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Note

High-sensitivity amino acid analysis of stained peptides and proteins from a sodium dodecyl sulphate-polyacrylamide slab gel

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The automated amino acid analyser based on ion-exchange chromatographic procedures was first devised by Moore *et al.*[1]. The separated amino acids were determined by colorimetry after post-column derivatization with ninhydrin[1,2]. This device was widely employed for quantitative amino acid analysis and the chemical characterization of peptides and proteins in the living system.

More recently, reversed-phase separation procedures and high-performance liquid chromatography (HPLC) have been developed. The classical method of amino acid analysis was first subjected to the HPLC system, and subsequently ninhydrin has often been replaced by fluorescamine[3,4] or *o*-phthalaldehyde[7–9] offers high sensitivity but suffers from disadvantages relating to the lack of the proline reaction, instability of the fluorescent products and difficulties in quantitation owing to the sensitivity to quenchers. Quantitation of proline requires special oxidative procedures[8] for opening the ring, thus greatly complicating the design of *o*-phthalaldehyde-based analysers. This method requires improvement for the determination of proline, which is one of the important amino acids constituting biologically active peptides.

Pre-column derivatization for amino acid analysis has been reported with dansyl[10,11] and phenylthiohydantoin (PTH)[12,13] derivatives of amino acids. The HPLC analysis of the pre-column derivatives (PTH- and dansyl-amino acids) is relatively rapid and exhibits good efficiency and sensitivity. However, the methods suffer from a lack of stability of the derivatives, low quantitative yields of PTH-amino acids and interference by peaks of byproducts originating from dansyl chloride.

More recently, high-sensitivity analysis of amino acids, including proline, derivatized quantitatively with phenyl isothiocyanate (PITC) has been described[14,15]. This technique, especially with reversed-phase columns, offers higher efficiency, greater ease of use and a higher speed of analysis than the conventional ion-exchange techniques. This method is not suitable, however, for samples that contain a large amount of ammonium chloride, a byproduct occurring during acid hydrolysis of peptides and proteins, because the separation of phenylthiocarbamoyl (PTC)-proline and a large amount of phenylthiourea derived from ammonium chloride is not achieved.

The purpose of this study was to establish a method for the separation of

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PTC-amino acids, especially PTC-proline and phenylthiourea, and to apply it to the amino acid analysis of acid hydrolysates of insulin B-chain, insulin, cytochrome *c* and other peptides and proteins extracted from a polyacrylamide slab gel after electrophoresis and staining with Coomassie brilliant blue. They were considered to produce a large amount of ammonium chloride on acid hydrolysis. We achieved their separation by the addition of 0.1% of sodium dodecyl sulphate (SDS) to the mobile phase. The results of amino acid analysis showed that the numbers of amino acids in these samples were each close to those calculated from their amino acid sequences, with a few exceptions. This method may be suitable for the routine microanalysis of the hydrolysates of peptides and proteins which are separated and stained on SDS-polyacrylamide slab gels.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: acetonitrile, triethylamine (TEA), amino acid standards and constant-boiling hydrochloric acid, Pierce (Rockville, IL, U.S.A.); sodium acetate, glacial acetic acid, PITC, acrylamide, Coomassie brilliant blue, and cytochrome *c* (Cyc, from horse heart), Wako (Osaka, Japan); oxytocin, bradykinin, angiotensin I and insulin B-chain, Protein Research Foundation (Osaka, Japan); SDS, BDH Chemicals (Poole, U.K.); and insulin (from bovine pancreas, crystalline), Sigma (St. Louis, MO, U.S.A.). High-purity water was prepared with a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus and chromatography

