MICROSEQUENCE ANALYSES: II. DABTH-AMINO ACID IDENTIFICATION BY HIGH-PERFORMANCE LIQUID AND THIN-LAYER CHROMATOGRAPHY

Kenneth J. WILSON, Kenneth RODGER and Graham J. HUGHES+

Biochemisches Institut der Universität Zürich, CH-8032 Zürich and *+Laboratorium fur Biochemie der Eidgenössischen Technischen Hochschule, CH-8092 Zürich, Switzerland*

Received 2 October 1979

1. Introduction

Of the various methods available for the identification of amino acid derivatives arising during sequential degradation of proteins or peptides probably the best suited is high-performance liquid chromatography. This method offers rapid analysis, high sensitivity and essentially baseline separation; thus the possibility of quantitation. HPLC systems for isocratic separation [1–3] and gradient elution [4–10] of PTH-amino acids have been described.

The use of DABITC, as a substitute for PITC in Edman sequence analysis, has been introduced [11–14]. DABTH-derivatives have an advantage in that:

(i) They absorb in the visible (340–580 nm) and thus ultraviolet absorbing by-products of the reaction and/or solvents do not interfere with identification

(ii) Their extinction coefficients ($\epsilon_{mM}^{436\ nm} = 34$ [15]) are ~2-times higher than their PTH-analogues ($\epsilon_{mM}^{254} = 16$ [16]). The identification of DABTH-amino acids by TLC at the level of ~5–25 pmol/spot has been reported [11–15,17,18]. Here we report the separation of these derivatives by isocratic and gradient HPLC systems and by TLC on silica gel. In [19–21] we indicate their application in automatic solid- and liquid-phase sequencing.

Abbreviations: DABITC, 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate; DABTC, 4-*N,N*-dimethylaminoazobenzene 4′-thiocarbamyl; DABTH, 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoin; DABTZ, 4-*N,N*-dimethylaminoazobenzene 4′-thiozolinone; DMSO, dimethylsulfoxide; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PITC, phenylisothiocyanate; PTH, phenylthiohydantoin; TLC, thin-layer chromatography

2. Materials and methods

DABTH-amino acids were synthesized according to [11] with minor modifications:

(i) Drying was performed at <45°C by rotary evaporation under vacuum;

(ii) Conversions from the DABTC to the DABTH-derivatives were done using 1 N HCl–methanol [22];

(iii) The DABTH-amino acids, following N_2-drying of HCl–methanol, were extracted with ethyl-acetate from either 1 N HCl or 1 N NH_4OH.

Isocratic HPLC was performed at room temperature on a Waters Chromatograph (model ALC/GPC-204) equipped with a model 440 absorbance detector. The apolar DABTH-derivatives were chromatographed on a Lichrosorb (5 μM particle size) column (3.9 × 300 nm) equilibrated in dichloromethane to which 8 ml/l isopropanol–DMSO mixture (47.73:1, v/v) had been added. Similarly, the polar derivatives were separated using a Partisil (11 μm particle size) column (3.9 × 300 mm) equilibrated in dichloromethane to which 36 ml methanol, 12 ml DMSO and 7 ml 57% aqueous acetic acid had been added per liter.

Gradient HPLC was carried out on an instrument comprised of two Altex pumps (model 110), an Altex microprocessor (model 420), a Rheodyne sample-inject valve and the Uvikon LCD 725 detector. A

Zorbax ODS (5 μm particle size) column (4.6 X 250 mm) was developed with the solvent systems (*A*) 700 ml formate buffer (5 ml formic acid/liter to pH 3.0 with NaOH) to 1 liter with 1-propanol and (*B*) 500 ml formate buffer (made as in (*A*) to 1 liter with 2-propanol. The L-amino acids were purchased from Sigma; DABITC from Fluka or Pierce; all other chemicals were of analytical grade and purchased from either Fluka or Merck. The silica plates for TLC (Kieselgel 60 F_{254}) and HPTLC (Kieselgel 60) were from Merck. Amino acid analysis were carried out on a Durrum D-500 analyzer following 6 N HCl hydrolysis.

