Returning to the initial condition required minimum of 53 min, after which injection of the sample was possible; i.e., one cycle of determination required 53 min. Flow-rate was 1.0 ml/min, and the column inlet-pressure was 11.5–19.0 MPa (1664–2760 p.s.i. or 117–194 kg/cm^2). Degassing by helium gas was not necessary due to prior mixing of solvents. The chromatogram was recorded on a Shimadzu C-R6A, and chromatographic background due to gradient elution was subtracted by C-R6A (base line corrected).

2.5. Effect of column-temperature on the separtion of PTH–amino acids using 60 Å ODS column

Daiso ODS silica gel (60 Å pore diameter, 5 μm particle diameter) was packed into a stainless steel tube (150×2.0 mm I.D.) according to the procedure described previously [20]. The methanol concentration in acidic phosphate buffer was linearly increased from 23.6% (v/v) to 52–58% (v/v) over 20–30 min, at a flow-rate of 0.2–0.23 ml/min and at fixed temperatures of 4, 20, 35, 47, 60, and 70°C. Column temperature was manually controlled by a water-bath (Gebrüder Haake, Germany). The effect of temperature gradient from 4 to 85°C was also tested as shown in Fig. 1. In these tests, baseline correction was not performed.

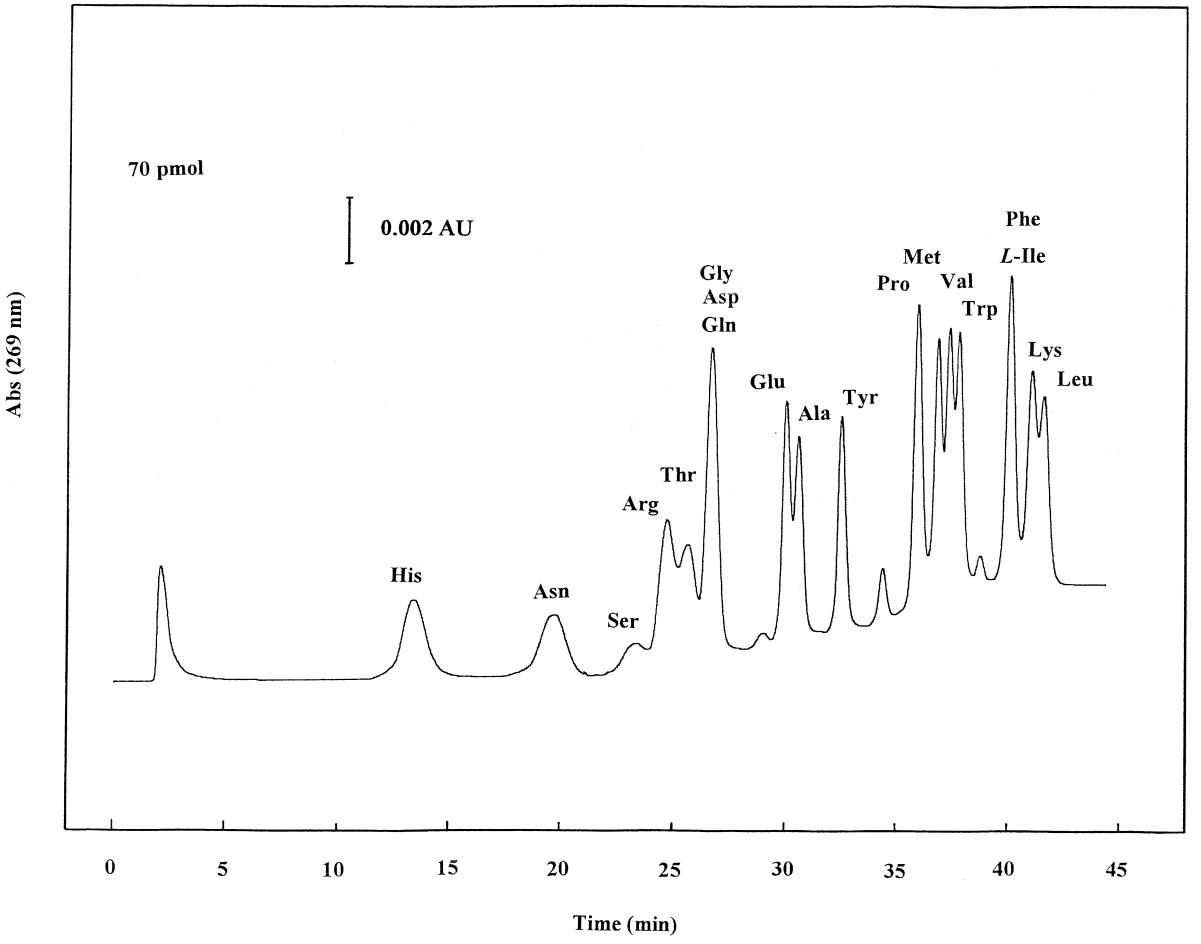

Fig. 1. Typical separation trial of PTH–amino acid with 60 Å pore diameter ODS column using temperature gradient. 70 pmol of PTH–amino acid mixed standard were injected, and amino acids were detected at UV 269 nm. Initially temperature of the water-bath was 4°C. After 20 min, the temperature of the water-bath was adjusted to 70°C. And it was adjusted to 85°C at 30 min. A linear gradient was applied; the initial eluent being the mixture of 0.1 *M* sodium phosphate buffer (pH 2.1)–methanol (76.4:23.6, v/v) and final eluent being the mixture of 0.1 *M* sodium phosphate buffer (pH 2.1)–methanol (47:53, v/v). The gradient was terminated after 20 min, and the terminal condition maintained for 30 min. The flow-rate was 0.2 ml/min. Other conditions were as described in the Experimental (section 2.5). The chromatogram was recorded on a Shimadzu C-R6A, and chromatographic background due to gradient elution was not subtracted.

