A complete separation of dimethylaminoazobenzenesulphonyl-amino acids

Amino acid analysis with low nanogram amounts of polypeptide with dimethylaminoazobenzenesulphonyl chloride

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A chromatographical system has been developed to give a complete baseline separation of all dimethylaminoazobenzenesulphonyl (DABS)-amino acids for high-sensitivity amino acid analysis [Chang, Knecht & Braun (1981) *Biochem. J.* **199**, 547–556]. The system, which uses a Merck RP-18 column with phosphate buffer (12 mM, pH 6.5)/acetonitrile mixture, allows reliable analysis of DABS-amino acids at the 1–2 pmol level. The accuracy of this new system is demonstrated by the composition analysis of two immunoglobulin light chains (214 amino acid residues) with differences at only three amino acid residue positions.

With the advancement of high-sensitivity sequencing techniques, there is a growing demand for amino acid analysis with sub-microgram amounts of polypeptide. For instance, when one has only 1 nmol of pure peptide, it would be desirable to spend less than 10% of the material for amino acid analysis and use the remaining part for sequence determination.

Pre-column formation of derivatives of amino acid with dimethylaminoazobenzenesulphonyl chloride (DABS-Cl) (Lin & Chang, 1975) followed by reversed-phase h.p.l.c. separation (Chang *et al.*, 1981) allows detection of DABS-amino acid at the sub-picomole level in the visible region. The efficiency and reliability of the DABS-Cl method have been demonstrated by amino acid analysis of 10–30 ng of protein hydrolysates.

The present paper describes the complete separation of all DABS-amino acids, including cysteic acid, carboxymethylcysteine, asparagine, glutamine and tryptophan, by a single gradient system. Additional amino acid analysis information is given to demonstrate the reliability of the DABS-Cl method.

Materials and methods

Chemicals and instrumentation

Details of chemicals and h.p.l.c. instrument were described previously (Chang *et al.*, 1981). Eglin C was provided by Dr. U. Seemüller (University of Munich, Munich, Germany). Immunoglobulin light

chains were derived from monoclonal antibodies raised against azobenzenearsonate (S. S. Alkan, R. Ball, J.-Y. Chang & D. G. Braun, unpublished work).

Hydrolysis of protein and dabsylation of amino acids

Those were performed in the same tube (4 mm internal diam. × 10 cm) by the procedure described previously (Chang *et al.*, 1981). It is important that an amino acid standard (250 pmol, about 0.5 µg) was also hydrolysed with the same batch of 6 M-HCl in order to correct for the possible amino acid destruction due to acid hydrolysis, notably of aspartic acid, serine, threonine and methionine. A blank sample (with the same batch of 6 M-HCl and dabsylation buffer) should also be processed to monitor the amino acid contaminations (Fig. 2).

Results and discussion

Separation of DABS-amino acids

Complete baseline separation of all DABS-amino acids is shown in Fig. 1. The findings that led to these selected conditions will be useful to those who are interested in establishing this technique. They are as follows: (1) the Merck Lichrosorb RP-18 column gave a much sharper peak of DABS-arginine than did the Dupont Zorbax-ODS column, perhaps owing to the lower silanol content on the stationary phase; (2) the Bio-Rad Bio-Sil ODS-5S column gave a similar separation pattern of DABS-amino acids to that with the Merck RP-18 column; (3) higher column temperature generally gave sharper

Fig. 1. Separation of 21 DABS-amino acids (5 pmol each) on a Merck RP-18 column

Solvent A was phosphate buffer (12 mM, pH 6.5) containing 4% (v/v) of dimethylformamide. Solvent B was acetonitrile containing 4% (v/v) of dimethylformamide. The gradient was 12%–37% solvent B/0–22 min, 37%–60% solvent B/22–35 min, kept at 60% solvent B at 35–40 min and then 60%–12% solvent B/40–45 min. The flow rate was 1 ml/min. The column temperature was 50°C. The chart speed was 40 cm/h. Key: C₁, DABS-cysteic acid; D, DABS-Asp; E, DABS-Glu; C₂, DABS-carboxymethylcysteine; N, DABS-Asn; Q, DABS-Gln; S, DABS-Ser; T, Dabs-Thr; G, DABS-Gly; A, DABS-Ala; P, DABS-Pro; V, DABS-Val; R, DABS-Arg; M, DABS-Met; I, DABS-Ile; L, DABS-Leu; W, DABS-Trp; F, DABS-Phe; NH₃, DABS-NH₂; K, bis-DABS-Lys; H, bis-DABS-His; Y, bis-DABS-Tyr.

resolution of peaks and lower flow pressure; (4) one should be aware of the possible precipitation of phosphate buffer at the high percentage of solvent B (acetonitrile), since this may clog the column; (5) inclusion of dimethylformamide is not absolutely necessary, but, however, it helps to expand slightly the complete chromatogram; (6) the elution time of DABS-arginine can be adjusted by changing the salt concentration of phosphate buffer; (7) the elution order of acidic DABS-amino acids is particularly sensitive to the pH of the phosphate buffer, e.g. when the pH of the phosphate buffer changes from 6.5 to 6.7 the elution times of DABS-aspartic acid and DABS-glutamic acid decrease by approx. 20 s and the elution times of DABS-cysteic acid and DABS-carboxymethylcysteine decrease by less than 4 s.

It is necessary that each new column be optimized by slight adjustment of the above-mentioned variables.