3. Results and discussion

Figure 1 shows the separation of the apolar and polar standard DABTHs by the described isocratic HPLC systems. Those peaks which have not been marked, using the standard one letter abbreviations for the amino acids, are side products from the synthesis of the DABTH-derivatives. Prior to preparation of the mixtures, the individual derivatives were injected to assure that none of the contamination co-chromatographed with the DABTH-amino acids. It should be noted that the DABTCs were converted to the DABTHs in 1 N HCl–methanol (see section 2), a procedure which in our work has been found to be suboptimal for these derivatives. The standard mixtures, prepared by drying down (N_2) an aliquot containing ~1 nmol of each derivative in plastic microcentrifuge tubes, are stable for >6 months at −20°C. This isocratic HPLC system, which is quite similar to that described [1] for PTH identification, is most convenient to use with the DABITC/PITC double-coupling methodology [13]. Since the

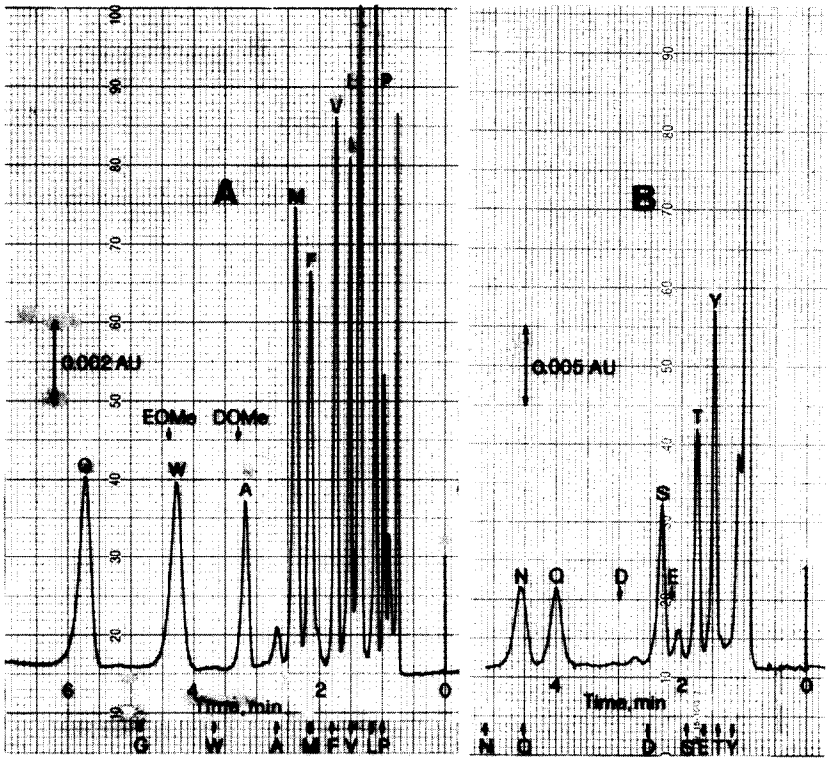

Fig.1. Isocratic separation of DABTH-amino acids. (A) Apolar derivatives. Pressure 3000 lb/in²; flowrate 3 ml/min; sample size 200–400 pmol of each DABTH; EOMe and DOMe, methylesters of E and D; absorbance measured at 436 nm (B) Polar derivatives. Conditions essentially identical to (A). The arrows and the one letter abbreviations under each chromatogram indicate the elution points for the PTH-derivatives.

unreacted amino groups are subsequently blocked with PITC (and overlap thus reduced) the ratio of the two derivatives (DABTH:PTH), where quantity or degree of background permit, is a convenient means of controlling the extent of the DABITC reaction. The elution positions of the PTH-derivatives are also shown in fig.1.

Reverse phase HPLC separation of the DABTH-amino acids is shown in fig.2. Due to the apparent complexity of the chromatogram the apolar and polar standards have been injected separately. It is important to note, however, that each peak elutes at a discrete point in the gradient with only the derivatives of Asp and Gln as well as Glu and Thr overlapping. DABTH-Arg and -His elute between the Ala- and Trp-derivatives; the Lys-derivative sometimes exhibits multiple peaks in the vicinity of DABTH-Trp (see fig.2 in the following article [20]).

We have also attempted to take advantage of the higher absorption properties of the DABTHs when protonated, by introducing a dilute HCl solution following chromatographic separation and prior to detection at 550 nm. Although the system functioned the increase in absorbance was roughly equalized by the dilution caused by the acid and thus of no advantage over that carried out at 436 nm.

