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Note

A simple ultra-micro method for the separation and identification of dipeptides in mixtures obtained during polypeptide sequence determination with dipeptidylaminopeptidase I

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The development of methods for sequencing polypeptides by means of their dipeptide spectrum, as generated by dipeptidylaminopeptidase I[1], has led to the difficulty of the unambiguous identification of the dipeptides produced by this enzyme. It has been suggested that these dipeptides could be separated and identified by combined ion-exchange and paper chromatography and a comparison of the observed chromatographic behaviour with that of standard dipeptides[2]. Although certain regularities in the migration properties and elution times can be expected and have been observed, the standardization of the analytical system with a sufficient number of pure dipeptides is a tedious and expensive task. It is therefore desirable to identify the dipeptides in a given mixture by *ab initio* methods, *i.e.*, by determining the N-terminal and C-terminal amino acids of each single dipeptide, and to be able to do this with very small amounts of material.

In this paper, a method is described for the separation and identification of ultra-micro amounts of dipeptides. All of the steps involved in this procedure are performed with dansylated dipeptides and amino acids. The principle of the method is outlined in Fig. 1.

MATERIALS AND METHODS

Dipeptides were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and dansyl chloride from Serva (Heidelberg, G.F.R.). Pre-coated SIL-G silica gel sheets (20×20 cm) were obtained from Macherey, Nagel & Co. (Düren, G.F.R.). The polyamide plates were Mikropolyamid F-1700 (15×15 cm) from Schleicher & Schüll (Dassel, G.F.R.) and were cut into 5×5 cm pieces. All other chemicals were of reagent grade. Glassware was carefully cleaned with dichromate–sulphuric acid solution and kept in closed containers. The reactions were carried out in Reacti-vials with PTFE screw-caps (Pierce Chemical Co., Rockford, Ill., U.S.A.).

Dansylation

The mixture of dipeptides (1 nmole) is dissolved in 20 μl of 0.2 *M* $NaHCO_3$ and the pH is checked (it should be above 7.5–8.0, otherwise another aliquot of the $NaHCO_3$ solution is added). Then 20 μl of a 0.3% solution of dansyl chloride in acetone are added and thoroughly mixed, and the mixture is incubated at 37° for

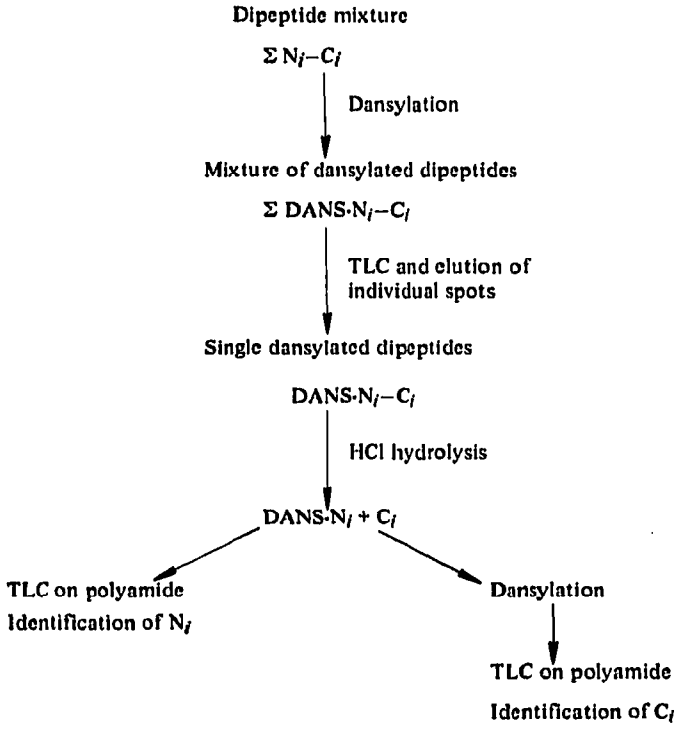

Fig. 1. Scheme for the separation and identification of ultramicro amounts of dipeptides. N_i and C_i are the N-terminal and C-terminal amino acids, respectively, of the ith dipeptide.

1 h. After cooling the mixture, the unreacted dansyl chloride is extracted with two 50-μl portions of toluene. The aqueous phase is evaporated to dryness and the dansylated dipeptides are extracted into 100 μl of a solution prepared by mixing 19 ml of acetone and 1 ml of 1 *N* HCl. The extract is concentrated to a small volume under a stream of nitrogen.

