MICROSEQUENCE ANALYSIS: IV. AUTOMATIC LIQUID-PHASE SEQUENCING USING DABITC

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Received 2 October 1979

1. Introduction

Since the introduction of automatic liquid-phase sequencing by Edman and Begg [1], methods for either increasing the number of residues determined per run [2–4] or for the sequence analysis of limited amounts of material, 'microsequencing' (<20 nmol), have been reported [5–7]. However, the greater proportion of recent publications indicate that most laboratories are still starting with 50–500 nmol of material in the liquid-phase sequencer and most, if not all, instruments have remained with the reagent phenylisothiocyanate. The recently introduced methods for microsequencing with [^{35}S]PITC [7] or [^{125}I]PITC [8] have attempted to circumvent the main drawback of the reagent, i.e., the difficulty inherent in PTH-amino acid identification at the micro-level. Although various isothiocyanate reagents have been commercially available for some time, there has been little or no attempt to find a suitable substitute. The introduction of DABITC [9] and its successful application to manual liquid- [10,11] and solid-phase [12,13] sequence analysis suggested that the adaption of this reagent to automatic sequencers might be feasible [9]. In the preceeding articles we have shown how HPLC can be employed for peptide separation at the micro-level [14], DABTH detection at the 100 pmol level [15] and the application of DABITC to automatic solid-phase sequencing [16]. Here the use of DABITC in automatic liquid-phase sequencing is presented.

Abbreviations: ANS, 2-amino-1,5-naphthalenedisulfonic acid; DABITC, 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate; DABTH, 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoin; DABTZ, 4-*N,N*-dimethylaminoazobenzene 4′-thiazolinone; DMF, dimethylformamide; DMSO, dimethylsulfoxide; EDC, 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide hydrochloride; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PITC, phenylisothiocyanate; polybrene, hexadimethrin bromide; PTH, phenylthiohydantoin; TLC, thin-layer chromatography

2. Materials and methods

Automatic sequencing was performed with a Beckman Sequencer model 890B (updated). The program employed (see fig.1) was a slightly altered form of the Beckman protein program 060275. Reagents and solvents (sequencer grade and from Fluka, unless otherwise noted) were arranged in the following manner: R_1-PITC, 5% in heptane; R_2-Quadrol, 0.25 M; R_3-DABITC (purum), 0.5% in DMF (puriss); R_4-empty; R_5-HFBA; S_1-benzene (Merck); S_2-butylacetate (puriss); and S_3-butylchloride.

Due to the instability of DABITC in solution, an attempt was made to reduce the volumes of reagent which would be exposed to light and/or remain standing in tubing for extended times. Thus, the bottle for R_3 was removed from its normal position within the access door to the vacuum pumps, mounted outside of the sequencer (on the left side) and the tubing to the 3-way R_3–S_3 valve was replaced with a shorter, smaller diameter teflon tube. A test tube containing a freshly prepared DABITC solution, sufficient for 20–30 cycles (~350 μl/cycle), was placed in the reagent bottle, the teflon tube inserted

into the solution and the system closed. Flushing with N_2 and setting the N_2 flowrate through the bottle was performed as normal; the reagent bottle, as well as the teflon tube to the 3-way valve, was wrapped in aluminium foil. The cell temperature was maintained between 57–59°C and the DABTZs dried down from chlorobutane just prior to conversion.

Peptides of known concentrations and compositions, determined by analysis on a Durrum D-500 analyzer following 6 N HCl hydrolysis, were sequenced either in the unmodified form, after reaction with EDC and ANS [17], or in the presence of polybrene which had been cleaned by chromatography over the TFA-form of Dowex-1 (G. Frank, personal communication). The samples were dried using the Beckman application program 031872 and sequencing started immediately. Conversion of DABTZ- to DABTH-derivatives was according [1] in 1 N HCl or with 20% TFA as described [18]. It should be noted that both the 1 N HCl–methanol [19] and the acetic acid saturated with HCl [9] conversion methods have given extremely variable results. HPLC and HPTLC identifications were performed as in [15].