The advantages and disadvantages of both systems can be summarized as follows: isocratic elution requires no gradient-forming device, columns are readily packed and separation of Asp/Gln and Glu/Thr is obtained. However, eluant composition is critical, column life is low relative to the reverse phase column and sensitivity of latter eluting derivatives are decreased. The major advantage of the reverse phase system is that, when necessary, 100% of the sample can be injected. This is illustrated in [20] where the entire DABTH sample originating at each cycle between residues 33 and 44 was injected and thus the

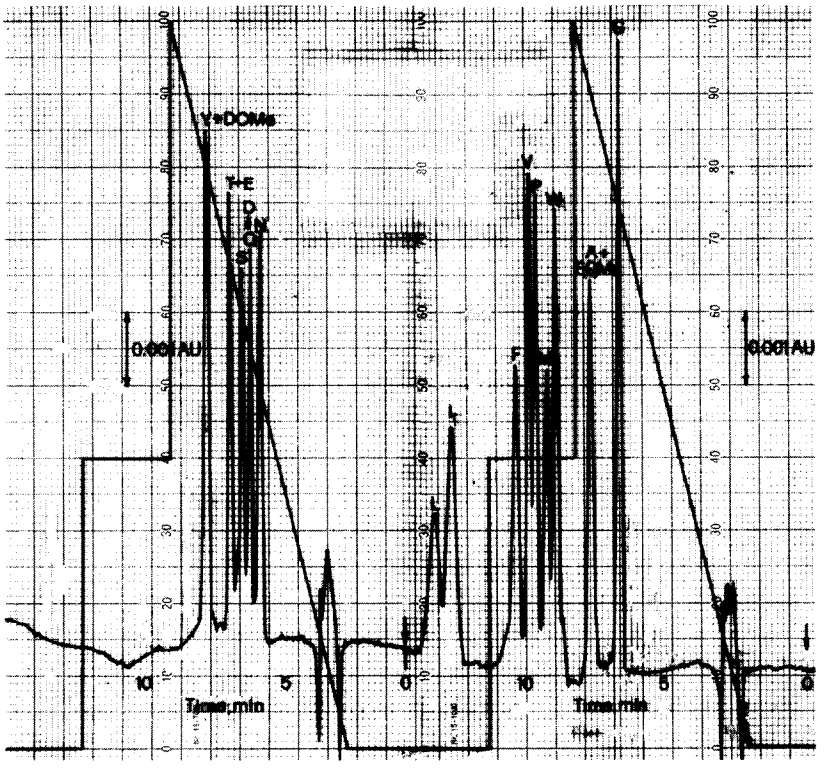

Fig.2. Gradient separation of DABTH-amino acids. Pressure 500 lb/in^2; flowrate 1 ml/min, sample size 100–200 pmol of each DABTH; column temp. 60°C; absorbance measured at 436 nm. Solid line indicates the gradient as % buffer (*B*) in (*A*) (see section 2).

sequence information extended.

Both these HPLC methods for DABTH identification are exceedingly rapid, 8 and 5 min for apolar and polar isocratic columns, respectively, and 15 min for the gradient elution. A combination of both methods therefore provides an unambiguous identification of all common amino acid derivatives with the exception of the Lys-, His- and Arg-DABTHs which at present require, as the second means of distinction, chromatography on polyamide sheets [12].

As an independent means of identification, including those derivatives not well separated by HPLC (see above), chromatography on silica plates is most convenient (fig.3). Using the plates for either standard TLC or those for HPTLC excellent resolution of all derivatives can be accomplished within 15 min at the <100 pmol level. The best separation of the apolar DABTHs occurs in solvent III on HPTLC plates and for the polar derivatives either plate type in solvent II. It is also possible to combine the systems, i.e., chromatography in solvent III followed by either solvent II or IV. Identification of DABTH-Arg, -His and -Lys can be done on polyamide sheets according to [12] or by HPLC as previously shown. In those cases where the identification of a DABTH-derivative is dubious, back-hydrolysis and amino acid analysis can be carried out. The hydrolysis conditions, similar to those in [23] for PTHs, are 6 N HCl containing 0.2% $SnCl_2$ at 150°C under vacuum for 8–12 h.

As will be illustrated in [19–21], the above identification methods have been extensively used during solid- and liquid-phase sequencing. In conjunction with the advantages of DABITC- over PITC-sequencing [11–15, 17–21], the HPLC systems offer:

(i) Quantitative results at <100 pmol level;
(ii) A means of controlling the extent of the DABITC reaction (DABTH:PTH ratio);
(iii) A degree of reliability which is difficult and tedious to achieve by any other method, e.g., TLC or back-hydrolysis.

Acknowledgements

The authors would like to thank H. Lutz for his valuable help. This research was supported by grant 3.049-0.76 (to K. J. W.) from the Swiss National Science Foundation and grant 0.330.077.98-3 from the Eidgenössischen Technischen Hochschule, Zürich (to K. H. Winterhalter).

References

[1] Frank, G. and Strubert, W. (1973) Chromatographia 6, 522–524.
[2] Frankhauser, P., Fries, P., Stahala, P. and Brenner, M. (1974) Helv. Chim. Acta 57, 271–277.
[3] Schlesinger, D. H., Weiss, J. and Audhya, T. K. (1979) Anal. Biochem. 95, 494–496.