Separation of the dansylated dipeptides

A 20×20 cm SIL-G plate is activated at 105° for 30 min. Then the residual extract from the previous step is applied and two-dimensional chromatography is performed according to the method of Zanetta *et al.*[3]. The solvents used are toluene–pyridine–acetic acid (150:50:3.5) for the first dimension and toluene–2-chloroethanol–25% ammonia solution (30:24:2) for the second dimension. After drying the plate, the fluorescent spots are marked under UV light, keeping the exposure time as short as possible.

Elution and hydrolysis

The individual DANS-dipeptide spots are scraped off the plate and extracted successively with 500 μl each of acetone–water (1:1), acetone–1 *N* HCl (19:1), acetone–acetic acid (3:2), absolute ethanol, pyridine–water (1:1) and ethyl acetate–water (1:1, upper phase). The combined extracts are evaporated to dryness and treated overnight with 100 μl of 6 *N* HCl at 105°. The hydrochloric acid is removed

completely by evacuation over KOH and P_2O_5. The residue is dissolved in 100 μl of acetone–water (1:1) and divided into two equal portions.

Identification of N-terminal amino acids

One 50-μl portion is evaporated almost to dryness and applied to one corner of a 5×5 cm Mikropolyamide F-1700 plate with the aid of a very fine capillary (made from disposable 5-μl glass capillaries). The spot is kept as small as possible (diameters much less than 1 mm can easily be obtained). Two-dimensional chromatography is performed with the solvents used by Woods and Wang[4], *i.e.*, water–formic acid (200:3) for the first dimension and benzene–acetic acid (9:1) for the second dimension. As the Mikropolyamid F-1700 plates are coated on both sides, the identification of amino acids is greatly facilitated if a standard mixture is applied on the reverse side of the plate.

Identification of C-terminal amino acids

The second 50-μl portion of the hydrolyzate is evaporated to dryness and dansylated as described above. The dried residue, after toluene extraction, is treated with 100 μl of acetone–1 *N* HCl (19:1) and the extract evaporated to dryness. The residue is evacuated overnight over KOH and P_2O_5 and then dissolved in a small amount of acetone–water (1:1). Polyamide chromatography, as described above, reveals two fluorescent spots, one of which corresponds to the identified N-terminal amino acid while the other represents the C-terminal amino acid of the dipeptide.

RESULTS

A mixture composed of Gly–Leu, Leu–Gly, Gly–Gly, Gly–Lys, Gly–Asp and Gly–Asn was analyzed by the above procedure. Some of the results obtained are

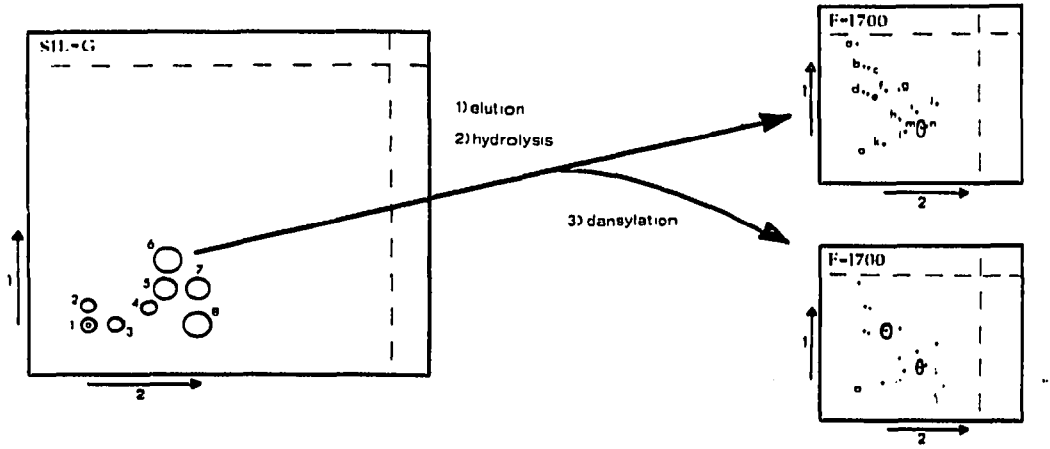

Fig. 2. Identification of Leu–Gly in a mixture of dipeptides. SIL-G chromatogram: first dimension, toluene–pyridine–acetic acid (150:50:3.5); second dimension, toluene–2-chloroethanol–25% ammonia solution (30:24:2). The spots correspond to the dansyl derivatives of (1) Gly–Leu; (2) Gly–Asp; (3) Gly–Asn; (4) Gly; (5) Gly–Gly; (6) Leu–Gly; (7) Gly–Lys; (8) DANS–OH. Mikropolyamid F-1700 chromatograms: first dimension, water–formic acid (200:3); second dimension, benzene–acetic acid (9:1). The spots indicated in the upper chromatogram are the dansyl derivatives of (a) Arg; (b) Ser; (c) Thr; (d) Asp; (e) Glu; (f) Gly; (g) Ala; (h) unknown; (i) Val; (j) Pro; (k) di-Lys; (l) Phe; (m) Leu; (n) Ile.

