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Determination of the unusual amino acid hypusine at the lower picomole level by derivatization with 4-dimethylaminoazobenzene-4′-sulphonyl chloride and reversed-phase high-performance or medium-pressure liquid chromatography

Dirk Bartig and Friedrich Klink

Institut für Biochemie, Universität Kiel, Olshausenstrasse 40–60, 2300 Kiel (Germany)

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ABSTRACT

Hypusine, an unusual amino acid formed by post-translational modification of lysine, is normally determined by specific metabolic labelling followed by measurement of released radioactivity after protein hydrolysis. This paper describes a sensitive non-radioactive method for the determination of hypusine, involving complete protein hydrolysis and precolumn derivatization of the released amino acids with 4-dimethylaminoazobenzene-4′-sulphonyl chloride, followed by reversed-phase high-performance or medium-pressure liquid chromatography of the dabsylated derivatives. The detection limit of hypusine was about 500 fmol. Additionally, the hypusine-containing protein from the archaebacterium *Sulfolobus acidocaldarius* was purified. By applying the dabsylation method to the analysis of tryptic peptides derived from this protein, it was possible to determine the correct positioning of the hypusine residue in the amino acid sequence, which was not possible by the amino acid sequencing procedure alone.

INTRODUCTION

The post-translationally formed amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] occurs specifically in the eukaryotic initiation factor eIF-5A (formerly eIF-4D) [1]. The biosynthesis of this amino acid is performed in two steps. Firstly, in a transferase reaction, the aminobutyl moiety of spermidine is coupled to the ε-amino group of a specific lysine residue [2]. The second step consists in the hydroxylation of the newly formed deoxyhypusine by an unusual type of dioxygenase [3]. Until a few years ago, the occurrence of hypusine was considered to be a specific feature of the eukaryotic kingdom [4]. However, it was recently demonstrated that the synthesis of deoxyhypusine and hypusine is also common to all archaebacterial species so far investigated, but not to eubacteria [5,6]. In contrast to the eukaryotic hypusine-containing protein (HP), attempts to label specifically the archaebacterial HP by various *in vivo* [4,5] or *in vitro* [7] methods have not been successful until now. Considering this and the fact that there is as yet no functional test for the archaebacterial HP, we developed a sensitive non-radioactive method for the determination of hypusine, thus allowing the purification and analysis of the archaebacterial HP from *Sulfolobus acidocaldarius*. The details of the purification procedure and other results will be published elsewhere [8]. In this paper we report a procedure for the determination of picomole amounts of the HP by a precolumn derivatization method with 4-dimethylaminoazobenzene-4′-sulphonyl chloride (DABS-Cl). The de-

Correspondence to: Dirk Bartig, Institut für Biochemie, Universität Kiel, Olshausenstrasse 40–60, 2300 Kiel, Germany.

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tection limit of this method, its applicability in high-performance (HPLC) and medium-pressure liquid chromatographic (MPLC) systems and its use for the analysis of a hypusine-containing tryptic peptide is described.

EXPERIMENTAL

Materials

DABS-Cl was purchased from Fluka (Neu-Ulm, Germany) and recrystallized from boiling acetone as described [9]. Trifluoroacetic acid (TFA) and constant-boiling (c.b.) hydrochloric acid were obtained from Sigma (Deisenhofen, Germany), Amberlite CG 120 II (200–400 mesh) from Serva (Heidelberg, Germany), acetonitrile, isopropanol and water (all of HPLC grade) from Biomol (Hamburg, Germany) and trypsin (sequencing grade) from Boehringer (Mannheim, Germany). All other chemicals (analytical-reagent grade quality) were obtained from Merck (Darmstadt, Germany). Synthetic hypusine was a kind gift from T. Shiba, Osaka University, Japan.

Chromatographic equipment

The HPLC system consisted of a Model 2248 pump, a Model 2252 controller, a Rheodyne Model 7125 injection valve, a UV-M II detector (Pharmacia–LKB, Freiburg, Germany), an ERC 7215 UV detector and an ERC 3522 degasser (ERC, Alteglofsheim, Germany).