The apparatus consisted of a Waters new sample preparation module (Pico-Tag Workstation) and a Waters liquid chromatograph (Pico-Tag amino acid analyser) (Waters Assoc., Milford, MA, U.S.A.). The former incorporated two separate functions in a compact design for the batchwise preparation of protein and peptide hydrolysates and for derivatization of the free amino acids. The latter was an HPLC system that consisted of two Model 6000A solvent delivery systems and a Model 440 fixed-wavelength detector (254 nm) controlled with a Model 680 controller. The temperature was controlled at 38 + 1°C with a column heater (Waters Assoc.). Sample were injected in volumes ranging from 1 to 40 μl using a Waters Model 710B auto-sample injector. The chromatographic separation was achieved in the presence of 0.1% of SDS on a Pico-Tag octadecylsilane column (particle size 5 μm, 150 $\times$ 3.9 mm I.D.) with a solvent system consisting of two eluents: eluent A, 131 m*M* sodium acetate buffer (pH 6.4) containing 6% of acetonitrile and 0.05% of TEA; eluent B, 60% acetonitrile in water. The gradient controller was programmed as follows: eluent B set to 0–46% in 10 min using a convex curve (No. 5), 46–100% in 0.5 min using a linear gradient (No. 6) and 100% for 2 min.

Hydrolysis (110°C, 24 h in 6 *M* HCl gas) of peptides and proteins and derivatization of the resultant amino acids with PITC were carried out according to the method of Bildingmeyer *et al.*[15].

Polyacrylamide gel electrophoresis

Polyacrylamide slab gels, 12 $\times$ 13 cm in size and 1–2 mm average thickness,

were prepared according to the method of Laemmli[16]. Prior to electrophoresis, the gels were subjected to an electric current (40 mA, 100 V, 3 h) in the absence of peptide and protein samples in order to remove unpolymerized materials.

Samples, each of 10 μg dissolved separately in 10 μl of deionized water, and 10 μl of deionized water alone for reference gels were boiled in the loading buffer[16] for 3 min and applied, after cooling, to the top of the same slab gel at 20-mm intervals in a volume not exceeding 50 μl. After electrophoresis (40 mA, 100 V, 2 h), the gel was stained with 0.2% Coomassie brilliant blue for 3 h. The stained gel was destained with 500 ml of methanol–acetic acid–water (5:7:88) and rinsed successively with 500 ml each of deionized water and high-purity water, five times in each instance. During the destaining procedures, the glycine in the gel from the running buffer (Tris–glycine) was also eliminated. The stained bands of peptides and proteins and the reference gels corresponding to the running positions of the stained bands were cut, each in a size of 2 × 8 mm, from the slab gel. The cut-off gels were kept in 200 μl of 2% TEA with gentle shaking at 37°C for 30 min in Pyrex glass tubes in order to extract the stained peptides and proteins. The supernatants, lightly coloured with Coomassie brilliant blue, were removed from the tubes with a microsyringe and used as samples for amino acid analysis. The drying and hydrolysis (110°C, 24 h, in 6 *M* HCL gas) of the samples and derivatization of the resultant amino acids with PITC were carried out in the Pico-Tag Workstation according to the method of Bidlingmeyer *et al.*[15]. Prior to the above experiment, all the glass tubes were heated in an electric oven at 550°C for 1 h.

RESULTS AND DISCUSSION

Separation of PTC-amino acids

Fig. 1 shows the separation of PTC-amino acids at the 250 pmol level. PTC-proline and phenylthiourea (shown as NH_3 in the figures) were chromatographed with the same retention time in the absence of 0.1% SDS at a flow-rate of 1 ml/min. PTC-β-alanine (shown as β-A in the figures) and PTC-histidine were also eluted at identical positions (Fig. 1A). The addition of 0.1% of SDS to the mobile phase and a simultaneous decrease in the flow-rate from 1.0 to 0.7 ml/min led to the clear separation of PTC-proline and phenylthiourea and also of PTC-β-alanine and PTC-histidine, as shown in Fig. 1B. PTC-arginine was eluted before PTC-threonine in the absence of SDS (Fig. 1A). In the presence of SDS, it was chromatographed with an increased retention time, appearing before and after PTC-alanine and PTC-proline, respectively (Fig. 1B). All the PTC-amino acids were thus separated completely by the addition of 0.1% of SDS.

Detection limits

Fig. 2 shows the analysis of standard amino acid mixtures at 25–250 pmol levels. In other experiments, 5 and 10 pmol of the standard amino acids could be detected on the chromatograms, but their peaks were all very small (not shown). Quantitation of less than 10 pmol is not practical because of background contributions from the solvents, chemicals, glasswares and pipette tips.

