Note

Separation of Dns-amino acids using reversed-phase high-performance liquid chromatography: a sensitive method for determining N-termini of peptides and proteins

STEPHEN WEINER*

Isotope Department, The Weizmann Institute of Science, Rehovot (Israel)

and

ARYE TISHBEE

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot (Israel)

(Received March 17th, 1981)

Dns chloride (Dns-Cl) has proved to be a very convenient reagent for "providing fluorescent handles for the study of proteins"[1]. It is commonly used for N-terminus analysis of peptides and proteins. The reacted substrate is hydrolysed to release the dansylated amino acids. The N-terminus is usually identified by two- or three-dimensional thin-layer chromatography[1-3]. In this paper we present an alternative method of identifying the N-terminus using high-performance liquid chromatography (HPLC). In addition, the method may also be useful for obtaining semi-quantitative estimates of the amino acid compositions of small peptides.

Using reversed-phase liquid chromatography, almost all the amino acids commonly found in protein or peptides hydrolysates are separated as their Dns (5-dimethylaminonaphtalene-1-sulfonyl) derivatives. Dns derivative analyses have previously been reported using HPLC on both silica and reversed-phase columns[4-6]. These methods use extreme pH buffer systems (with accompanying problems of silica dissolution in the case of the silica gel system), and many of the relevant amino acids for peptide or protein N-terminus analyses are not separated. In addition, the application of these methods to N-terminus analysis has not been demonstrated.

MATERIALS

Reagents

Dns-Cl, Dns-amino acid standards and egg-white lysozyme (Grade 1) were obtained from Sigma (St. Louis, MO, U.S.A.) and an amino acid standard hydrolysate from Pierce Chemical Co. (Rockford, IL, U.S.A.). All the solvents and chemical reagents used for preparing buffers were spectra grade or analytical grade, respectively.

Chromatographic apparatus and conditions

A high-performance liquid chromatograph with a Waters fluorescent detector (Model 420 using standard filters: excitation filter, 340 ± 6 nm; emission filter, 425 nm long pass), an Eldex pump (Model A-30-S), a Rheodyne injector (Model 7125) and a simple, low-cost gradient programmer[7] was used. A reversed-phase column

(230 × 4.6 mm I.D.) self-packed with Nucleosil C_{18} (5 μm) (Machery, Nagel & Co., Düren, G.F.R.) was equilibrated with buffer (A) comprising 0.05 M sodium acetate pH 6.3, 12.5% acetonitrile and 5% isopropanol. The second buffer (B) was composed of 0.05 M sodium acetate pH 6.5, 50% acetonitrile and 1% isopropanol. The pH of both buffers was readjusted to the required values with glacial acetic acid and then degassed under reduced pressure in an ultrasonic bath for a few minutes. The appropriate gradient for separating the amino acids is shown in Fig. 1, and the program introduced into the programmer is shown in Table I. When using the low-pressure gradient maker, care must be taken to maintain both buffers under equal hydrostatic pressure. The gradient program was initiated 12 min after sample injection. The flowrate was 0.6 ml/min. The final buffer mixture was pumped for 10 min, and re-equilibration with the starting buffer at the end of the run was achieved in 15 min.
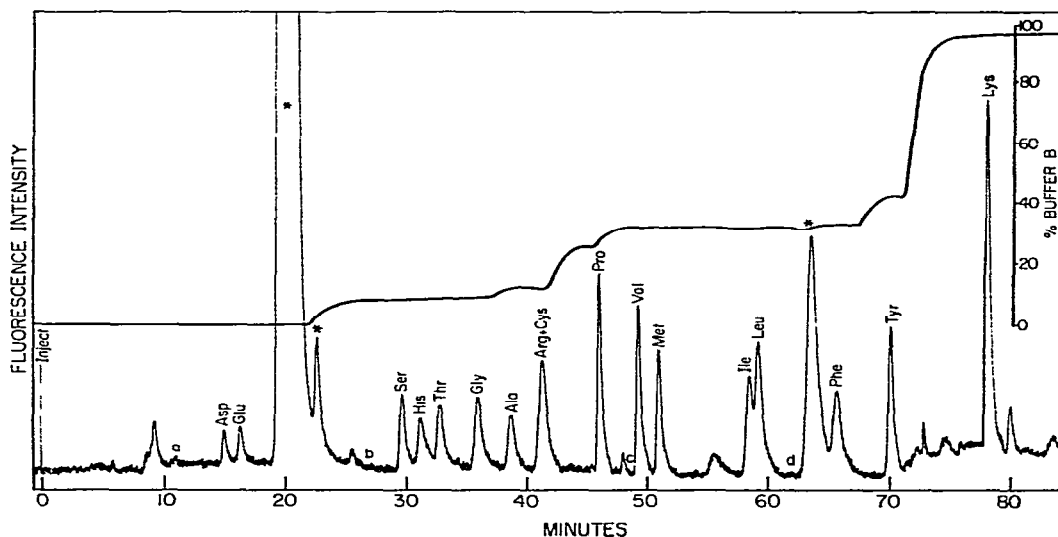

