Isolation and characterization of polypeptide at the picomole level

Pre-column formation of peptide derivatives with dimethylaminoazobenzene isothiocyanate

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Polypeptides coupled with dimethylaminoazobenzene isothiocyanate through their amino groups to form dimethylaminoazobenzenethiocarbamoyl- (DABTC-)peptides can be separated by reversed-phase high-pressure liquid chromatography and detected in the visible region (436 nm). As little as 1 ng (2 pmol) of a DABTC-pentapeptide can be identified against a stable base-line with the signal-to-noise ratio of 10. The DABTC-peptides can also be recovered from the column, and their *N*-terminal amino acids (obtained by direct treatment with aqueous acid) and amino acid compositions and sequences can be all analysed at the picomole level. The power of this method is demonstrated by the complete separation and characterization of model peptides, peptide hormones and peptides derived from enzymic fragmentation of proteins. This new technique should provide a sensitive and efficient tool for peptide analysis at the nanogram level.

Isolation and characterization of polypeptides at the picomole level relies on a sensitive and non-destructive detective system. Detection based on the absorption by the amide bond in the u.v. region (200–230 nm) (Hancock *et al.*, 1978; Fullmer & Wasserman, 1979) is non-destructive, but the sensitivity is limited in practice to 1 nmol because of the solvent interference. The sensitivity based on the fluorescent reagents (Lai, 1977; Rubinstein *et al.*, 1979) is at the low-picomole level, but the fluorophore-reacted peptide is no longer suitable for further structure analysis.

Dimethylaminoazobenzene isothiocyanate (DABITC) (Chang *et al.*, 1976; Chang, 1977) is a modified Edman reagent, a coloured compound for both sequence (Chang *et al.*, 1978; Chang, 1979) and *N*-terminal (Chang, 1980) analysis of polypeptide. Pre-column formation of the polypeptide derivative with DABITC, followed by separation of DABTC-peptide on reversed-phase h.p.l.c., offers the following advantages: (1) incorporation of a hydrophobic DABITC group on to a generally hydrophilic peptide enables the peptide to be a suitable candidate for reversed-phase h.p.l.c., whose

mechanism of separation is mainly based on the hydrophobic interaction (Horvath *et al.*, 1976); (2) introduction of a dimethylaminoazobenzene chromophore enables the peptide derivative to be detected in the visible region at less than 5 pmol sensitivity; (3) the DABTC-peptides can be recovered, and their structure, including *N*-termini and amino acid compositions and sequences, can be determined.

The potential of this new technique is demonstrated by the isolation of a 20-residue peptide from 500 pmol (8.5 µg) of sperm-whale myoglobin tryptic digest. Sequence analysis of this peptide (corresponding to residues 119–139 of the intact myoglobin) by the micro DABITC/PITC method (Chang, 1981) confirms that residue 122 is aspartic acid (Herrera & Lehmann, 1974) instead of asparagine (Edmundson, 1965).

Materials of methods

Materials

The sources of DABITC, pyridine and trifluoroacetic acid (sequential grade) as well as other chemicals used in both *N*-terminal amino acid and *N*-terminal sequence analysis are described in the accompanying paper (Chang, 1981). Polypeptides and peptide hormones were supplied by Sigma (dipeptides, glucagon, neurotensin, enkephalins, angiotensin I and angiotensin II), Serva (oxidized

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; DABITC, dimethylaminoazobenzene isothiocyanate; DABTC, dimethylaminoazobenzenethiocarbamoyl; DABTH, dimethylaminoazobenzene-thiohydantoin; PITC, phenyl isothiocyanate.

insulin A- and B-chains, vasopressins) or Beckman (sperm-whale apomyoglobin). Other chemicals were commercial analytical grade provided by Fluka and by Merck.

