Complete separation and quantitative determination of DABTH-amino acid derivatives by isocratic reversed-phase high performance liquid chromatography

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The isocratic separation by reversed-phase HPLC of all DABTH-amino acid derivatives which are released by degradation of polypeptides with 4-*N*,*N*-dimethylaminobenzene-4′-isothiocyanate is described. The method allows an easy and quick identification and the quantitative determination of all derivatives in 30 min and includes the separation of isoleucine and leucine. Detection limit is 5 pmol at 436 nm. The solvent mixture is continuously recycled through the column, thereby reducing the solvent consumption considerably.

DABTH-amino acid *Reversed-phase high performance liquid chromatography (HPLC)* *Isocratic separation* *Quantitative determination*

1. INTRODUCTION

In recent years the sensitive manual Edman-type degradation of peptides and proteins by coupling with the reagent 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate (DABITC) has been widely applied in microsequence analysis. Degradation with this reagent [1,2] releases coloured amino acid derivatives, the DABTH-amino acids, which are red when treated with acid. They are therefore visible with the eye and can be differentiated from byproducts of the reaction because these are blue coloured or invisible.

The degradation method uses a double coupling, first with DABITC, followed by phenylisothiocyanate (PITC), to ensure complete reaction of all terminal amino groups as described [3–5]. In combination with the identification of the released derivatives by 2-dimensional thin-layer chromatography on small polyamide sheets [6] the method allows the degradation of as little as 500 pmol of polypeptide; detection limit of the derivatives on the sheets is 20 pmol.

Furthermore, reversed-phase HPLC has been used to separate the DABTH-amino acid derivatives by applying gradient solvent systems in order to enable an additional and independent identification of the derivatives and to obtain quantitative results [4,7,8]. The detection limit of this approach was reported to be 1 pmol if the absorbance at 436 nm was monitored [4].

The drawbacks of gradient systems demanded the development of a simpler and less expensive HPLC separation technique that allows complete separation of all DABTH-amino acid derivatives and their quantitative determination. We describe an isocratic system based on a reversed-phase C8-coated silica column, which is sensitive, gives high resolution of the DABTH-amino acids and has the advantage that the solvent mixture can be recycled for at least a week.

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2. MATERIALS AND METHODS

DABITC was purchased from Fluka or the Pierce Chemical Company and recrystallized from boiling acetone; melting point of the obtained brown flakes was 169–170°C. Acetonitrile, LiChrosolv grade, and 1,2-dichloroethane, Uvasol grade, purchased from Merck, Darmstadt, were used without further purification. A DNA-binding protein of *Bacillus sulfulobus* was isolated by Dr R. Reinhardt and a cyanogen bromide peptide from ribosomal protein L5 of *Bacillus stearothermophilus* by Dr M. Kimura.

2.1. *Preparation of standard DABTH-amino acids*

Standard DABTH-amino acids were prepared according to [7] but on a larger scale in order to allow further purification of the derivatives. For this purpose 200 μM each of the amino acids were dissolved in 10 ml of water/acetone/acetic acid/triethylamine (50:50:0.06:0.4, by vol.) and adjusted to a final pH of 10.4 by the addition of triethylamine. The reaction was performed under nitrogen with 50 μM DABITC in 10 ml of acetone, for 60 min at 60°C with stirring. The solvent was removed in a rotary evaporator; the remainder, after further drying over KOH in vacuo, was reacted under nitrogen with 6 ml of 40% trifluoroacetic acid (TFA) for 60 min at 50°C with stirring (asparagine and glutamine were reacted at 45°C). The DABTH-amino acids were dried over KOH in vacuo, dissolved in 5–15 ml of acetonitrile/water (1:1, by vol.) and repurified by injecting 1 ml portions to a preparative HPLC column (250 × 8 mm, Knauer). The column was filled with Hypersil MOS, 5 μm at 300 bar. Elution was made with a water/acetonitrile/1,2-dichloroethane solvent mixture as indicated in table 1 for the different amino acid derivatives, at a flow rate of 2.0 ml/min and 45°C. The effluent was monitored at 436 nm and 1.0 AUFS (absorbance units full scale). The combined fractions for each amino acid were concentrated, diluted with 0.5 ml of water and lyophilized. The derivatives were stored at −20°C and portions used as reference DABTH-amino acids (determination by weight). DABTH-Arg and -His were repeatedly dissolved in water and lyophilized. The additional DABTH-products of serine, threonine and lysine which are released after Edman degradation with DABITC/PITC were obtained from degradations of natural or synthetic peptides.

