CHAPTER 35

The Dansyl Method for Identifying N-Terminal Amino Acids

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1. Introduction

The reagent 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride, DNS-Cl) reacts with the free amino groups of peptides and proteins as shown in Fig. 1. Total acid hydrolysis of the substituted peptide or protein yields a mixture of free amino acids plus the dansyl derivative of the N-terminal amino acid, the bond between the dansyl group and the N-terminal amino acid being resistant to acid hydrolysis. The dansyl amino acid is fluorescent under UV light and is identified by thin-layer chromatography on polyamide sheets. This is an extremely sensitive method for identifying amino acids and in particular has found considerable use in peptide sequence determination when used in conjunction with the Edman degradation (see Chapter 36). The dansyl technique was originally introduced by Gray and Hartley (1), and was developed essentially for use with peptides. However, the method can also be applied to proteins (see Note 1).

2. Materials

1. Dansyl chloride solution (2.5 mg/mL in acetone). Store at 4°C in the dark. This sample is stable for many months. The solution should be prepared from concentrated dansyl chloride solutions (in acetone) that are commercially available. Dansyl chloride available as a solid invariably contains some hydrolyzed material (dansyl hydroxide).

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2. Sodium bicarbonate solution (0.2M, aqueous). Store at 4°C. Stable indefinitely, but check periodically for signs of microbial growth.
3. 5N HCl (aqueous).
4. Test tubes (50 × 6 mm) referred to as "dansyl tubes."
5. Polyamide thin layer plates (7.5 × 7.5 cm). These plates are coated on both sides, and referred to as "dansyl plates." Each plate should be
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numbered with a pencil in the top corner of the plate. The origin for loading should be marked with a pencil 1 cm in from each edge in the lower left-hand corner of the numbered side of the plate. The origin for loading on the reverse side of the plate should be immediately behind the loading position for the front of the plate, i.e., 1 cm in from each edge in the lower right-hand corner.

6. Three chromatography solvents are used in this method.
   Solvent 1: Formic acid:water, 1.5:100 (v/v);
   Solvent 2: Toluene:acetic acid, 9:1 (v/v);

7. An acetone solution containing the following standard dansyl amino acids. Pro, Leu, Phe, Thr, Glu, Arg (each approx 50 μg/mL).

8. A UV source, either long wave (265 μm) or short wave (254 μm).

3. Method

1. Dissolve the sample to be analyzed in an appropriate volume of water, transfer to a dansyl tube, and dry in vacuo to leave a film of peptide (1–5 nmol) in the bottom of the tube.

2. Dissolve the dried peptide in sodium bicarbonate (0.2M, 10 μL) and then add dansyl chloride solution (10 μL) and mix (see Note 2).

3. Seal the tube with parafilm and incubate at 37°C for 1 h, or at room temperature for 3 h.

4. Dry the sample in vacuo. Because of the small volume of liquid present, this will only take about 5 min.

5. Add 6N HCl (50 μL) to the sample, seal the tube in an oxygen flame, and place at 105°C overnight (18 h).

6. When the tube has cooled, open the top of the tube using a glass knife, and dry the sample in vacuo. If phosphorus pentoxide is present in the desiccator as a drying agent, and the desiccator is placed in a water bath at 50–60°C, drying should take about 30 min.

7. Dissolve the dried sample in 50% pyridine (10 μL) and, using a microsyringe, load 1-μL aliquots at the origin on each side of a polyamide plate. This is best done in a stream of warm air. Do not allow the diameter of the spot to exceed 3–4 mm (see Note 3).

8. On the reverse side only, also load 0.5 μL of the standard mixture at the origin.

9. When the loaded samples are completely dry, the plate is placed in the first chromatography solvent and allowed to develop until the solvent front is about 1 cm from the top of the plate. This takes about 10 min, but can vary depending on room temperature.
10. Dry both sides of the plate by placing it in a stream of warm air. This can take 5–10 min since one is evaporating an aqueous solvent.

11. If the plate is now viewed under UV light, a blue fluorescent streak will be seen spreading up the plate from the origin, and also some green fluorescent spots may be seen within this streak. However, no interpretations can be made at this stage (see Note 4).

12. The dansyl plate is now developed in the second solvent, at right angles to the direction of development in the first solvent. The plate is therefore placed in the chromatography solvent so that the blue "streak" runs along the bottom edge of the plate.