Instrumentation

The h.p.l.c. system (Waters Associates) comprises two 6000A pumps, a 720-system controller, a U6K sample injector and a model 440 detector (fixed wavelength at 436 nm). Sensitivity of the detector is routinely set at 0.01 AUFS (absorption unit, full scale) unless otherwise indicated. DABTC-peptides were separated on a μ -Bondapak C-18 column (Waters) and DABTH derivatives of amino acids were analysed on a Zorbax-ODS column (Dupont). Sample sizes of 5–25 μ l were usually injected.

Irreversible blocking of protein amino groups with methyl isocyanate

Sperm-whale apomyoglobin (50 μ g; approx. 3 nmol) was dissolved in 150 μ l of aq. 50% (v/v) pyridine, and 30 μ l of methyl isocyanate (98%; Fluka) was added. After incubation at 53°C for 30 min, the excess of reagent and organic solvent were extracted with 3 $\times$ 500 μ l of heptane/ethyl acetate (2:1, v/v). The aqueous phase was freeze-dried, and the dried sample was used for enzymic digestion. Methyl isocyanate was chosen as blocking reagent for several reasons: (1) the reagent is volatile (b.p. 36–38°C) and can be removed under reduced pressure; (2) the reagent irreversibly blocks the original *N*-terminus, thus leaving one less DABTC-peptide after enzyme digestion; (3) the methyl isocyanate-modified myoglobin is completely soluble in water.

Enzyme digestion of proteins

Digestions of proteins (1–3 nmol) by both trypsin (Merck) and staphylococcal (*Staphylococcus aureus*) proteinase V8 (Miles) were performed in 100 μ l of 0.1 M-ammonium bicarbonate buffer, pH 8, at 38°C for 20 h. The substrate/enzyme weight ratio was 25:1. Digested proteins were freeze-dried overnight.

Coupling of peptide with DABITC

Peptide or peptide mixture (1–2 nmol) was dissolved in 20 μ l of water, and 40 μ l of DABITC solution (2 mg/ml in pyridine, freshly prepared) was added. The mixture was heated at 70°C for 1 h. Excess of DABITC and by-products were extracted by mixing the reaction mixture with five 250 μ l portions of heptane/ethyl acetate (2:1, v/v) in a vortex mixer and centrifuging. The organic phase was removed by a 5 ml syringe. Hydrophilic DABTC-peptides remain soluble in the aqueous phase, whereas hydrophobic DABTC-peptides tend to form a precipitate at the interface. The DABTC-peptides were freeze-dried and redissolved in a suitable volume of aq. 50%

(v/v) acetonitrile for h.p.l.c. analysis. Some very hydrophobic DABTC-peptides are only soluble in aq. 50% (v/v) pyridine.

Recovery of DABTC-peptide from h.p.l.c.

Depending on the sharpness of the peak, most DABTC-peptides were eluted within the volume 200–500 μ l. They were collected with a RediRac fraction collector (LKB) at 50–100 μ l/fraction. For partially overlapping DABTC-peptides, manually controlled collection is recommended. Since it is difficult to measure the absorbance of each tube after collection, a precise timing and measurement of the dead volume between the collector and flow cell of the detector should first be established. The collected DABTC-peptides were dried *in vacuo* and stored at –20°C for further structure analysis.

N-Terminal amino acid analysis of DABTC-peptide before or after h.p.l.c. separation

The dried DABTC-peptide (200–400 pmol) was dissolved in 20 μ l of water, and 40 μ l of acetic acid saturated with HCl was added; the sample was incubated at 53°C for 50 min. The aqueous acid was evaporated, and the sample (containing the released amino acid DABTH derivative and shortened peptide) was dissolved in 100–200 μ l of 70% (v/v) ethanol for h.p.l.c. analysis of amino acid DABTH derivatives (Chang, 1981). However, with this protocol, a shortened peptide with a lysine side chain also coupled with DABITC could possibly interfere with the h.p.l.c. analysis. An alternative procedure to circumvent this is as follows. The dried acid sample was dissolved in 80 μ l of water, and the mixture of amino acid DABTH derivatives was extracted into 80 μ l of butyl acetate. The bottom aqueous phase (after centrifugation) was removed by a micro-syringe, and the organic phase was evaporated off and the residue was redissolved in 100–200 μ l of 50% acetonitrile for h.p.l.c. analysis of amino acid DABTH derivatives. The cleavage of *N*-terminal amino acid could also be performed in aq. 50% (w/v) trifluoroacetic acid. However, in some cases the yield of amino acid DABTH derivatives by this method was unsatisfactory, for unknown reasons.