The synthetic peptides RKDVY, RVYIHPF, GEGFLGFL, and WAGGNASGE were kindly supplied by Dr P. Grogg, Bachem, AG. The CNBr peptide of human α-globin residues 1–32, was isolated as in [14]. The peptide PS-1 was isolated as in [20]; tryptic peptides were from chicken heart mitochondrial aspartate aminotransferase (U. Hausner, P. Christen, K.J.W., unpublished results) and *Aeromonas proteolytica* aminopeptidase (K.J.W., unpublished results). Polybrene and ANS were from Aldrich and EDC from Sigma.

3. Results and discussion

The adaption of DABITC to the liquid-phase sequencer was comparatively easy once the solubility and stability characteristics of the compound were known. It was found to be totally soluble as a 0.5% solution, in benzene, butylacetate and DMF, less soluble in acetone and DMSO, and sparingly in ethanol and methanol. As judged by TLC in chloroform:ethanol (98:2) only a single spot (R_F = 0.67) was visible in freshly prepared solutions. Following 48 h in the dark under N_2 two additional spots

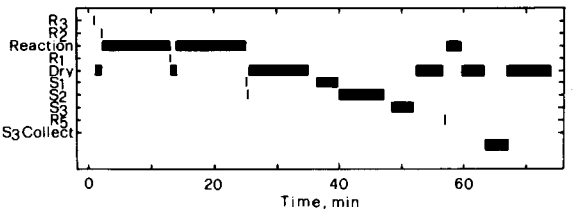

Fig.1. The liquid-phase DABITC/PITC double-couple program. The contents of each reagent and solvent bottle are described in section 2. Vent and pressurize steps, as well as the different vacuum stages for drying, have been omitted for the sake of clarity.

appeared (R_F = 0.57 and 0.27), the latter of which can approach the intensity of the DABITC spot. Only negligible degradation was noted in acetone, benzene, butylacetate and ethylacetate; slightly more occurred in DMF, DMSO and methanol.

In designing the program given in fig.1 it was found that consecutive washes with benzene (S_1) and butylacetate (S_2) were not sufficient to remove the excess DABITC and thus the chlorobutane (S_3) wash

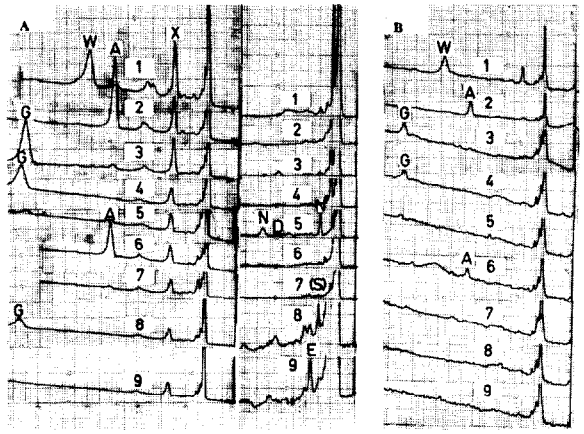

Fig.2. Isocratic HPLC analysis from the liquid-phase degradation of the peptide WAGGNASGE with DABITC (A) and PITC (B). Degradations of the peptide (6.2 nmol) were performed in the presence of polybrene (3 mg) using for (A) the program in fig.1. For (B) the Beckman program 060275 was used with a 5% PITC solution in *n*-heptane. Conversions were in 1 N HCl and the DABTHs extracted into ethylacetate and dried under N_2 at 40°C. The detection wavelengths are 436 nm and 254 nm for (A) and (B), respectively; chromatography was performed as in [15].

was added. The 2 s pulse of DABITC (R_3) followed by 90 s of restricted vacuum delivers ~350 μl of the 0.5% solution into the reaction cell. Neither the addition of a larger volume (≤6 s) nor increasing the concentration of the DABITC to 1.0% were found to improve the results. The double-couple, i.e., with PITC, has been included since it appears to be necessary when performing manual DABITC degradations [11]; no attempt was made to find conditions which might remove this step. The cleavage with HFBA was left as given in the Beckman program (120 s).