given in Fig. 2. The complete analysis could easily be carried out in 3 days, the major time-consuming steps being those intended to remove completely all traces of hydrochloric acid before dansylation and polyamide thin-layer chromatography (TLC). The results were unambiguous, provided that all contamination was avoided. In general, this requirement did not cause too severe difficulties, and it was always sufficient to use glassware that had been treated with dichromate–sulphuric acid and rinsed with distilled water. The removal of HCl before dansylation and polyamide chromatography has been found to be achieved most effectively by evacuating the vials overnight over KOH and P_2O_5. Direct application of the acetone–1 *N* HCl (19:1) extract results in hydrolysis of the polyamide layer. Non-acidic extraction with acetone–water (1:1) dissolves the salts present, and these salts cause abnormal behaviour during chromatography.

DISCUSSION

The main advantages of the dansylation method for the identification of amino acids lie in its sensitivity and simplicity. Under properly slected conditions and by the use of micro-methods, the dansylation reaction permits the detection of amino acids at levels well below the nanomole range. This detection depends, of course, on the type of thin layer and the solvent system used. In general, 1 nmole of a dansylated amino acid is easily detected on a 250-μm silica gel layer when the solvent system of Zanetta *et al.*[3] is used. On the other hand, the detection limit on polyamide microplates is much lower (10^{-11}–10^{-12} mole) and this limit, considering the procedure described above, leaves enough latitude for the losses that arise during extraction, etc. In fact, the procedure described was carried out with 0.5–1 nmole of each initial dipeptide and no difficulties due to the low amount of peptide material present occurred.

There are, however, some inherent difficulties as follows, some of which can be overcome relatively easily, while others may require the use of additional data obtained by other methods.

(1) It may be that the solvent systems used for the separation of the dansylated dipeptides are not suitable for certain combinations of dipeptides. However, a large variety of such systems is described in the literature[5] and solvents capable of separating a given mixture can be selected. In general, it may be useful to analyze the DANS-dipeptide spots obtained by the application of two or more solvent systems in order to ensure that each dipeptide of the sample has been found.

(2) In the solvent systems used in this work, the reciprocal dipeptides Gly–Leu and Leu–Gly show different chromatographic behaviour. This is probably not the case with all dipeptides of this type, but other solvent systems may be found that give the desired separations.

(3) The amide bonds of Asn and Gln are hydrolyzed during acid hydrolysis. A decision on the identification of Asp (Glu) peptides and Asn (Gln) peptides can be made, however, by considering the chromatographic behaviour of their dansyl derivatives. At this stage during the procedure described above, the amide bonds originally present are still intact. Fig. 2 shows the different behaviour of Gly–Asp and Gly–Asn (spots 2 and 3, respectively).

(4) The method as described above does not allow one to discriminate directly

between, for example, Gly and Gly–Gly. However, dipeptides with the same residues can be distinguished from the corresponding single amino acids (a) by taking into account the migration properties of DANS-X and DANS-X–X on silica gel layers — Fig. 2 demonstrates the differences between DANS-Gly (spot 5) and DANS-Gly–Gly (spot 4)— and (b) by performing the second dansylation with radioactively labelled dansyl chloride —the detection of radioactivity in the single spot (found on the C-terminal chromatogram) would indicate the presence in the sample of X–X and not X. However, quantitative aspects are important, as the first dansylation was eventually not complete and as contaminating amino acids will also contribute to the radioactivity.

(5) Tryptophan will not be found, as is the case with all methods that involve acid hydrolysis.

It appears, therefore, that by the use of the technique described above the complete qualitative analysis of a dipeptide mixture can be performed with very small amounts of material. Such mixtures are produced by the enzyme dipeptidyl-aminopeptidase I[1] during the digestion of polypeptides. The method permits a large reduction in the amount of polypeptide necessary for the determination of its dipeptide spectrum, which will be of advantage in the analysis of the structures of some biologically active polypeptides.

REFERENCES

1 P. X. Callahan, J. K. McDonald and S. Ellis, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 31 (1972) 1105.
2 P. X. Callahan, J. A. Sheppard, T. Reilly, J. McDonald and S. Ellis, *Anal. Biochem.*, 38 (1970) 330.
3 J. P. Zanetta, G. Vincendon, P. Mandel and G. Gombos, *J. Chromatogr.*, 51 (1970) 441.
4 K. R. Woods and K.-T. Wang, *Biochim. Biophys. Acta*, 133 (1967) 369.
5 N. Seiler, *Methods Biochem. Anal.*, 18 (1970) 259.