2.6. Attachment of proteins and peptides to the glass fiber disks

Some proteins and peptides were immobilized to glass fiber disks according to procedures described by Machleidt et al. (DITC method) [21] and Salnikow et al. (EDC method) [22] with some modifications. Proteins and peptides were dissolved in serum dilution solution [23] at 3.0 mg/ml and peptides in serum dilution solution without addition of EDTA at 1.0 mg/ml. Dephosphorylated β-casein was dissolved in an aqueous–organic solvent mixture: ethanol–2-propanol–distilled water–orthophosphoric acid (16:16:67:1, v/v). A 0.07 ml volume was used to bind onto two or three glass fiber disks.

2.7. Preparation of phenylthiocarbamyl (PTC)–cystine

PTC–cystine was prepared according to the method of Bidlingmeyer et al. [24].

3. Results and discussion

3.1. Effect of column temperature

Since the alcohol acidic phosphate buffer system of Mönch and Dehnen [3–5] as the eluent affords stable and reproducible results, we chose this eluent system. In order to clarify the separation characteristics of phenylthiohydantoin (PTH)–amino acid, the effect of temperature was first tested using a manually packed 60 Å pore diameter ODS column as a model gel support. The result suggested that the temperatures greater than or equal to 47°C were necessary to separate Glu and Ala, and Met and Val (data not shown). However, lower temperatures were effective in separating the early eluting amino acids such as His, Asn and Ser (data not shown). Therefore, the use of temperature gradient was effective in separating PTH–amino acid with a 60 Å pore diameter ODS column as shown in Fig. 1. From this qualitative result of 60 Å pore diameter ODS column with a high carbon content (20.4%; polymeric type), we next tried a lower carbon content (17.6%) monomeric type column in order to distinguish between Gly, Asp, and Gln and between Phe and Ile; i.e., the Develosil ODS UG-5 column.

3.2. Separation of PTH–amino acids without using acetonitrile

Although we separated Ser, Arg, Thr, Gly, Asp and Gln, and Phe and Ile using a Devolosil ODS UG-5 column at 35°C, another isocratic condition was required to separate Val and 1,3-diphenyl-2-thiourea (DPTU) (data not shown).