Table 2

Quantitative data on the areas obtained for the reference DABTH-amino acids by isocratic HPLC separation

DABTH-amino acid	Area (mm^2)
Asp	130
Asn	126
Gln	122
Ser	119
Thr	125
Gly	123
Tyr	112
Ala	127
Met	111
Val	137
Pro	134
Trp	137
Phe	131
Ile	127
Leu	132

Isolation and purification of the reference DABTH-amino acids are as given in section 2 and table 1. HPLC separation as given for fig.1. Listed are the areas obtained (in mm^2), averaged from 3 injections of 100 pmol/DABTH-amino acid

2.2. *Isocratic separation of DABTH-amino acids*

HPLC was performed with a Gynkothek constant flow pump, model 600/200, a Beckman model 160 absorbance detector, a Rheodyne injection valve and a Kipp and Zonen BD9 chart recorder. Empty stainless steel columns (250 × 4 mm, Knauer) were filled with Hypersil MOS (C8), 5 μm (Shandon, Cheshire) in a slurry of heptane at 30 MPa (300 bar) and were rinsed with heptane and methanol as described [9]. A precolumn (40 × 4.6 mm, i.d.) was filled with the same support. The conditions for the isocratic DABTH-amino acid separation were: column temperature, 45°C (maintained by a thermostatted aluminium block); flow rate, 1.2 ml/min; column pressure, 40–50 bar; measurements at 436 nm, sensitivity range 0.02–0.005 AUFS; recorder speed, 5 mm/min; solvent mixture was 12 mM sodium acetate, pH 5.0/acetonitrile/dichloroethane (50:50:0.5, by

vol.). Depending on the MOS-batch used the solvent mixture had to be varied between 48 and 49% sodium acetate and 52 and 51% acetonitrile to arrive at the same separation profile. The mixture was freshly prepared weekly and degassed by sonication. The sodium acetate buffer was made by adjusting 12 mM acetic acid with NaOH, both pro analysis grade from Merck. The solvent reservoir was equipped with a 2.0 μm steel filter.

To rinse the column a solvent mixture of 12 mM sodium acetate, pH 5.0/acetonitrile/dichloroethane (20:80:0.5, by vol.) was employed. This solvent also eluted DABTH-arginine which otherwise remained trapped on the column.

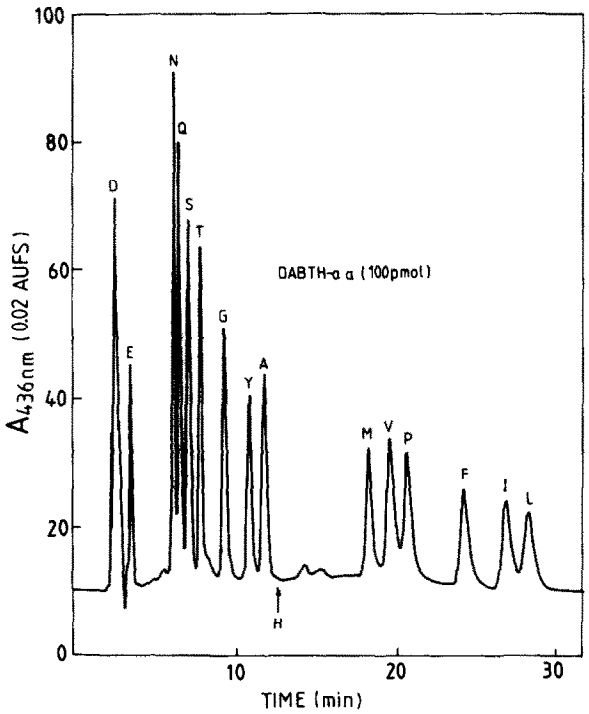

Fig.1. Isocratic separation of DABTH-amino acids by reversed-phase HPLC. A mixture containing 100 pmol each of the DABTH-amino acids was injected into a Hypersil MOS column (250 × 4 mm), self-packed with 5 μm support. Chromatography was performed at 45°C, a flow rate of 1.2 ml/min with the solvent mixture: 12 mM sodium acetate (pH 5.0)/acetonitrile/dichloroethane (50:50:0.5, by vol.). Detection was at 436 nm and 0.02 AUFS; the recorder speed was 5 mm/min (further details see section 2).