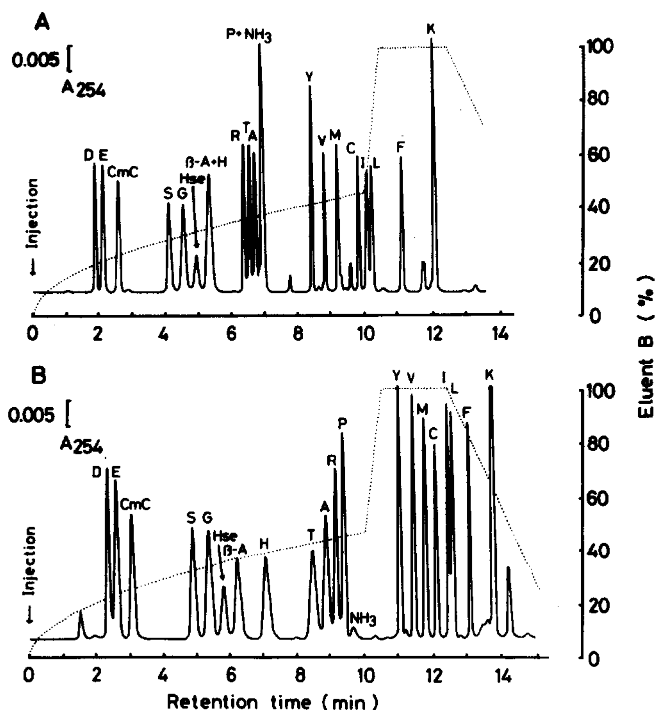

Fig. 1. HPLC of PTC-amino acids in the presence and absence of 0.1% of SDS. Standard amino acids (all 250 pmol) were derivatized with PITC according to the method of Bidlingmeyer *et al.*[15]. Eluent A: 131 m*M* sodium acetate (pH 6.4) containing 6% acetonitrile and 0.05% TEA. Eluent B: 60% acetonitrile in water. Gradient: 0–46% B in 10 min on a concave curve (No. 5), 46–100% in 0.5 min on a linear gradient (No. 6) and 100% B for 2 min. Column: Pico-Tag. Detector: 254 nm at 0.05 a.u.f.s. The one-letter symbols for amino acids are shown in Table I. (A) In the absence of SDS: flow-rate, 1.0 ml/min. (B) In the presence of 0.1% of SDS: flow-rate, 0.7 ml/min. CmC, carboxymethylcysteine; Hse, homoserine; β-A, β-alanine. -----, Gradient profile.

High-sensitivity analysis of peptides and proteins

The method was applied to the analysis of acid hydrolysates of some peptides and proteins at picomole levels, and its practicability was investigated. Aliquots (250 pmol) of the hydrolysed samples were injected into the amino acid analyser. The results are shown in Table I. For the low-molecular-weight peptides oxytocin, bradykinin, angiotensin I, and insulin B-chain, the numbers of residues per molecule determined by the amino acid analysis were each very close to those calculated from their amino acid sequences. With insulin and Cyc, however, the number of half-cystine residues observed was smaller than that in their sequences. Cysteine, generally determined as half-cystine or a cysteine derivative (cysteic acid or carboxymethylcysteine, is usually obtained with a value lower than that expected because of its instability to acid hydrolysis. In this experiment, cysteic acid was chromatographed before aspartic acid (data not shown).