Fig. 1. Separation of the seventeen Dns-amino acids using reversed-phase HPLC. About 500 pm of each amino acid in a standard mixture was derivatized and injected. The peaks marked with an asterisk are Dns hydrolysis products. The elution positions of (a) Dns-cysteic acid, (b) Dns-hydroxyproline, (c) Dns-ε-lysine and (d) Dns-tryptophan are shown. Unmarked peaks are due to unknown compounds.

TABLE I

SOLVENT PROGRAM USED TO SEPARATE THE Dns AMINO ACIDS SHOWN IN FIGS. 1–3

Time after injection (min)	Buffer B
12	0
37	13
42	16
46	33
68	40
72	50
82	97

METHODS

Dns derivatization of a standard mixture of amino acids was carried out according to the method of Zanetta et al.[3]. A 40-μl volume of the standard hydrolysate (i.e., 20 nmoles per amino acid) was made up to 0.3 ml with 0.2 M sodium phosphate buffer. The pH was adjusted to 8.9 with dilute sodium hydroxide before the 0.3 ml of 10 mg/ml Dns-Cl solution was added. Care was taken to ensure complete desalting by repeated extractions of the residue into 500 μl of acetone–1 M HCl (19:1). The supernatants were dried under a stream of nitrogen and the residue re-extracted with acetone: 1 M HCl until all the salt was removed. An appropriate volume (ca. 2.5 %) was injected in the acetone–1 M HCl (19:1) mixture.

Dns derivatization for N-terminus analysis of proteins and peptides was carried out according to the method of Gray for peptides[1]. Although complete derivatization of the N-termini of proteins may not take place under these conditions, the small volumes of buffer and Dns-Cl solution allow for direct injection (in the acetone–1 M HCl (19:1) mixture) of the dried products without any desalting. Hydrolysis to release the N-termini was also performed according to the method of Gray[1]. As an example, ca. 7 nmoles of lysozyme were reacted under the appropriate conditions, hydrolysed for 16 h at 108 C, and evaporated to dryness under vacuum; 10 % of the sample was injected into the HPLC in 3 μl of acetone–1 M HCl (19:1).

Dns derivatization of a peptide or protein hydrolysate was performed according to the procedure described by Zanetta et al. for amino acids[3]. As an illustration of the method, lysozyme was hydrolysed in vacuo at 108°C for 20 h in 0.5 ml of 6 M HCl after flushing twice with nitrogen. Following hydrolysis, the sample was dried under vacuum and then derivatized according to the method of Zanetta et al.[3] and chromatographed under the conditions described above.

RESULTS

Separation of the standard mixture of Dns-amino acids is shown in Fig. 1, together with the gradient required for separation. All the amino acids are completely or partially separated, except for Dns-Arg and Dns-Cys. Dns-cysteic acid, Dns-hydroxyproline, Dns-ε-lysine and Dns-tryptophan are also separated, and their elution positions are indicated in Fig. 1.

As the solution derivatized contained equimolar amounts of amino acids, the peak areas all effectively represent equal amounts of Dns-amino acids. Under the derivatization conditions described, only lysine appears to form the di-Dns derivative.