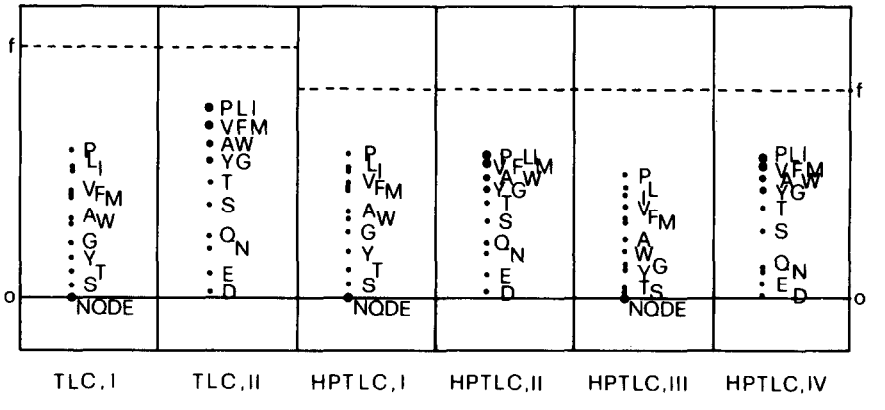

Fig.3. TLC and HPTLC separations of the DABTH-amino acids. Solvent systems I (chloroform: ethanol, 92:2 (v/v)) and II (chloroform: ethanol: methanol, 88.2:1.8:10 (v/v/v) are according to [24]. This DABTH separation has been described [18] and is given here as a means of comparison. System III is chloroform:ethylacetate, 90:10 (v/v) and IV, chloroform:isopropanol, 90:10 (v/v). Chromatography was performed for 15 min on 10 cm high plates, followed by drying and exposure to HCl fumes [11]: (o) origin; (f) solvent front.

[4] Matthews, E. W., Byfield, P. G. H. and MacIntyre, I. (1975) J. Chromatog. 110, 369–373.
[5] Downing, M. R. and Mann, K. G. (1976) Anal. Biochem. 74, 298–319.
[6] Zimmermann, C. L., Appella, E. and Pisano, J. J. (1976) Anal. Biochem. 75, 77–85.
[7] Zimmermann, C. L., Appella, E. and Pisano, J. J. (1977) Anal. Biochem. 77, 569–573.
[8] Margolies, M. N. and Brauer, A. (1978) J. Chromatog. 148, 429–439.
[9] Bhown, A. S., Mole, J. E., Weissinger, A. and Bennett, J. C. (1978) J. Chromatog. 148, 532–535.
[10] Devillers-Thiery, A. and Blobel, G. (1978) Anal. Biochem. 87, 66–70.
[11] Chang, J. Y., Creaser, E. H. and Bentley, K. W. (1976) Biochem. J. 153, 607–611.
[12] Chang, J. Y. (1977) Biochem. J. 163, 517–520.
[13] Chang, J. Y., Brauer, D. and Wittmann-Liebold, B. (1978) FEBS Lett. 93, 205–214.
[14] Chang, J. Y. (1979) Biochim. Biophys. Acta 578, 188–195.
[15] Chang, J. Y. (1979) Biochim. Biophys. Acta 578, 175–187.
[16] Edman, P. and Henschen, A. (1975) in: Protein Sequence Determination (Needleman, S. B. ed) pp. 232–279, Springer-Verlag, Berlin, New York.
[17] Chang, J. Y. and Creaser, E. H. (1977) J. Chromatog. 132, 303–307.
[18] Chang, J. Y., Creaser, E. H. and Hughes, G. J. (1977) J. Chromatogr. 140, 125–128.
[19] Hughes, G. J., Winterhalter, K. H. and Wilson, K. J. (1979) FEBS Lett. 108, 81–86.
[20] Hughes, G. J., Winterhalter, K. H., Lutz, H. and Wilson, K. J. (1979) FEBS Lett. 108, 92–97.
[21] Wilson, K. J., Hunziker, P. and Hughes, G. J. (1979) FEBS Lett. 108, 98–102.
[22] Tarr, G. E. (1975) Anal. Biochem. 63, 361–370.
[23] Mendez, E. and Lai, C. Y. (1975) Anal. Biochem. 68, 47–53.
[24] Bridgen, J., Graffeo, A. P., Karger, B. L. and Waterfield, M. D. (1975) in: Instrumentation in Amino Acid Sequence Analysis (Perham, R. N. ed) pp. 111–145, Academic Press, London, New York.