Amino acid analysis of DABTC-peptide recovered from h.p.l.c.

The dried DABTC-peptide (0.5–1 nmol) was dissolved in 30 μ l of 6 M-HCl and transferred to a hydrolysis tube (3 mm internal diam. $\times$ 10 cm) with a micro-syringe. The tube was sealed under vacuum, and hydrolysis was performed at 110°C for 24 h. The amino acid composition of the DABTC-peptide was analysed by using either a standard analyser (Biotronik, LC 6000E) or a newly developed dimethylaminoazobenzenesulphonyl chloride method

(Chang *et al.*, 1981). The *N*-terminal amino acid of DABTC-peptide was normally quantitatively destroyed (Table 1).

N-Terminal sequence analysis of DABTC-peptide recovered from h.p.l.c.

The dried DABTC-peptide (0.5–1 nmol) was dissolved in 100 μ l of anhydrous trifluoroacetic acid and transferred with a micro-syringe to a sequencing tube (Chang, 1981). The *N*-terminal sequence of the DABTC-peptide was determined by the DABITC/PITC double-coupling method in accordance with the protocol described in the accompanying paper (Chang, 1981). The first cycle was started with anhydrous acid cleavage.

Results

Formation of peptide derivative with DABITC

The structure of DABTC-peptide is presented in Scheme 1. To release the *N*-terminal amino acid, two pathways are available. In the single *N*-terminal analysis (Chang, 1980), DABTC-peptide is treated directly with aqueous acid, and the *N*-terminal amino acid is obtained as amino acid DABTH

derivative (pathway 1). In the extended sequence determination (Chang, 1981), a separated cleavage (in anhydrous acid) and conversion (in aqueous acid) reaction should be used.

One of the main concerns with the column separation of DABTC-peptides is the stability of the thiocarbamoyl bond. Although there is no detectable degradation of DABTC-peptide during the chromatographic run with aqueous buffer at pH 5, the recovery of DABTC-peptide (as judged by *N*-terminal analysis) is usually only 30–70%. It is probable that oxidation of thiocarbamoyl bond (Ilse & Edman, 1963) may occur during the chromatographic run. Another concern is the extraction of excess of reagent (which is mainly composed of DABITC and its hydrolysed amino product) after formation of the derivative. Under the conditions described in the Materials and methods section approx. 100–150 pmol of reagent excess will remain unextracted. These excess reagents generally have a greater retention time than most DABTC-peptides and therefore do not interfere with DABTC-peptide identification. However, it is recommended that a blank run be performed with each new gradient system used.

Scheme 1. Formation of the DABTC-peptide and the release of *N*-terminal amino acid as the DABTH derivative from the DABTC-peptide

For full experimental details see the text.

Separation of DABTC derivatives of peptides and of peptide hormones

DABTC derivatives of dipeptides (Fig. 1) and of peptide hormones (Figs. 2 and 3) are readily separated by h.p.l.c. at the sensitivity level of 2–5 pmol. Resolution of overlapping DABTC-peptides can be improved by a slight change of buffer or gradient systems. For example, DABTC-Ala-Tyr can be completely separated from DABTC-Ala-His (Fig. 1) by simply decreasing the concentration of acetate buffer to 5 mM or by expanding the gradient system from 20% solvent B to 75% solvent B in 15 min (results not shown). The results (Fig. 2) also demonstrate that h.p.l.c. can separate DABTC-peptides with only one amino acid substitution, e.g. those between [Met]enkephalin and [Leu]enkephalin and between [Lys]vasopressin and [Arg]vasopressin.