A comparison of the results from the degradation of a nonapeptide at the 6 nmol level with DABITC (fig.2A) and PITC (fig.2B) illustrates the usefulness of this system. In the presence of polybrene the DABTH-Gly_8 was readily identifiable whereas the PTH-Ala_6 was questionable. As reported [21,22] the necessity of polybrene was absolute, i.e., the peptide was washed out after 4 cycles in its absence (results not shown). The modification of the peptide with ANS–EDC [17] prior to the start of sequencing yielded results similar to those where polybrene was omitted. Attempts to sequence with 'uncleaned' polybrene failed due to the presence of a huge artifact peak which eluted in the position marked X on the first apolar chromatogram in fig.2A. Note that peak X remains more or less constant throughout the DABITC experiment (fig.2A) but has totally disappeared following the first cycle with PITC (fig.2B). At the present there is no explanation for this observation.

The degradation of 18 nmol of the amino-terminal 32 residue-long CNBr peptide from the α-chain of human hemoglobin, pool C in [14], using DABITC, is illustrated in fig.3. Although positive identification of Ala_{28} and Leu_{29} is possible there are a number of

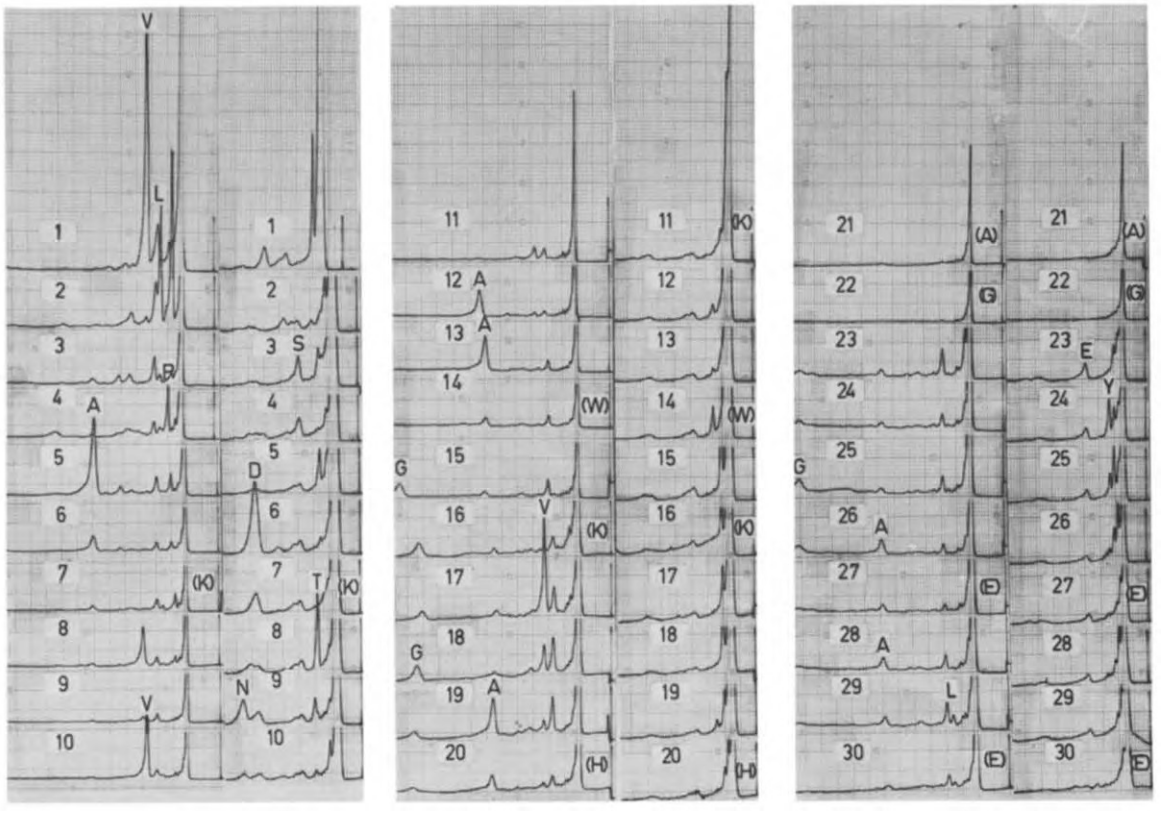

Fig.3. Isocratic HPLC analysis from the DABITC sequencing of the amino-terminal CNBr peptide of human α-globin. 17.9 nmol peptide, isolated as in [14], was degraded for 33 cycles (the peptide is 32 residues long), in the presence of 3 mg polybrene. Conversions were performed as in fig.2 and the HPLC effluent monitored at 436 nm as in [15].

'gaps' existing. The absences of Lys at positions 7, 11 and 16 and the His at 20 are clear since they cannot be identified using the isocratic system [15]. The appearance of an additional peak on the apolar column, eluting where the DABTH-Phe elutes, is most likely the DABTH-Thr$^{\Delta}$ derivative [23]. Absence of peaks at positions 14 (Trp), 17 (Glu) and 30 (Glu) is due to destruction during conversion and can be prevented to a large degree by converting either automatically (see results in fig.2 of [16]) or manually as soon as possible following DABTZ formation. Due to an instrument failure no identifications could be made for positions 21 (Ala) and 22 (Gly). The residues at 30 (Arg) and the carboxy-terminal 31 (homoserine/homoserine lactone) were analyzed and, as expected, neither derivative was identified. The results from various sequencing attempts on different peptides using the described system are summarized in table 1. Although polybrene was included at the beginning of each experiment, two of the peptides were quite readily lost which supports the notion that it is still not possible to sequence all peptides automatically at the < 10 nmol level. Sperm whale myoglobin and α-lactoglobulin, as well as other proteins, have been sequenced at the < 20 nmol level with excellent results. This suggests that the described DABITC–Quadrol program (fig.1) is also applicable to protein sequencing in the low nanomolar range and experimentation is underway to clarify this point. In summary, we have shown that the reagent DABITC can be easily substituted for PITC in the liquid-phase sequencer and employed for peptide–protein sequencing at the micro-level in the presence of polybrene. In conjunction with the micro-HPLC methods for peptide isolation [14], DABTH-amino acid identification [15], and automatic DABITC solid-phase methodology [16] two of the inconveniences inherent to protein sequencing have been reduced; that is:

(i) The time involved in peptide fractionation or isolation;
(ii) The sensitivity or detection limits at the various stages of sequencing.

Acknowledgements

The authors would like to thank H. Lutz and K. Rodger for their 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).

Table 1
Peptides sequenced by the automatic DABITC liquid-phase system

Peptide[a]	Amount[b] (nmol)	Source[c]
RKDVY	5.7	Synthetic
RV (YIHPF)	8.7	Synthetic
GE (GFLGFL)	7.8	Synthetic
SENNFQP (K)	12.8	AAP
WAGGNASGE	6.2	Synthetic
GSQDLANQY (K)	10.2	AAP
IASLILNTPEL (R)	17.5	AAT
ASAELALGENSEAF (K) SG (R)	19.0	AAT
SLLL (H) ACA (H) NPTGVDP (R)	14.4	AAT
D$^{V}_{L}$PSANANANNQ (R) TAAA (K) PQANAQA (SS)	16.2	PS-1
VLSPAD (K) TNV (K) AA (W) G (K) VGA (H) AGEYGA (E) AL (ERM)	17.9	α-globin

[a] The parenthesis denote those portions of the sequences which were not identified. All experiments were performed in the presence of polybrene (3 mg)
[b] Determined by amino acid analysis, see section 2
[c] AAP and AAT are abbreviations for *Aeromonas* aminopeptidase and aspartate aminotransferase, see section 2

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