3. RESULTS AND DISCUSSION

The separation of 100 pmol of the reference DABTH-amino acids by isocratic HPLC on reversed-phase C8-bonded silica support is shown in fig.1 and quantitative estimates are listed in table 2. The HPLC chromatogram shows the excellent resolution of virtually all derivatives with simple HPLC equipment and a solvent mixture which can be recycled; one a liter is sufficient for

Table 1

Conditions for the purification of DABTH-amino acids by HPLC

DABTH-amino acid	I Solvent (water/AcCN)	II HPLC system (water/AcCN/DCE)
Asp	40:60	40:60:0.5
Glu	40:60	40:60:0.5
Asn	40:60	40:60:0.5
Gln	40:60	40:60:0.5
Ala	50:50	52:48:0.5
Ser (+ dehyd. Ser)	50:50	40:60:0.5
Thr (+ dehyd. Thr)	50:50	40:60:0.5
Gly	50:50	40:60:0.5
Tyr	50:50	40:60:0.5
Met	50:50	40:60:0.5
Val	50:50	30:70:0.5
Pro	50:50	30:70:0.5
Phe	50:50	30:70:0.5
Cys	50:50	30:70:0.5
Ile	50:50	30:70:0.5
Leu	50:50	30:70:0.5
Trp	25:75	30:70:0.5
His	[a]20:80	[a]20:80:0.5
Arg	[a]20:80	[a]20:80:0.5

[a] Means, water was replaced by 8 mM sodium acetate buffer, pH 5.0

The parameters for the isocratic HPLC purification were: column, 250 × 8 mm; support, Hypersil MOS, 5 μm; flow rate, 2.0 ml/min; temperature, 45°C. The crude DABTH-amino acids (for preparation see section 2) were dissolved in water/acetonitrile (concentration, column I) and chromatographed with a water/acetonitrile (AcCN)/dichloroethane (DCE) solvent mixture (column II)

a week of chromatography. As a result, the elution profile is constant and the sensitivity high. Depending on the sensitivity of the detector, the detection limit for the DABTH-derivatives was 1–5 pmol at 0.005 AUFS. Especially at higher sensitivities the isocratic separation is better than the gradient HPLC elution reported [4,7,8] as the stability of the baseline is improved. Further, compared with the common thin-layer detection of the DABTH-amino acids on polyamide sheets and the reported HPLC gradient elutions the isocratic system described here enables excellent separation of DABTH-isoleucine/leucine which otherwise cause problems.

DABTH-histidine and DABTH-tryptophan elute after alanine and between methionine and valine, respectively, as indicated in fig.1, depending on the ionic strength of the mobile phase. α-DABTH,-ϵ-PTC-lysine elutes between valine and proline but is only obtained if liquid-phase peptide degradation is performed without any attachment, or if peptide attachment is made via the C-terminal carboxyl-group by means of a water-soluble carbodiimide [10]. Under the chromatographic conditions employed DABTH-homoserine gives a characteristic peak between DABTH-serine and -threonine and can be easily differentiated from them as the latter amino acids give rise to two peaks on the chromatogram, resulting from the dehydrated and authentic forms. Typical serine and threonine containing degradation steps are illustrated in fig.2.

Under the elution conditions used only DABTH-arginine is trapped on the column. It can be identified by rinsing the column with higher acetonitrile concentrations as indicated in fig.3. All degradation cycles which do not show any new amino acid are therefore separately checked for arginine. This was done by washing the column with acetonitrile/12 mM sodium acetate buffer/dichloroethane (80:20:0.5, by vol.); DABTH-arginine elutes 15 min after injection. As this solvent mixture was also used to rinse the column after about 10 normal injections, the arginine identifications were usually made after several normal injections, thereby simultaneously cleaning the column which increased its lifetime considerably. The self-packed columns which were used were stable for at least 1500 injections.