The N-termini of peptides or proteins can be determined by chromatographing the hydrolysate under the same conditions as described for the standard amino acid mixture. An analysis of the N-terminus of lysozyme is shown in Fig. 2. Lysine is easily identified as the N-terminus. As expected, ε-Dns-lysine is present as a major peak. None of the other minor peaks corresponds to derivatized amino acids. It is often convenient to inject the sample together with a small amount of the standard Dns-amino acid mixture. The sensitivity of the technique is dependent on the relative fluorescence values of the different Dns-amino acids and varies from approximately a few hundred picomoles for Dns-Asp to tens of picomoles for amino acids such as Dns-Val or di-Dns-Lys.
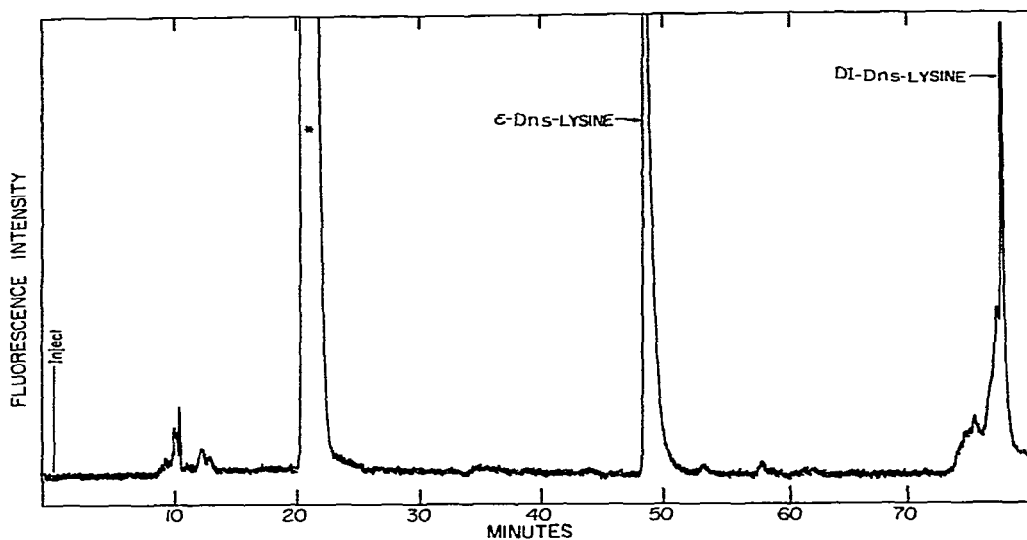
Fig. 2. Identification of lysine as the N-terminus of lysozyme. About 300 pm of Dns-derivatized and hydrolysed lysozyme were chromatographed under the same conditions as those used for the standard Dns-amino acid separation shown in Fig. 1. Unmarked peaks are due to unknown compounds which have different retention times from any known Dns-amino acids. The peaks marked with an asterisk are Dns hydrolysis products.
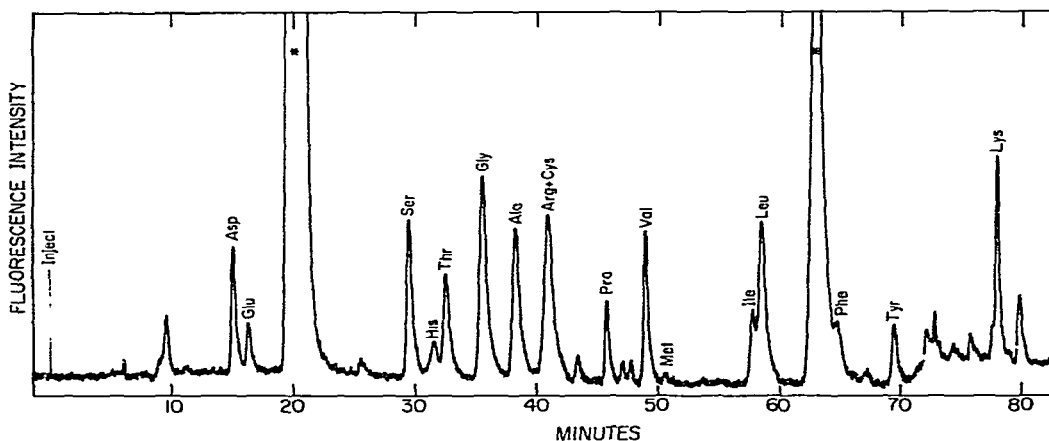

Fig. 3. Separation of a Dns-derivatized total hydrolysate of lysozyme under the same conditions as those used for the standard Dns-amino acid separation shown in Fig. 1. The peaks marked with an asterisk are Dns hydrolysis products. Unmarked peaks are due to unknown compounds.