An exhaustive procedure (70°C for 1 h) is necessary for formation of derivatives of peptides that contain lysine side chains, since the ϵ -amino group is less reactive than the α -amino group towards DABTC in the aqueous pyridine. Incomplete formation of the glucagon derivative (containing only one lysine residue, at position 12) resulted in two peaks (Fig. 4). These two peaks were collected (500 pmol) and characterized. The collected fractions were dried, incubated with aqueous acid to release the *N*-termini, redissolved in 70% ethanol and analysed by polyamide t.l.c. (Chang *et al.*, 1978). Peak 1 gives only one red colour spot of DABTH-His. Peak 2 shows, in addition to DABTH-His, a blue tailing spot along the direction of solvent 1 (33% acetic acid). These results suggest that peak 1 contains α -DABTC-glucagon and peak 2 contains $\alpha\epsilon$ -bis-DABTC-glucagon. A similar result has also been observed with insulin B-chain, which contains only one lysine residue.

Isolation and characterization of insulin peptide

Insulin contains four glutamic acid residues (two in the A-chain and two in the B-chain). Digestion of oxidized insulin by staphylococcal proteinase V8 (an enzyme specifically cleaving the glutamyl bond at pH 8; Houmard & Drapeau, 1972) should generate six peptides. Separation of the DABTC coupled insulin digest by two solvent systems (Fig. 5) revealed only five major peptides (A1 and A2 of the A-chain, and B1, B2 and B3 of the B-chain) and one minor peptide (A3). Those DABTC-peptides were collected and their *N*-terminal amino acids (Fig. 6) and amino acid compositions (Table 1) were determined. The results indicate that peptide A1 is Asn-Tyr-Cya-Asn (residues 18–21 of the A-chain), peptide A2 is Gly-Ile-Val-Glu (residues 1–4 of the A-chain), peptide B1 is Ala-Leu-Tyr-Leu-Val-Cya-Gly-Glu (residues 14–21 of the B-chain), peptide B2 is Phe-Val-Asn-Gln-His-Leu-Cya-Gly-Ser-His-Leu-Val-Glu (residues 1–13 of the B-chain) and peptide B3 is Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (residues 22–30 of the B-chain). The *N*-terminus of peptide A3 is leucine. It is clear that all four glutamyl bonds were nearly quantitatively cleaved and there is also a partial cleavage between peptide bond of residues 12–13 of the A-chain (Chang, 1980). The absence of the peptide corresponding to residues 5–17 of the A-chain is apparently due to the cyclization (*N*-terminal blocking) of its *N*-terminus glutamine (Doolittle, 1972).

Separation of these five DABTC-peptides was achieved within 18 min, and the loading capacity of each run can be varied from 5 pmol (analytical run) to 1 nmol (preparative run). Owing to the sharpness of the peaks, each DABTC-peptide was only collected in a volume of 200–300 μ l. A 200–300 pmol sample of the collected DABTC-peptide is sufficient for *N*-terminal analysis. However, 0.5–

Fig. 1. Separation of approx. 10 pmol of DABTC-dipeptides on a reversed-phase column (μ -Bondapak C-18). Solvent A was 10 mM-acetate buffer, pH 5.0, solvent B was acetonitrile, and the gradient was from 25% solvent B to 50% solvent B in 10 min (linear). The flow rate was 1 ml/min. The column temperature was 22°C. The chart speed was 40 cm/h. Other experimental details are given in the text. Key: AD, DABTC-Ala-Asp; AS, DABTC-Ala-Ser; PA, DABTC-Pro-Ala; AA, DABTC-Ala-Ala; AY, DABTC-Ala-Tyr; AH, DABTC-Ala-His; RY, DABTC-Arg-Tyr; AI, DABTC-Ala-Ile; WL, DABTC-Trp-Leu.