When retention times were compared between the 100 m*M* and 200 m*M* phosphate buffer, retention times of Arg, Gln, and PeC were longer when the 200 m*M* buffer was used as compared to the 100 m*M* (data not shown). The result indicates that a mechanism of salting-out or the solvophobic effect [2] is at work in the acidic phosphate buffer eluent system.

With respect to the effects of pore diameter, narrower pore diameter silica gels seem to require eluents with higher ionic strength and drastic temperature differences (Fig. 1). This may possibly be due to the predominant effect of the silanol groups of silica gel [1]. However, it has also been found that the use of the narrower pore-size silica gel is important in elucidating the separation mechanisms of various compounds. Thus, we recently found that a previously developed high-performance gel-permeation chromatographic (HPGPC) serum protein assay using 100 Å pore diameter Diol gel [23] was also applicable to the protein determination of pancreatic juice using a narrower 50Å pore diameter Diol gel and higher ionic strength acidic eluent containing an organic modifier at low temperature [25]. Since the separation of PTH–amino acids with acidic phosphate buffer system using the polymeric-type ODS gel (Develosil ODS HG-5) has not been successful, it seems that the monomeric-type structure of ODS gel (Develosil ODS UG-5) may provide the answer.

3.3. Temperature controlled single-run method

It was found that acetonitrile as an organic modifier together with a temperature gradient is effective

in separating Val and DPTU using Develosil ODS UG-5 columns as shown in Fig. 2, with programme detailed in Table 1.

Contamination of Running solvent A* with acetonitrile prevented separation among Asp, Gln and PeC (data not shown). In such a situation, only exchange to new Running solvent A* and washing the pump is sufficient to recover. In Fig. 3, the pattern of measured column temperature together with a gradient curve (Table 1) is shown. As expected, this method was reproducible (Table 2) and quantitative (Table 3). Since retention times of PTH–amino acids are stable, their peak heights are measurable using within-day RSDs for most of amino acids within a 1.73–4.54% range, except for delta T and Glu. This may be due to the lability of these two amino acids during storage in aqueous 20% acetonitrile solution at −20°C. Day-to-day RSDs for most of amino acids were within a 0.46–4.86% range. The relatively higher RSD for Leu is due to a separation problem, and improved separation is expected to give a lower RSD value. PTH–amino acids were safely measurable at 1.0 pmol level except for delta Thr (data not shown). As