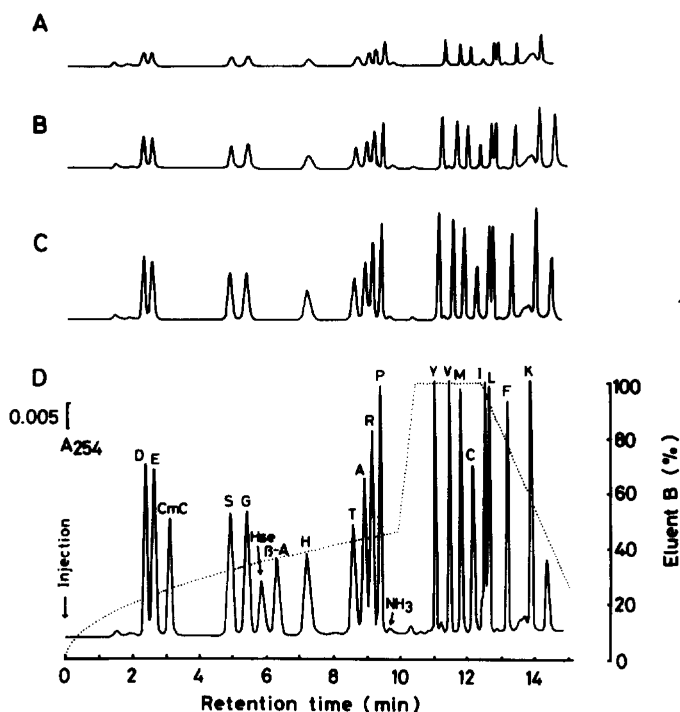

Fig. 2. High-sensitivity analysis of different amounts of standard amino acids. (A) 25; (B) 50; (C) 100; and (D) 250 pmol. Other conditions and abbreviations as in Fig. 1.

TABLE I

AMINO ACID ANALYSIS OF PEPTIDES AND PROTEINS

Amino acid	*Oxy-tocin*	*Brady-kinin*	*Angio-tensin I*	*Insulin B-chain*	*Insulin*	*Cyc*
Asp (D)	1.0(1)*		0.4(1)	0.6(1)	3.2(3)	8.1(8)
Glu (E)	1.0(1)			2.4(3)	6.8(6)	12.5(12)
Ser (S)		0.9(1)		0.9(1)	2.6(3)	
Gly (G)	1.0(1)	1.0(1)		3.2(3)	5.0(5)	11.3(12)
His (H)			2.1(2)	2.0(2)	1.9(2)	2.9(3)
Thr (T)				1.0(1)	0.9(1)	10.2(10)
Ala (A)				2.0(2)	3.0(3)	6.0(6)
Arg (R)		1.9(2)	1.0(1)	1.1(1)	1.0(1)	2.2(2)
Pro (P)	1.0(1)	3.0(3)	1.2(1)	1.0(1)	1.0(1)	4.2(4)
Tyr (Y)	0.9(1)		1.1(1)	2.2(2)	3.7(4)	4.2(4)
Val (V)			1.1(1)	4.0(3)	4.6(5)	3.7(3)
Met (M)						1.8(2)
½-Cys (C)	1.0(1)			0.7(2)**	4.7(6)	1.0(2)
Ile (I)	0.9(1)		1.1(1)		0.6(1)	5.8(6)
Leu (L)	1.1(1)		1.2(1)	4.4(4)	5.7(6)	5.8(6)
Phe (F)		1.9(2)	1.2(1)	3.4(3)	3.6(3)	3.9(4)
Lys (K)				1.0(1)	0.8(1)	19.2(19)

* Residues per molecule determined by amino acid analysis. Numbers in parentheses are those calculated from the sequences.

** Half-cystine calculated as cysteic acid.

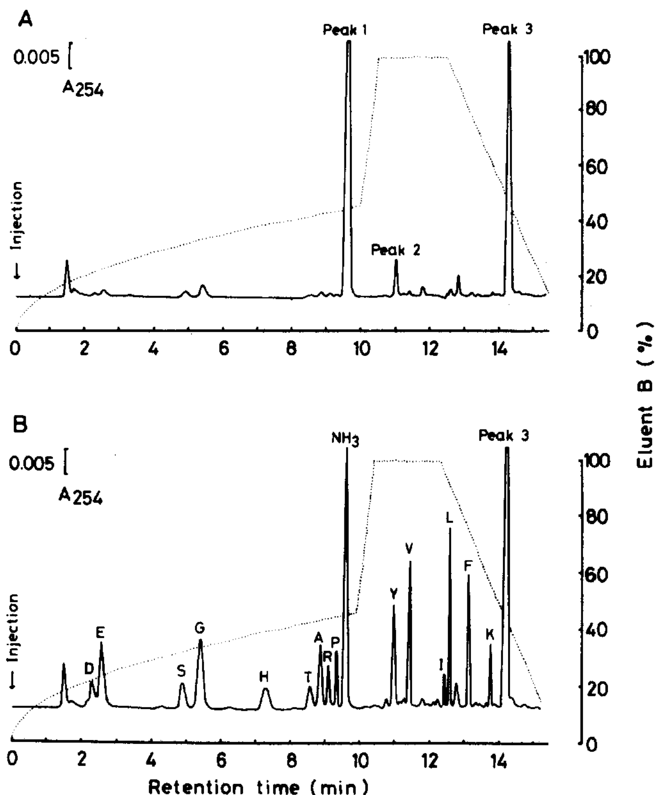

Fig. 3. High-sensitivity amino acid analysis of stained insulin from SDS-polyacrylamide slab gel. (A) Hydrolysate of the extract from reference gel; (B) hydrolysate of stained insulin extracted from SDS-polyacrylamide slab gel. -----, Gradient profile.