Fig. 2. Separation of approx. 10 pmol (5–10 ng) each of DABTC-peptide hormones on h.p.l.c.

The quantities are based on the weight claimed by the suppliers (Sigma and Serva) without correction against extraction losses. Solvent A was 35 mM-acetate buffer, pH 5.0, solvent B was acetonitrile, and the gradient was from 20% solvent B to 85% solvent B in 15 min (linear). The flow rate was 1 ml/min. The column temperature was 22°C. The chart speed was 40 cm/h. Other experimental details are given in the text. Key: YGGFM, DABTC-Tyr-Gly-Gly-Phe-Met; YGGFL, DABTC-Tyr-Gly-Gly-Phe-Leu.

Fig. 3. Separation of 12.5 pmol of DABTC derivatives of oxidized insulin A-chain and B-chain and glucagon on a μ -Bondapak C-18 column

The quantities are based on the amino acid analysis without correction for extraction losses. Conditions for column chromatography were as described in Fig. 2 except for the use of a different gradient system of 20% solvent B to 75% solvent B in 15 min (linear).

1 nmol is needed for determination of amino acid composition, since contamination (notably glutamic acid and glycine) becomes significant when less material was used.

Isolation and characterization of sperm-whale myoglobin tryptic peptides

The primary structure of sperm-whale myoglobin was first determined by Edmundson (1965), but residue 122 was later reported to be aspartic acid (Herrera & Lehmann, 1974) instead of asparagine. To resolve this ambiguity, an attempt was made to isolate and sequence the peptide containing residue 122 by the DABITC method. Searching for this peptide from a tryptic mixture (a total of more than 23 peptides) has proved to be laborious. However, a

selective cleavage at arginine residues is an efficient approach. Myoglobin contains four arginine residues and one of them is located at position 118. An irreversible blocking of *N*-terminal amino acid and lysine side chains of myoglobin, followed by trypsin digestion, should give five peptides, but only four with free *N*-terminal amino acid residues, these being leucine, phenylalanine, histidine and lysine respectively. A 50 μ g (3 nmol) sample of apomyoglobin was treated with methyl isocyanate, digested with trypsin and then coupled with DABITC. One-tenth of the DABTC-peptides mixture was removed for *N*-terminal analysis by direct incubation with aqueous acid solution (see the Materials and methods section) and, as expected, only four major *N*-terminal amino acids were found. An optimum

Fig. 4. Separation of α -DABTC-glucagon and α -bis-DABTC-glucagon (peak 2) by h.p.l.c.

To achieve predominance of bis-DABTC-glucagon requires a more exhaustive procedure for formation of the polypeptide derivative. Conditions for derivative formation were: (a) 53°C, 1.5h, DABITC concentration 1mM, DABITC/glucagon molar ratio 10:1; (b) 53°C, 1.5h, DABITC concentration 4mM, DABITC/glucagon molar ratio 40:1; (c) 70°C, 1h, DABITC concentration 10mM, DABITC/glucagon molar ratio 100:1. Conditions for column chromatography were as described in the legend to Fig. 3.

condition for separating those DABTC-peptides (Fig. 7) was established by using less than 3% (100pmol) of the total materials. Accordingly, a preparative separation for characterizing the *N*-terminal amino acid of each DABTC-peptide was performed with 300pmol of the sample. Peak 3 material (Fig. 7) was found to possess histidine as the *N*-terminus and thus should contain the peptide carrying residues 119–139 of the intact myoglobin. Finally, another preparative run (500pmol) was performed to collect the DABTC-peptide in peak 3 for sequence analysis by the micro DABITC/PITC method (Chang, 1981). After four cycles of degradation, the *N*-terminal sequence was established as His-Pro-Gly-Asp (Fig. 8). The result confirmed the findings obtained by Herrera & Lehmann (1974). The complete analysis consumed only 1.2nmol of myoglobin tryptic peptide.