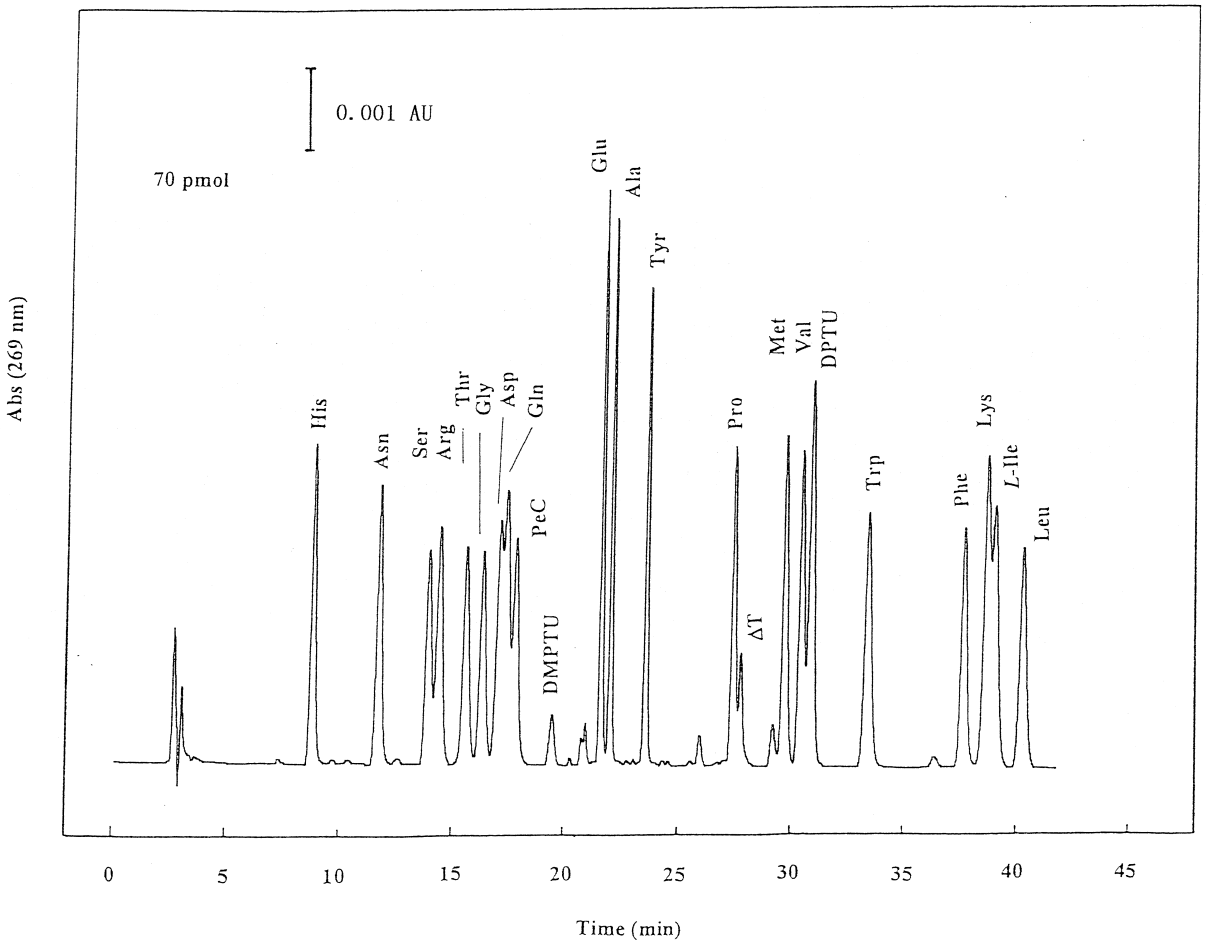

Fig. 2. Separation of PTH–amino acids in a single run with Develosil ODS UG-5 column. Gradient and column temperature were controlled as shown in Table 1. 70 pmol of PTH–amino acids mixed standard (20 amino acids) together with PTH–PeC, and DPTU and DMPTU were injected; i.e., 21 PTH–amino acids. Other conditions were as described in the Experimental (Section 2.3 and 2.4). The chromatogram was recorded on a Shimadzu C-R6A, and chromatographic background due to gradient elution was subtracted by C-R6A (base line corrected).