Stability of DABS-amino acids and reproducibility of dabsylation

DABS-amino acids are stable in the phosphate (40 mM, pH 6.5)/ethanol (1:1, v/v) solution (a dilution buffer for h.p.l.c. injection) at room temperature over months. Quantitative analysis of the same dabsylated Hamilton standard (left at room temperature) over a period of 5 days shows no appreciable degradation of all DABS-amino acids ($\pm 4\%$), including bis-DABS derivatives of histidine and tyrosine. There is also a strict linear rela-

tionship between the peak response and amount of amino acid subjected to dabsylation for all amino acids (Chang *et al.*, 1981), despite the fact that dabsylation of aspartic acid, glutamic acid, serine and threonine was not quantitative.

Amino acid analysis with low nanogram amounts of protein hydrolysate

Amino acid compositions of the proteinase inhibitor eglin C (Seemüller *et al.*, 1980) and two immunoglobulin light chains were determined by the DABS-Cl method (Figs. 2 and 3 and Table 1). The methionine content of the Hamilton standard had totally disappeared after 24 h acid hydrolysis with 6 M-HCl (Pierce, no. 24309) under reduced pressure, perhaps owing to slight contamination with oxidant, and this may also account for the low tyrosine yield found with eglin C (Table 1).

The variable-region sequences of immunoglobulin light chains 10K26-12A1 and 10K44-7A1 have recently been determined (J.-Y. Chang, R. Knecht & D. G. Braun, unpublished work). They differ in only three amino acid residue positions, namely valine, tyrosine and serine in light chain 10K44-7A1 were replaced by isoleucine, histidine and asparagine respectively in light chain 10K26-12A1. Amino acid analysis by the DABS-Cl method clearly detected the quantitative differences between aspartic acid, valine, isoleucine and histidine. Amino acid compositions of light chains 10K26-12A1 and 10K44-7A1 calculated on the basis of peak height in

Fig. 2. Original chromatogram of amino acid analysis of 15 ng (1.85 pmol) of eglin C by the DABS-Cl method (a) Eglin C (0.75 μ g) was hydrolysed and dabsylated, and 2% (15 ng) of the dabsylated sample was injected. (b) Hamilton standard (250 pmol) was hydrolysed and dabsylated, and 2% (5 pmol) of the total dabsylated sample was injected. (c) A blank run (see the Materials and methods section). A second Merck RP-18 column was used and the chromatographic conditions were also slightly different from those described in the legend to Fig. 1. Solvent A was 17 mM-phosphate buffer, pH 6.5, containing 2% (v/v) of dimethylformamide. Solvent B was acetonitrile containing 4% (v/v) of dimethylformamide. The gradient was 15%–45% solvent B/0–25 min, 45%–75% solvent B/25–35 min and 70%–15%/35–40 min. The flow rate was 1 ml/min. The column temperature was 50°C. The chart speed was 40 cm/h. For key to symbols see the legend to Fig. 1. By-products originated from the excess reagent are marked by asterisks (*).

Table 1. Amino acid analysis of approx. 15 ng of eglin C by the DABS-Cl method

The two analyses were from two separate acid hydrolyses of 0.5 and 0.75 μ g of eglin C. The number of amino acids was calculated on the basis of mol.wt. 8100. The expected values were based on the sequence data (Seemüller *et al.*, 1980).

Amino acid	Analysis 1		Analysis 2		Expected no. of residues
	pmol	No. of residues	pmol	No. of residues	
Asp	11.64	7.3	13.14	7.4	7
Glu	10.84	6.8	12.26	6.9	7
Ser	4.46	2.8	5.86	3.3	3
Thr	7.33	4.6	9.95	5.4	5
Gly	7.81	4.9	9.58	5.3	5
Ala	1.50	0.9	2.30	1.3	1
Pro	9.88	6.2	11.19	6.3	6
Val	17.06	10.7	17.76	10.0	11
Arg	6.69	4.2	6.75	3.8	4
Leu*	8.29	5.2	9.06	5.1	5
Phe	8.45	5.3	9.06	5.1	5
Lys	3.80	2.4	4.26	2.4	2
His	5.42	3.4	5.33	3.0	3
Tyr	7.65	4.8	8.35	4.7	6

* The batch of Hamilton standard used in this study was contaminated by leucine. The leucine content of eglin C was calculated from the isoleucine standard.

Fig. 3. Original chromatograms of amino acid analysis of 14 ng (0.6 pmol) of oxidized immunoglobulin light chains 10K44-7A1 (a) and 10K26-12A1 (b)

The histidine contents of light chains 10K44-7A1 and 10K26-12A1 (values not shown) were calculated from the analysis of 40 ng of the dabsylated hydrolysates. Chromatographic conditions were as described in the legend to Fig. 2. For key to symbols see the legend to Fig. 1.

Fig. 3 are comparable with those obtained from a commercial analyser.

However, despite the fact that 10 ng of dabsylated protein hydrolysate is sufficient for complete amino acid analysis, it is risky to start acid hydrolysis with less than 50–100 ng of protein without resorting to the stringent purity control of acid and buffer solutions. The limitation stems from the amino acid contamination that resides in both 6 M-HCl and the dabsylation buffer (especially with glycine and serine, which can be as much as 20 pmol in the total dabsylated sample). It is safer to inject less than 5% of the total dabsylated sample for each analysis, and this would require 0.2 μ g of protein to start with.

In general, the results convincingly demonstrated that amino acid analysis in the picomole range by the DABS-Cl method is as reliable as those in the nanomole range obtained from the commercial amino acid analyser (Spackman *et al.*, 1958). The

attributes that make this new technique particularly attractive are that it is more sensitive, more versatile and more economical than the commercial amino acid analyser. The system can be easily adapted to the use of micro peptide isolation as well as analysis of amino acid thiohydantoin derivatives.

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