The described isocratic separation was used to

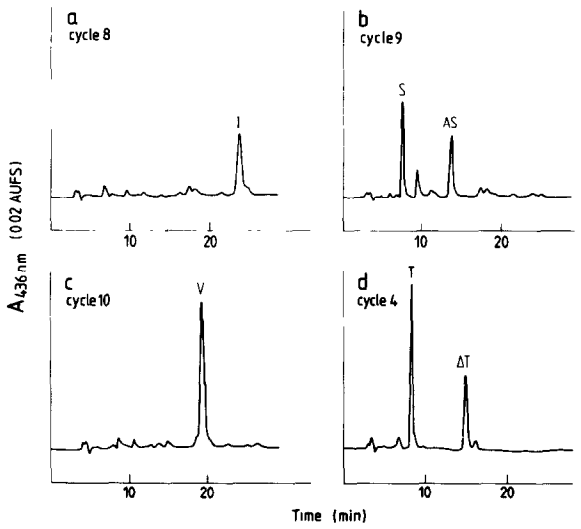

Fig.2. Identification of some degradation cycles of natural polypeptides by the isocratic HPLC method given in fig.1. The polypeptides were attached to amino glass and sequenced by the DABITC/PITC method in a LKB solid-phase sequencer [10]. a–c show DABTH-Ile, DABTH-Ser (and its dehydrated form, ΔS) and DABTH-Val from degradation steps 8–10 of a homoserine containing cyanogen bromide peptide from *B. stearothermophilus* ribosomal protein L5, attached by the lactone method to the solid support. d shows a typical threonine step (DABTH-Thr and its dehydrated form, ΔT) released in step 4 of the degradation of a DNA-binding protein derived from *Bacillus sulfulobus*; in this case attachment was made via the ϵ-amino groups of the lysine residues of the protein.

differentiate DABTH-isoleucine/-leucine and for additional identification of manually performed degradations, which were initially identified by polyamide thin-layer chromatography. Further, it was applied for the identification of most degradation steps, obtained from solid-phase sequencing of peptides and proteins which were isolated from ribosomes of different bacteria. It was also useful for the identification of degradation steps produced from peptides covalently bound to a solid support, but sequenced manually.

In the past, a large number of degradation steps from dozens of proteins and peptides derived from the ribosomes of Halobacteriae, Bacillus and methanogenic bacteria were identified employing this procedure [5]. The degradation cycles of peptides which were sequenced manually in the liquid

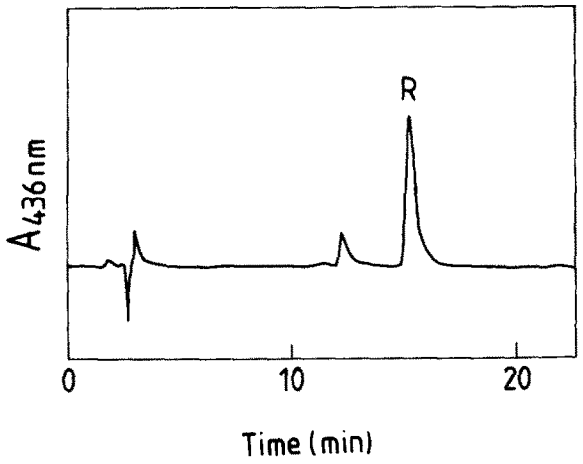

Fig.3. Identification of DABTH-arginine. After isocratic elution of all the DABTH-amino acids under the conditions in fig.1 the trapped DABTH-Arg is eluted by rinsing the column with 12 mM sodium acetate (pH 5.0)/acetonitrile/dichloroethane (20:80:0.5, by vol.). The other conditions are as given in fig.1.

phase, contained impurities which gave extra peaks on the HPLC chromatogram; on the other hand, the solid-phase degradation cycles were almost contamination-free as the extraction of by-products of the Edman reaction can be performed more efficiently if peptides are linked to solid supports.

In conclusion, it must be stressed that the simple isocratic system described suffices for the complete separation and quantitative determination of DABTH-amino acids which are generated during microsequence analysis of peptides and proteins. This holds especially true as the quick and easy identification by the sensitive polyamide thin-layer technique of these derivatives is also possible. Further, the more sophisticated and expensive HPLC gradient systems proposed before for the separation of DABTH-amino acids may then be applied to other problems, such as peptide or protein purifications. Progress in the design of detectors, the new silica-bonded alkyl supports of spherical shape and the availability of micro-bore columns will increase the resolution and sensitivity of the method. Therefore, microsequencing on femtomole quantities of polypeptides will become feasible in the near future.

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