Fig. 5. Separation of DABTC-peptides mixture of oxidized insulin digested by staphylococcal proteinase. Peptides A1, A2, and A3 were derived from the A-chain, and peptides B1, B2 and B3 were derived from the B-chain. All six peptides can be obtained in pure form by combined use of two solvent systems. In (a) solvent A was 20mM-phosphate buffer, pH6.03, and in (b) solvent A was 35mM-acetate buffer, pH5.0. Other conditions for column chromatography were as described in the legends to Figs. 2 and 3.

Discussion

For column chromatography of polypeptide at the picomole level, pre-column formation of peptide derivatives with DABITC offers distinct advantages over those conventional methods in which detection is based on either u.v. absorption (Hancock *et al.*, 1978; Fullmer & Wasserman, 1979) or post-column reaction with fluorescent reagents (Lai, 1977; Rubinstein *et al.*, 1979): (1) detection in the visible region completely avoids interference by u.v.-absorbing contaminants or base-line rise due to gradient change; (2) pre-column formation of derivatives limits the source of contaminations to only the sample and the coupling buffer.

The mechanism of separation of DABTC-peptides by reversed-phase h.p.l.c. is mainly based on the reversible association of the solutes with the hydrophobic ligand of the bonded stationary phase, the so-called solvophobic interaction (Horvath *et al.*, 1976; Karger *et al.*, 1976). The elution order of DABTC-peptides is therefore dependent on their hydrophobicity, with hydrophobic peptides possessing a greater capacity factor, K' (longer retention time) (Brown & Krstulovic, 1979).

Fig. 6. H.p.l.c. analysis of amino acid DABTH derivatives released from the N-termini of DABTC-peptides A1, A2, A3, B1, B2 and B3 (Fig. 5)

Approx. 500 pmol of each DABTC-peptide (except for A3) was collected, treated with aqueous acid and redissolved in 70% ethanol. Only one-twentieth to one-fifteenth of each sample was injected. Recoveries of DABTC-peptides as judged by their N-termini yield are about 40–60%. Conditions for the separation of amino acid DABTH derivatives were as follows. Solvent A was 35 mM-acetate buffer, pH 5.0, solvent B was acetonitrile, and the gradient was 42% solvent B to 70% solvent B in 10 min (linear). The column was Zorbax-ODS. The flow rate was 1 ml/min. The column temperature was 22°C. The chart speed was 40 cm/h. Other experimental details are given in the text. Key: N, DABTH-Asn; G, DABTH-Gly; L, DABTH-Leu; A, DABTH-Ala; F, DABTH-Phe; R, DABTH-Arg.

This hydrophobic interaction mechanism, however, does not entirely account for the elution behaviour of basic solutes, which usually exhibit an extraordinarily greater retention time. The result has been explained and substantiated as an interaction between the positively charged basic solute with the anionic silanol groups on the silica-gel surface of the stationary phase, and this ionic interaction can be suppressed by increasing the ionic strength (salt concentration) of the mobile phase (Schmidt *et al.*, 1980). It can therefore be predicted that increasing the salt concentration of the mobile phase would decrease the retention time of basic solute and increase the retention time of acidic solute (Table 2). In addition, increasing the pH of the mobile phase would deprotonate the carboxy group of the peptide, render the DABTC-peptide less hydrophobic and thus decrease its retention time. Thus there are a number of adjustable parameters including (1) the nature of the mobile phase as well as gradient selections (Schoenmakers *et al.*, 1979; Melander *et al.*, 1979a,b,c), (2) the nature of the stationary phase (Berendsen & Galan, 1980), (3) the temperature of the column (Gant *et al.*, 1979) etc., which could all be explored to achieve the maximum separation of a peptide mixture, and it is indeed interesting to see how a slight change of a gradient system can effect the separation of overlapping DABTC-peptides (Fig. 5).