The MPLC system included two P-500 FPLC pumps, an LCC-500 controller, an MV-7 injection valve and a UV-M detector (Pharmacia–LKB). The column used for peptide chromatography was Hypersil C_{18}, 5-μm particle size (120 × 2 mm I.D.). A Spherisorb C_{18}, 5-μm particle size column (100 × 4.2 mm I.D., plus guard column) was employed in the separation of DABS derivatives.

Analysis of tryptic peptides derived from HP

Purified HP from *S. acidocaldarius* was dissolved in 100 μl of 100 m*M* NH_4HCO_3–0.02% Tween 20 (pH 7.3) and trypsin was added in a weight ratio of 1:50 (trypsin:substrate). The reaction was performed at 37°C for 6 h and was stopped by the addition of formic acid. The peptide mixture was separated by reversed-phase HPLC using a 70-min linear gradient from 5 to 80% acetonitrile in 0.1% TFA at a flow-rate of 0.4 ml/min. The absorbance of the eluate was measured at 214 nm and peaks were collected manually.

Hydrolysis procedures

Protein amounts of more than 20 μg were hydrolysed in small flame-sealed glass tubes (60 × 4 mm I.D.) after adding 30–50 μl of c.b. HCl. Aliquots of pure HP or tryptic peptides were pipetted into the bottom of small glass tubes, dried under vacuum and then subjected to gas-phase hydrolysis in a vacuum hydrolysis vessel (Waters, Eschborn, Germany) after addition of 300 μl of c.b. HCl and applying a vacuum of $\leqslant$0.2 mbar. All hydrolyses were carried out at 110°C for 24 h.

Prepurification of hypusine

Because of the large amounts of other amino acids ($\geqslant$300-fold excess) in hydrolysates, the direct determination of hypusine required the incorporation of a prepurification step. This procedure was performed on a small Amberlite CG 120 II column (4 ml of resin, NH_4^+ form). The protein hydrolysate was bound to the resin previously equilibrated with water and eluted stepwise with 7 ml of 0.5 *M* NH_4^+ and 5 ml of 1.5 *M* NH_4^+. The eluate from the last step was collected in fractions of 0.5 ml and lyophilized.

Dabsylation procedure

The derivatization reaction was carried out in a small glass tubes (40 × 4 mm I.D.) previously pyrolysed overnight at 500°C in a muffle oven. A 4 m*M* stock solution of DABS-Cl in acetonitrile was always prepared freshly. Dried aliquots of samples or standard amino acids (2 nmol) were dissolved in 10 μl of 100 m*M* $NaHCO_3$ (pH 8.4) and 20 μl of DABS-Cl solution were added. The reaction mixture was shaken carefully, stoppered with a silicone-rubber plug and allowed to react for 10 min at 70°C. After cooling to room temperature, the solution was diluted by adding 50 m*M* phosphate (pH 7.0)–ethanol (1:1, v/v) [11,14].

Analysis of DABS derivatives

DABS–hypusine (DABS–Hpu) was separated from all other DABS derivatives using an elution system of 25 m*M* sodium acetate (pH 6.5) (buffer A) and acetonitrile (buffer B). For analysis of the hydrolysed tryptic peptides, the elution system con-

sisted of 33 m*M* sodium acetate (pH 6.3) (buffer A) and acetonitrile–isopropanol (4:1) (buffer B). DABS derivatives were detected by measuring the absorbance at 436 nm and concentrations were calculated by integration of peak areas.

RESULTS AND DISCUSSION

Reversed-phase HPLC and MPLC of DABS–Hpu

As shown in Fig. 1 DABS–Hpu is separated by reversed-phase HPLC within 25 min from all other DABS derivatives, specially from DABS–Lys, the only remaining basic amino acid after prepurification of the hydrolysates with a similar retention time to DABS–Hpu. Although the elution positions are not shown in Fig. 1, both DABS–His and DABS–Tyr would elute shortly after DABS–Lys, but still before DABS–Hpu, so that it is possible to analyse directly a hydrolysate of pure HP using this gradient system.