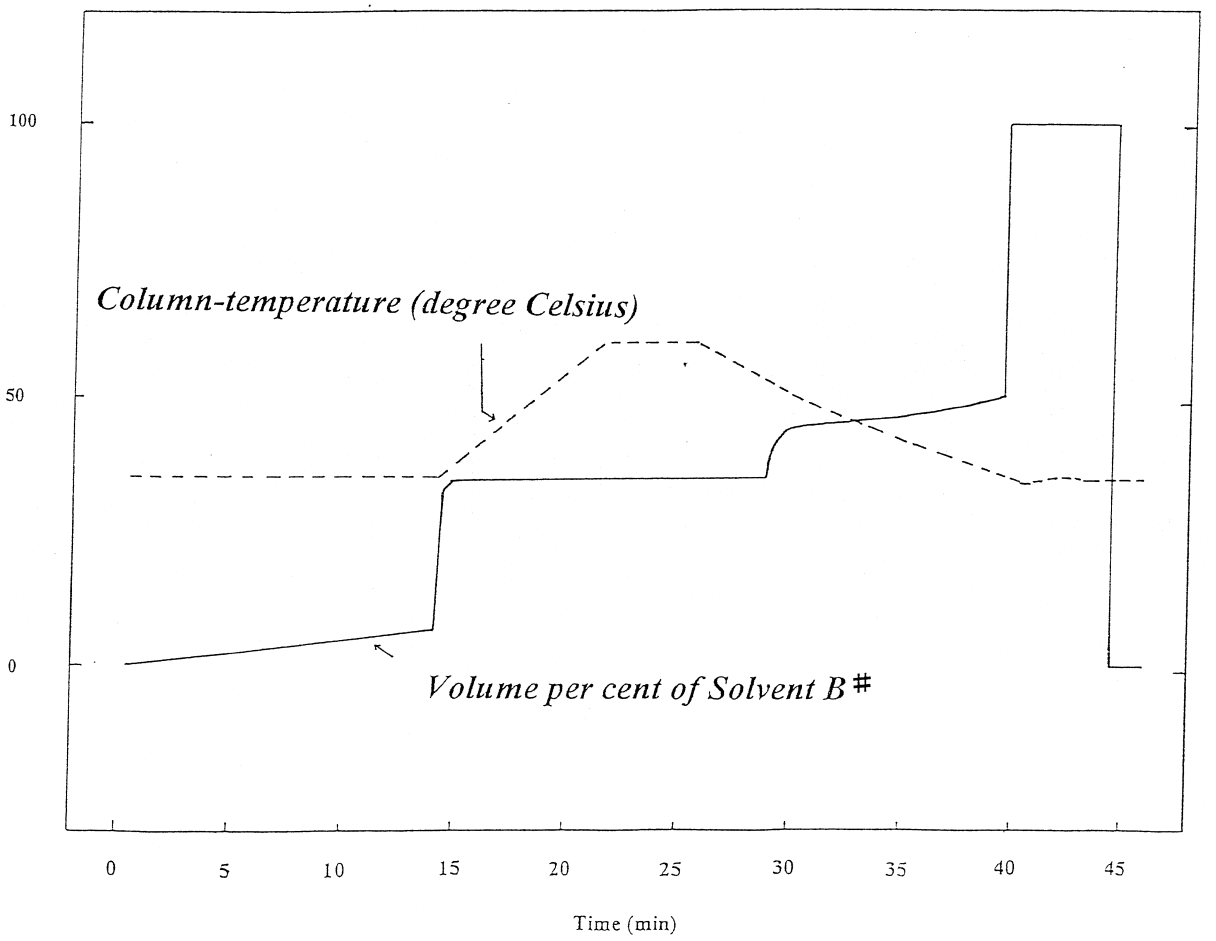

Fig. 3. Measurement of column temperature. Column temperature was measured and graphed using a dashed line. The gradient programme of Table 1 was performed.

shown in Table 3, graphs of the amount of PTH–amino acids injected versus peak heights were linear, intersecting the origin for all the amino acids in the range of 1–14 pmol, except for delta T and Leu which intersected the positive *y*-axis due to insufficient separation. Sufficient suppression of ionization [2] of Arg was achieved in this acidic phosphate buffer system as compared to the previous low ionic strength aqueous 0.1% trifluoroacetic acid eluent, where the graph of Arg intersected the positive *y*-axis [16].

With respect to the measurement of possible cystine residue, we have not yet been able to identify the PTH–cystine peak with this system. However, the PTC–cystine peak was identified and the correlation graph was obtained. It was found that although PTH–PeC was quantitative with this 250 mm column using phosphate buffer (Table 3), PTC–cystine was not; i.e., the graph of PTC–cystine intersected the positive *x*-axis ($y=-1.4960+1.0731x$; $r=1.000$: $n=3$) suggesting adsorbtion to the gel support. However, it was also found that a satisfactory graph was obtainable when it was measured with a 30 mm (3 μm particle diameter; Develosil ODS UG-3) column; i.e., regression line of $y=0.1900+0.9873x$ ($r=0.999$; $n=3$) was obtained. Such phenomenon has already been observed during the measurement of derivatized lipoic acid (thioctic acid), where two sulphur atoms are present [17]. Therefore, the use of such thiol compounds as ethanethiol in Reagent R3