The versatility of this separation mechanism, in combination with the high sensitivity of the DABTC-peptide detection (2–10 pmol), composes the most valuable facet of the DABTC technique. For instance, even with only 1 nmol of DABTC-peptides mixture, one can afford ten analytical runs and yet expend only 10% of the total material. The rest can therefore be used for preparative separation, and the desired peptide is collected for further structure analysis by using the micro sequencing method. This capability has been clearly demon-

Table 1. Amino acid composition of DABTC-peptides (A1, A2, B1, B2 and B3) recovered from h.p.l.c.

The peptides were derived from staphylococcal-proteinase digestion of oxidized insulin. N-Terminal amino acids of DABTC-peptides were quantitatively destroyed after acid hydrolysis, before h.p.l.c. For full experimental details see the text. Amino acids recovered at less than 0.2 mol/mol are not listed. (Cya represents cysteic acid.)

Peptide (minus N-terminus)	Amino acid composition (mol/mol)
A1*	Cya 1.12 (1), Asp 0.88 (1), Tyr 0.74 (1)
A2*	Glu 1.20 (1), Val 0.95 (1), Ile 1.10 (1)
B1*	Cya 1.13 (1), Glu 1.14 (1), Gly 1.12 (1), Val 0.86 (1), Leu 1.70 (2), Tyr 0.68 (1)
B1†	Cya 1.06 (1), Glu 1.06 (1), Gly 1.50 (1), Val 1.20 (1), Leu 1.78 (2), Tyr 0.64 (1)
B2*	Cya 1.40 (1), Asp 1.03 (1), Ser 0.99 (1), Glu 2.26 (2), Gly 1.30 (1), Val 2.09 (2), Leu 2.14 (2), His 1.97 (2)
B2†	Cya 1.05 (1), Asp and Ser (not determined), Glu 2.25 (2), Gly 1.22 (1), Val 2.25 (2), Leu 1.85 (2), His 1.78 (2)
B3*	Thr 0.97 (1), Pro 0.85 (1), Gly 1.14 (1), Ala 0.82 (1), Tyr 0.66 (1), Phe 2.40 (2), Lys 0.75 (1)

* Determined with a Biotronik analyser with approx. 1 nmol of DABTC-peptide.

† Determined by a newly developed dimethylaminoazobenzenesulphonyl chloride method (Chang *et al.*, 1981) with approx. 0.5 nmol of DABTC-peptide. Aspartic acid and serine were not separated in this system.

Fig. 7. DABTC-peptide 'map' of tryptic peptides of sperm-whale apomyoglobin selectively cleaved at arginine

The chromatograms at both analytical scale (15 pmol) (a) and preparative scale (300 pmol) (b) show the reproducibility of their separations. The *N*-terminal amino acid of each peak is indicated on the top of peak numbers (key: K, DABTH-Lys; H, DABTH-His; L, DABTH-Leu). There is no *N*-terminal amino acid found in peak 5, and peak 4 gives very low recovery of DABTH-Leu (10%). The average yield of *N*-terminal amino acids of peaks 1, 2, 3 and 6 was 50%. The peptide having phenylalanine as the *N*-terminus (73 amino acid residues) was not found, presumably owing to its insolubility in 50% acetonitrile after formation of the derivative. Conditions for column chromatography are as described in Fig. 2, except for use of a different gradient system of 30% solvent B to 70% solvent B in 30 min (linear).

strated by the isolation and characterization of a 20-residue peptide from sperm-whale myoglobin.

With the growing demand of a high-sensitivity peptide-separation technique, one may envisage several potential uses of the DABITC method in protein-chemistry research: (1) isolation of peptides from fragmented protein at the picomole level for sequence analysis; (2) a sensitive peptide-'mapping' technique; (3) isolation and characterization of physiologically interesting peptides that are available in only nanogram amounts; (4) a micro-method for antigenic structure analysis of protein antigen; for example, a defined peptides mixture was allowed to pass through the antibody (directed against the intact protein)-linked Sepharose; the bound peptides, which are presumed to carry the antigenic determinants within the intact protein, were then eluted and analysed by the DABITC method.