The determination of DABS–Hpu using a reversed-phase MPLC system is presented in Fig. 2 Owing to the lower pressure limit of the MPLC apparatus, the flow-rate had to be reduced to 0.3 ml/min, thus allowing the application of the same buffer–column system as used in reversed-phase HPLC. The disadvantage of the MPLC system is a comparatively noisy baseline, resulting in a detection limit poorer than 1 pmol of DABS–Hpu, and a much longer analysis time. However, it has been clearly demonstrated that the dabsylation method

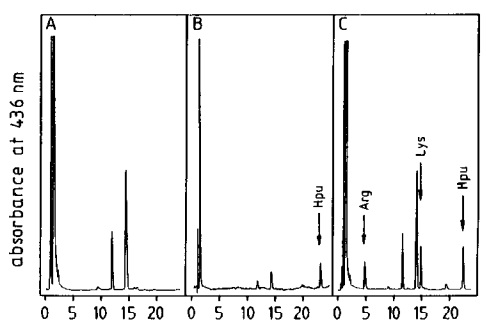

Fig. 1. Reversed-phase HPLC determination of DABS–Hpu on a Spherisorb ODS column. The column (100 × 4.2 mm I.D. plus guard column) was eluted with a 24-min linear gradient from 30–58% acetonitrile (buffer B) in 25 m*M* sodium acetate (pH 6.5) (buffer A) at a flow-rate of 1 ml/min and room temperature. (A) Blank; (B) 1 pmol of DABS–Hpu; (C) Amberlite CG 120 II prepurified protein hydrolysate.

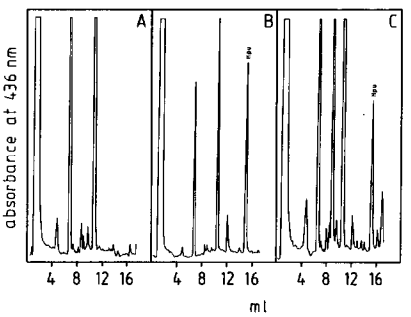

Fig. 2. Reversed-phase MPLC determination of DABS–Hpu on a Spherisorb ODS column. Column and buffers as in Fig. 1; flow-rate, 0.3 ml/min. The gradient shape was 2 ml isocratic at 35% B and then linear from 35 to 60% B in 15 ml. (A) Blank, (B) 4 pmol of DABS-Hpu; (C) Amberlite CG 120 II prepurified protein hydrolysate.

for the sensitive determination of hypusine is applicable using short HPLC columns in a system working in the medium pressure range.

Detection limit

The detection limit for DABS–Hpu in the HPLC system using the "fast" gradient run (Fig. 1) is 500 fmol of hypusine standard with an acceptable signal-to-noise ratio. This method is about 800 times more sensitive than the previously used ninhydrin system in a Biotronik LC 5001 amino acid analyser [5,6] and a ten-fold improvement on the most sensitive non-radioactive method described so far [10]. Owing to the low flow-rate resulting in a more unstable baseline, the sensitivity of the MPLC system was only half that of the HPLC system, although amounts of $\geqslant 1$ pmol DABS–Hpu could be readily determined. The overall detection limit for the determination of hypusine in a hydrolysate originating from pure HP or hypusine-containing peptides was about ten times lower in relation to hypusine standard as a maximum of 10% of the reaction mixture should be injected for analysis. Injections of larger amounts of reaction mixture impaired the resolution owing to the presence of interfering by-products.

The relationship between the amount of DABS–Hpu injected and the resulting peak area was linear over a broad range (Fig. 3). The detector response showed good reproducibility, with a maximum relative standard deviation of 2.4% ($n = 3$). The

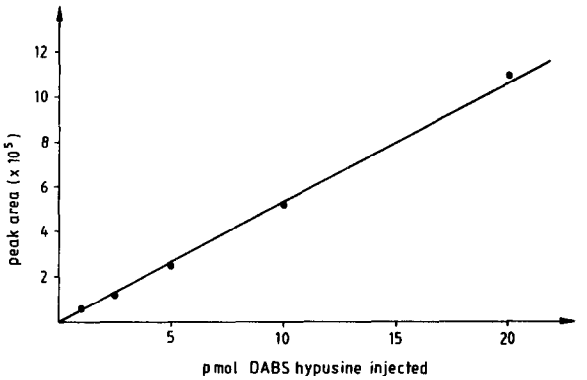

Fig. 3. Concentration–peak area relationship for different amounts of injected DABS–Hpu.

relative standard deviation for the retention time of DABS–Hpu was 0.26% (n = 12).