N-Terminus identifications of peptides are both more quantitative and more simple to perform than those of proteins. The presence of large amounts of ε-Dns-Lys in a protein hydrolysate may mask Dns-Val and possibly Dns-Met. A quantitative Dns derivatization of the N-terminus of a protein requires a special procedure using denaturing conditions and, as a result, desalting[1]. Using the simple method for de-

rivatizing peptides, however, the end-group of lysozyme is still clearly identifiable (Fig. 2).

Semi-quantitative estimates of peptide amino acid compositions can also be made by comparing the peak areas of a derivatized peptide hydrolysate with those of the standard amino acid mixture. Quantitation is affected by several factors.

(1) The reproducibility of the analytical procedure. Three successive injections of the same standard showed a mean percent standard deviation of $\pm 6\%$. Peak areas were calculated from the peak height multiplied by the peak width at half height. More sophisticated integration procedures for determining peak areas could certainly improve the reproducibility.

(2) The reproducibility of the Dns derivatization procedure. Three successive injections of independently derivatized standard mixtures increased the mean percent standard deviation to $\pm 10\%$.

The derivatized hydrolysate of lysozyme is shown in Fig. 3. A protein hydrolysate is used to illustrate the method, so that all the amino acids are represented.

DISCUSSION

This method provides a reliable and sensitive means of identifying the end-groups of peptides and proteins. It is, in our experience, convenient to separate peptides in the reversed phase column as their dansyl derivatives, using chromatographic conditions such as those described by Fullmer and Wasserman[8]. It is then possible to collect and hydrolyse (the peptides) and using the method described here, readily identify their N-termini. The analysis time is relatively long for HPLC separations. Most of the amino acids do show baseline separation, and there are adequate "windows" for tolerating even large excesses of Dns-Cl breakdown products. Only one pair of Dns-amino acids coelute, *viz*. Dns-Arg and Dns-Cys. This ambiguity can be resolved if the samples are oxidized prior to injection in order to convert Dns-Cys into Dns-Cysteic acid, which elutes before Dns-Asp.

The technique may also be used to obtain approximate amino acid compositions of peptides, where presumably the poor quantitation (compared with a commercial amino acid analyser) is not that critical. The high sensitivity, together with the fact that proline and hydroxyproline can be determined, provide certain advantages over the more commonly used post-column fluorescent derivatization methods using reagents such as o-phthalaldehyde or fluorescamine.

A third possible application of this method is in the analysis of mixtures of small peptides and amino acids which are commonly found in, for example, partial acid hydrolysates. Dns derivatized di- and tripeptides chromatographed under these, or similar, conditions are relatively easily separated from each other[9], and may be recognized even in the presence of a complex mixture of amino acids. This property may be particularly useful for the detection of small amounts of peptides in protein hydrolysates used in clinical nutrition[10,11].

REFERENCES

1 W. R. Gray, *Methods Enzymol.*, 11 (1967) 139.
2 C. Gros and B. Labovesse, *Eur. J. Biochem.*, 7 (1969) 463.

3 J. P. Zanetta, G. Vincendon, P. Mandel and G. Gombos, *J. Chromatogr.*, 51 (1970) 441.
4 E. Bayer, E. Grom, B. Kalteneggar and R. Uhmann, *Anal. Chem.*, 48 (1976) 1106.
5 J. N. Le Page, W. Lindner, G. Davies, D. E. Seitz and B. L. Karger, *Anal. Chem.*, 51 (1979) 433.
6 D. C. Olson, G. J. Schmidt and W. Slavin, *Chromatogr. Newslett.*, 7 (1979) 22.
7 R. V. Lewis, *Anal. Biochem.*, 98 (1979) 142.
8 C. S. Fullmer and R. H. Wasserman, *J. Biol. Chem.*, 254 (1979) 7208.
9 *Perkin-Elmer Technical Bulletin*, Vol. 2, No. 4 (1980) 11.
10 C. Garza, N. S. Serimshaw and Y. R. Young, *Amer. J. Clin. Nutr.*, 29 (1976) 280.
11 L. H. Edwin and E. Lohrey, *J. Nutrition*, 106 (1976) 382.