Despite all the merits and speculations described the DABITC method still has some limitations, as follows. (1) The recovered DABTC-peptide would almost certainly have lost its biological activity and thus is not suitable for bioassay. (2) There is a possible extraction loss for hydrophobic DABTC-dipeptides or -tripeptides during the washing out of excess of reagent. This shortcoming could be circumvented by adjusting the washing solvent with a higher proportion of *n*-heptane. (3) Peptides possessing more than two lysine residues are usually insoluble in 50% acetonitrile after coupling with DABITC and are not suitable for h.p.l.c. separation by the acetonitrile system. This limitation, how-

Fig. 8. Analysis of amino acid DABTH derivatives of the step-4 degradation from the sequence determination of DABTC-peptide carrying residues 119-139 of the intact sperm-whale myoglobin

The DABTC-peptide was collected from peak 3 of Fig. 7 and sequenced by the DABITC/PITC double-coupling method (Chang, 1981). The *N*-terminal sequence was found to be His-Pro-Gly-Asp. No trace amount of asparagine was found in step 4 (indicated by arrow), a result that confirms that residue 122 of sperm-whale myoglobin is aspartic acid (Herrera & Lehmann, 1974). Approx. 500 pmol of the DABTC-peptide was collected for sequencing and 30% of the extract of amino acid DABTH derivative of step 4 was applied. Conditions for analysing amino acid DABTH derivatives are as described in Fig. 6. Key: D, DABTH-Asp; N, DABTH-Asn. U is a by-product of the reagent (Chang, 1981).

Table 2. *Dependence of retention time of DABTC-peptide on the pH and salt concentration of solvent A of the mobile phase*
Solvent B was acetonitrile, and the gradient was from 20% solvent B to 75% solvent B in 15 min (linear). The flow rate was 1 ml/min. The column temperature was 22°C. Other experimental details are given in the text. The structures of the DABTC-peptides are as follows: A1, DABTC-Asn-Tyr-Cya-Asn; A2, DABTC-Gly-Ile-Val-Glu; A3, DABTC-Leu-Tyr-Gln-Leu-Glu; B1, DABTC-Ala-Leu-Tyr-Leu-Val-Cya-Gly-Glu; B2, DABTC-Phe-Val-Asn-Gln-His-Leu-Cya-Gly-Ser-His-Leu-Val-Glu; B3, DABTC-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (Cya represents cysteic acid).

Composition of solvent A	DABTC-peptide ...	Retention time (min)					
		A1	A2	A3	B1	B2	B3
20mm-Phosphate buffer, pH 5.0		10.44	13.38	13.83	12.90	13.40	18.93
20mm-Phosphate buffer, pH 6.03		10.00	11.61	12.38	11.96	12.38	18.30
20mm-Phosphate buffer, pH 7.05		9.90	11.07	11.94	11.70	11.70	18.15
20mm-Phosphate buffer, pH 8.10		9.60	10.70	11.62	10.65	10.50	18.50
10mm-Phosphate buffer, pH 6.03		9.30	10.95	11.70	11.10	11.85	18.38
30mm-Phosphate buffer, pH 6.03		10.28	11.85	12.60	12.23	12.56	18.06

ever, may be overcome by selection of other solvent systems for h.p.l.c. separation. For example, aqueous pyridine is a far more powerful solvent than aqueous acetonitrile for intractable peptides. (4) The recoveries of DABTC-peptides (usually assessed by *N*-terminal yield) is usually between 30 and 70%, depending on the amount applied. This low recovery may possibly be due to the oxidation of the thiocarbamoyl bond during chromatographic separation, since no precautions have been made to exclude oxygen from the system (except for degassing the aqueous buffer). If this is the case, then the recovery may be improved by addition of trace amount of reducing agent, such as mercaptoethanol, into the chromatographic solvent.

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