Discussing the detection limit of the hypusine determination in hydrolysates deriving from a mixture of proteins, the influence of the necessary prepurification step must also be considered. This prepurification procedure for hydrolysates was unavoidable if hypusine was present in only trace amounts compared with the other amino acids in the reaction mixture. Molar ratios above 1:300 (hypusine to other amino acids) resulted in incomplete derivatization of hypusine and other amino acids, because a critical excess of reagent over derivatizable groups is necessary in order to avoid artefact peaks in the reversed-phase chromatogram. On the other hand, large amounts of reagent in the injected sample gave rise to an unstable baseline, thus disturbing the determination of picomole amounts of hypusine. To solve this problem, the bulk of contaminating amino acids was eliminated by chromatography on a small Amberlite CG 120 II ion-exchange column. Most amino acids were eluted by the first step (0.5 M NH_4^+). Because of its basic properties, hypusine was bound very tightly to the resin and could be separated from all other amino acids by increasing the NH_4^+ concentration in the second elution step. Only minor amounts of the basic amino acids lysine and arginine were found in the hypusine-containing fraction(s). The use of this prepurification step was limited by the fact that the chromatography of very small amounts of hypusine on the Amberlite column resulted in loss of hypusine, probably caused by irreversible binding of hypusine to the resin or by dilution effects during elution. Lowering the volume of the column to minimize this problem resulted in a decrease in resolving power. The system described here can therefore be used in the purification of a minimum of 20 pmol of hypusine (equivalent to the same amount of HP) if the hydrolysate has to be prepurified. Screening other ion-exchange materials for the prepurification step may lead to the possibility of chromatographing smaller amounts of hypusine, thus enabling more advantage to be taken of the detection limit of the reversed-phase HPLC system.

Analysis of the hypusine-containing tryptic peptide.

As hypusine is formed by post-translational modification of a lysine residue, sequencing of the HP-gene can only reveal the position of the codon for the potential hypusine precursor. Moreover, direct amino acid sequencing yielded no signal for released hypusine, owing to the strong hydrophobicity of the PTH–hypusine [12]. Hence, the only way to verify the correct position of hypusine in the sequence is by comparison of sequence data with amino acid composition of defined short peptides (including the hypusine residue) resulting from chemical or enzymatic cleavage of HP.

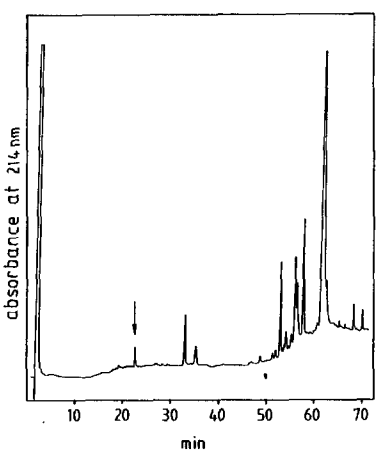

Fig. 4. Reversed-phase HPLC of tryptic peptides derived from HP of *S. acidocaldarius*. The fragments resulting from a tryptic digest of 10 μg of HP were separated on a Hypersil C_{18} column (120 × 2 mm I.D.) with a linear 70-min gradient from 5 to 80% acetonitrile in 0.1% TFA at a flow-rate of 0.4 ml/min. Peaks were collected manually. The tryptic peptide containing hypusine is marked with an arrow.