Table 2
Within-day and day-to-day reproducibility of PTH–amino acid analysis[a]

PTH –amino acid	Within-day mean RSD (%)±SD	Day-to-day RSD (%)
H (His)	1.852±0.789	1.133
N (Asn)	2.230±0.711	1.146
S (Ser)	2.610±1.025	1.841
R (Arg)	3.235±1.372	1.390
T (Thr)	1.727±0.456	2.197
G (Gly)	3.175±0.665	1.497
D (Asp)	2.995±1.779	1.320
Q (Gln)	2.735±0.665	1.142
PeC	3.850±1.847	2.160
E (Glu)	1.868±1.020	2.383
A (Ala)	2.375±1.121	1.121
Y (Tyr)	2.160±1.212	2.276
P (Pro)	1.913±0.971	0.462
delta T	6.943±2.567	8.979
M (Met)	3.203±2.784	1.893
V (Val)	3.465±1.574	1.771
W (Trp)	2.843±1.311	2.650
F (Phe)	3.352±1.920	1.138
K (Lys)	2.425±0.999	1.288
I (Ile)	3.135±1.163	0.679
L (Leu)	4.540±0.662	4.858

[a] 10 pmol Amount of PTH–amino acids were injected four times in one day, repeated for four days. Day-to-day RSD; variations of means (n=4) of four consecutive days are shown.

Table 3
Linearity of response for PTH–amino acids

PTH–amino acid	$y=ax+b$[a]		
	a	b	r
H (His)	0.9730	0.1948	0.999
N (Asn)	0.9262	0.1962	0.997
S (Ser)	0.9986	0.0516	0.999
R (Arg)	0.9500	0.4500	0.998
T (Thr)	0.9407	0.1503	0.997
G (Gly)	0.9307	0.4112	0.995
D (Asp)	0.9911	−0.1452	0.998
Q (Gln)	0.9769	0.0704	0.999
PeC	1.0343	−0.2677	0.999
E (Glu)	0.9824	0.1793	0.999
A (Ala)	0.9826	0.0642	0.999
Y (Tyr)	0.9599	0.0891	0.999
P (Pro)	1.0173	0.0510	0.999
delta T	0.9000	1.3600	0.979
M (Met)	0.9626	0.4011	0.999
V (Val)	1.0102	0.1088	0.999
W (Trp)	0.9807	−0.1388	0.998
F (Phe)	0.9881	−0.2269	0.997
K (Lys)	1.0605	−0.5715	0.998
I (Ile)	0.9936	−0.2180	0.996
L (Leu)	0.8914	1.1245	0.999

[a] Linear regression line. r: Correlation coefficient. Correlation between the amount of PTH–amino acid injected (pmol) and corrected peak height [10 mm at attenuation $1=1.33\cdot10^{-4}$ (AU: absorption unit)=10 pmol], calculated from eight sample points; i.e., 1, 2, 4, 6, 8, 10, 12 and 14 pmol.

and R4A, and dithiothreitol in Reagent S4B in the protein sequencer should be avoided to prevent the adsorption of thiol compounds to the 250 mm column. If fractions from the sequencer contain thiol compounds, they may be evaporated to dryness (40°C, 2 h) before storage in a centrifugal evaporator [15].

3.4. Application to some proteins and peptides

The above PTH–amino acid separation method was then applied to the micro-sequencing of several proteins and peptides after covalent bonding to glass fiber disks. Typical binding of 4.8% (as assessed by 2nd amino acid Ile from N-terminal) for lactoglobulin, 2.0% (as assessed by 2nd amino acid Glu) for dephosphorylated β-casein, 0.53% (as assessed by 3rd amino acid Trp) for Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg, and 0.89% (as assessed by 4th amino acid Leu) for Gly–Arg–Gly–Leu–Ser–Leu–Ser–Arg, to the two glass fiber disks by DITC method were observed. Binding of 0.10% (as assessed by 3rd amino acid Trp) for Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg was also observed by the EDC method. Micro-sequencing of DITC-bonded lactoglobulin of cow's milk (glycoprotein, molecular mass 18 400) showed a linear logarithmic-yield graph (data not shown). Similar result has already been reported in cytochrome *c* by Machleidt et al. [21]. Extrapolation of the graph suggested that over 60 steps of identifications of degradation of proteins by the DITC binding method is possible.