Amino acid analysis of stained peptides and proteins from SDS-polyacrylamide slab gel

Fig. 3 shows chromatograms obtained with insulin(B) and the corresponding reference gel(A). The PITC-derivatized hydrolysate of the extract from the reference gel gave two large peaks, 1 and 3, and several small peaks, including peak 2 (Fig. 3A). Peak 1 was identified as phenylthiourea, as both appeared at the same position. This phenylthiourea was probably formed by the reaction of PITC with ammonium chloride, which was produced from polyacrylamide gel on acid hydrolysis. Peak 3, not identified, was chromatographed after lysine. This peak also seems to have originated from polyacrylamide gel. Peak 2 eluted at the same position as PTC-tyrosine. The net amount of PTC-tyrosine in the peptide or protein sample was therefore obtained by subtracting the amount calculated from peak 2 from the amount of PTC-tyrosine observed in the sample. The first-appearing peaks having a retention time of 1.5 min in Fig. 3A and B can probaboly be ascribed to the same compound originally contained in the solvents or reagents used, as both peaks have similar heights. As Fig. 3B shows, PTC-proline and phenylthiourea (indicated by NH_3) were

TABLE II

AMINO ACID ANALYSIS OF STAINED PEPTIDES AND PROTEINS FROM SDS–POLYACRYLAMIDE SLAB GEL

Amino acid	*Insulin B-chain (7% recovery)*	*Insulin (14% recovery)*	*Cyc (5% recovery)*
Asp (D)	0.7(1)*	2.1(3)	7.3(8)
Glu (E)	2.0(3)	4.7(6)	11.8(12)
Ser (S)	0.7(1)	1.6(3)	0.6(0)
Gly (G)	3.1(3)	4.9(5)	16.4(12)
His (H)	1.7(2)	2.6(2)	2.5(3)
Thr (T)	0.8(1)	1.5(1)	9.0(10)
Ala (A)	2.0(2)	3.0(3)	6.0(6)
Arg (R)	0.9(1)	1.6(1)	2.0(2)
Pro (P)	1.3(1)	1.8(1)	3.8(4)
Tyr (Y)	4.4(2)	3.2(4)	2.3(4)
Val (V)	3.5(3)	4.8(5)	0.7(3)
Met (M)	0.5(0)	0.7(0)	0.7(2)
½-Cys (C)	N.D.**(2)***	0.7(6)	N.D.(2)
Ile (I)	0.4(0)	0.3(1)	4.4(6)
Leu (L)	4.0(4)	5.9(6)	5.3(6)
Phe (F)	3.2(3)	5.0(3)	3.0(4)
Lys (K)	1.0(1)	1.6(1)	13.3(19)

* Residues per molecule determined by amino acid analysis. Numbers in parentheses are those calculated from the sequences.

** N.D., not determined.

*** Half-cystine calculated as cysteic acid.

separated clearly by the addition of 0.1% of SDS to the moble phase. They could not be separated under the conditions examined unless SDS was added.

The amino acid compositions of stained insulin B-chain, insulin and Cyc are shown in Table II. Their recoveries from the SDS–polyacrylamide slab gel were in the range 5–14%, as calculated on the basis of amino acid analysis. The amino acid compositions of insulin B-chain, insulin and Cyc were close to the numbers calculated from their sequences, except for cystine and lysine. Quantitation of these two amino acids, which were considered to be sensitive to Coomassie brilliant blue, was very poor, probably because they were broken down during acid hydrolysis under the influence of the dye. This is similar to the poor lysine quantitation of fluorescamine-labelled peptides after acid hydrolysis, as observed in a protein kinase sequence study[17].

This highly sensitive method showed be widely applicable to the amino acid analysis of peptides and proteins separated by SDS–polyacrylamide gel electrophoresis.

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