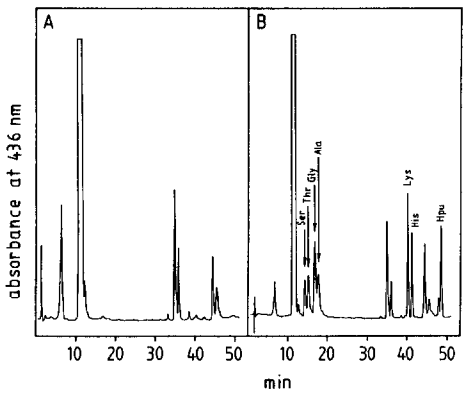

Fig. 5. Reversed-phase HPLC of DABS–amino acids resulting from the hypusine-containing tryptic peptide. The separated tryptic fragments (see Fig. 4) were lyophilized, gas-phase hydrolysed and dabsylated. The DABS derivatives were determined using a Spherisorb C_{18} column with an elution system of 33 m*M* sodium acetate (pH 6.3) (buffer A) and acetonitrile–isopropanol (4:1) (buffer B). The gradient was 2 min at 15% B, 3 min from 15 to 22% B, 9 min from 22 to 23% B, 1 min at 23% B, 5 min from 23 to 24% B, 20 min from 24 to 62% B and 2 min at 62% B at a flow-rate of 1 ml/min at room temperature. (A) Blank; (B) analysis of the hypusine-containing tryptic peptide.

Fig. 4 illustrates the separation of the hypusine-containing tryptic peptide. It can be seen that the hypusine-containing fragment was eluted in the earlier part of the gradient, thus suggesting a very hydrophilic composition of this peptide. This conclusion was confirmed by analysis of the DABS derivatives resulting from gas-phase hydrolysis of the isolated peptide. The determination of the amino acid composition of the tryptic hypusine-containing peptide is shown in Fig. 5 and Table I. The resulting sequence, after comparison of the data obtained with this method and those obtained from direct sequencing, is shown in Table II.

For the complete separation and determination of all amino acids derived from this peptide, the buffer composition and gradient shape in reversed-phase HPLC had to be altered. Because of their very similar elution behaviour in reverse-phase systems, DABS–Gly and DABS–Ala are not fully resolved, probably owing to the use of a short column. Several other workers have demonstrated the complete baseline separation of all proteinogenic DABS–amino acids using a longer column or higher column

TABLE I

AMINO ACID COMPOSITION OF THE HYPUSINE-CONTAINING TRYPTIC PEPTIDE FROM *S. ACIDOCALDARIUS*

Amino acid	Amount (pmol)	Residues
Ala	203	1
Gly	523	2
His	225	1
Lys	246	1
Ser	208	1
Thr	264	1
Hpu	220	1
Total	1889	8

TABLE II

COMPARISON OF HYPUSINE-CONTAINING TRYPTIC PEPTIDES FROM ARCHAEBACTERIA AND EUKARYOTES

Tryptic peptides derived from the HP of *S. acidocaldarius* were sequenced by direct amino acid sequencing [12] and by analysis of the amino acid composition after dabsylation. For comparison, the sequence of the corresponding eukaryotic tryptic peptide from human red blood cells is aligned (data taken from ref. 13).

Sequence[a]	Position of amino acid							
	1	2	3	4	5	6	7	8
A	Thr	Gly	Xaa[b]	His	Gly	Ser	Ala	Lys
B	Thr	Gly	Hpu	His	Gly	Ser	Ala	Lys
C	Thr	Gly	Hpu	His	Gly	His	Ala	Lys

[a] A = Sequence resulting from direct amino acid sequencing of the tryptic peptide from *S. acidocaldarius*. B = Sequence resulting from comparison of (A) with amino acid composition after analysis of DABS derivatives from this peptide (see Table I). C = Sequence of the corresponding eukaryotic peptide.

[b] Xaa = No signal during sequencing procedure.

temperatures [11,14–17]. It should thus be possible to combine and adapt these methods for the simultaneous determination of the common amino acids and hypusine, as the elution conditions can always be adjusted such that DABS–Hpu elutes after the last common DABS derivative (DABS–Tyr).

CONCLUSIONS

Use of the DABS-Cl precolumn derivatization method followed by reversed-phase HPLC or MPLC for the determination of hypusine can replace the need for radioactive labelling of HP and hypusine. This allows the isolation and characterization of further HPs from other archaebacteria on an analytical scale, as only picomole amounts of HP are needed for the determination of hypusine. In combination with narrow-bore reversed-phase HPLC, tryptic peptides from nanogram amounts of different HPs can be analysed for amino acid composition and compared with reference to the conservation of the special hypusine-surrounding sequence.

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