Peptide of Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg (an active fragment of myelin basic protein) was also bound and analyzed, as shown in Fig. 4. The yield of Ser (2nd amino acid) has been

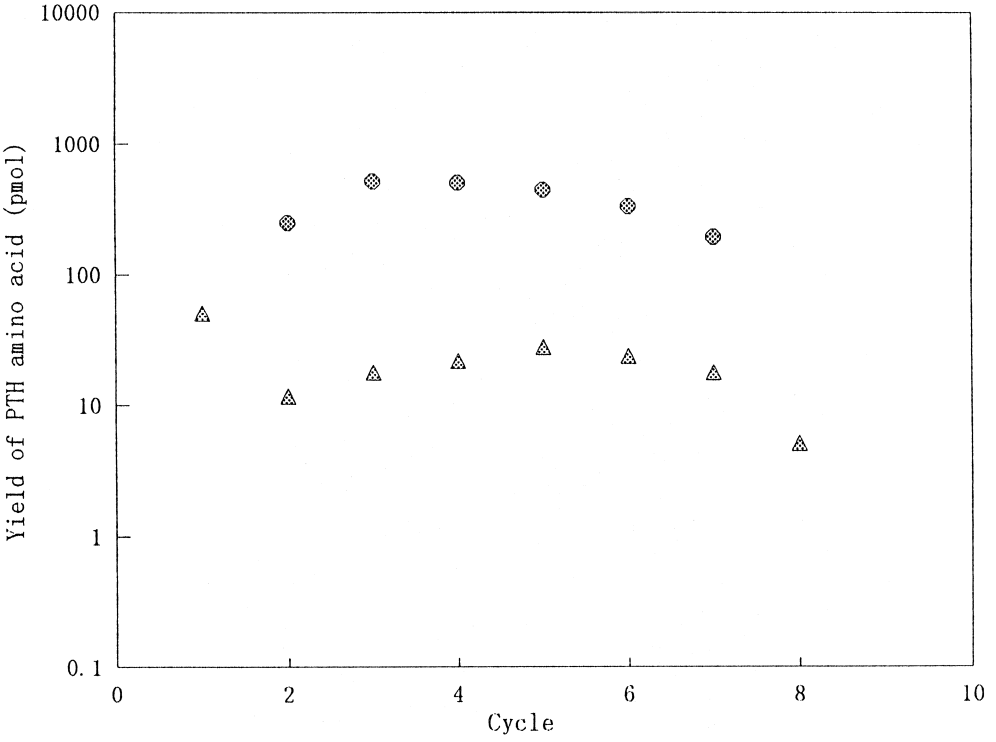

Fig. 4. Typical PTH-yields from the degradation of a peptide consisting of Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg. Closed circles: DITC method, Closed triangles: EDC method, Cycle: Edman degradation cycle. Other conditions were as described in the Experimental section.

proven satisfactory by this HCl cutting method [19]. Arg and Gln may be useful as anchor amino acids as Lys is in the DITC method [21]. Although these binding methods have not yet been perfected, the yield seems to be higher for the DITC method than the EDC method (Fig. 4).

The N-terminal amino acid was identifiable by the DITC method in the case of the protein sample of lactoglobulin (data not shown), although it was not in the case of the peptide sample (Fig. 4). Therefore, micro-sequencing of proteins by the DITC method together with the EDC method is expected to prove useful in the determination of the N-terminal amino acid.

Thus, this method for PTH–amino acids separation is shown to be applicable to micro-sequencing of proteins and peptides after covalent binding to glass fiber disks (aminopropyl glass).

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