13. The plate is now developed in the second solvent until the solvent front is about 1 cm from the top of the plate. This takes 10–15 min.

14. The plate is then dried in a stream of warm air. This will only take 2–3 min since the solvent is essentially organic. However, since toluene is involved, drying must be done in a fume cupboard.

15. The side of the plate containing the sample only should now be viewed under UV light. Three major fluorescent areas should be identified. Dansyl hydroxide (produced by hydrolysis of dansyl chloride) is seen as a blue fluorescent area at the bottom of the plate. Dansyl amide (produced by side reactions of dansyl chloride) has a blue–green fluorescence and is about one-third of the way up the plate. These two spots will be seen on all dansyl plates and seen as useful internal markers. Occasionally other marker spots are seen and these are described in the Notes section below. The third spot, which normally fluoresces green, will correspond to the dansyl derivative of the N-terminal amino acid of the peptide or protein. However, if the peptide is not pure, further dansyl derivatives will of course be seen. The separation of dansyl derivatives after solvent 2 is shown in Fig. 2. Solvent 2 essentially causes separation of the dansyl derivatives of hydrophobic and some neutral amino acids, whereas derivatives of charged and other neutral amino acids remain at the lower end of the chromatogram.

16. A reasonable identification of any faster-moving dansyl derivatives can be made after solvent 2 by comparing their positions, relative to the internal marker spots, with the diagram shown in Fig. 2. Unambiguous identification is made by turning the plate over and comparing the position of the derivative on this side with the standard samples that were also loaded on this side.

Note that both sides of the plate are totally independent chromatograms. There is no suggestion that fluorescent spots can be seen through the plate from one side to the other.
Fig. 2. Diagrams showing the separation of dansyl amino acids on polyamide plates after two solvents (A), and after three solvents (B): a = dansyl hydroxide; b = dansyl amide; 1 = tyrosine (o-DNS-derivative); 2 = lysine (ε-DNS-derivative); 3 = histidine (bis-DNS-derivative). The standard dansyl amino acids that are used are indicated as black spots.
17. Having recorded one's observations after the second solvent, the plate is now run in solvent 3 in the same direction as solvent 2. The plate is run until the solvent is 1 cm from the top, and this again takes 10–15 min.

18. After drying the plate in a stream of warm air (1–2 min), the plate is again viewed under UV light. The fast-running derivatives seen in solvent 2 have now run to the top of the plate and are generally indistinguishable (hence the need to record one's observations after solvent 2). However, the slow-moving derivatives in solvent 2 have now been separated by solvent 3 and can be identified if present. The separation obtained after solvent 3 is also shown in Fig. 2. The sum of the observations made after solvents 2 and 3 should identify the number and relative intensities of N-terminal amino acids present in the original sample (see Notes 5–12).

4. Notes

1. The dansylation method described here was developed for use with peptides. However, this method can also be applied quite successfully to proteins, although some difficulties arise. These are caused mainly by insolubility problems, which can limit the amount of reaction between the dansyl chloride and protein thus resulting in a lower yield of dansyl derivative, and the presence of large amounts of o-DNS-Tyr and ε-DNS-Lys on the chromatogram that can mask DNS-Asp and DNS-Glu. A modification of the basic procedure described here for the dansylation of proteins is described in ref. 3.

2. It is important that the initial coupling reaction between dansyl chloride and the peptide occurs in the pH range 9.5–10.5. This pH provides a compromise between the unwanted effect of the aqueous hydrolysis of dansyl chloride and the necessity for the N-terminal amino group to be unprotonated for reaction with dansyl chloride. The condition used, 50% acetone in bicarbonate buffer, provides the necessary environment. The presence of buffer or salts in the peptide (or protein) sample should therefore be avoided to prevent altering the pH to a value outside the required range.

3. Because of the unpleasant and irritant nature of pyridine vapor, loading of samples onto the dansyl plates should preferably be carried out in a fume cupboard.

4. The viewing of dansyl plates under UV light should always be done wearing protective glasses or goggles. Failure to do so will result in a most painful and potentially damaging conjunctivitis.

5. Most dansyl derivatives are recovered in high (>90%) yield. However, some destruction of proline, serine, and threonine residues occurs dur-
The sensitivity of the dansyl method is such that as little as 1–5 ng of a dansylated amino acid can be visualized on a chromatogram.

The side chains of both tyrosine and lysine residues also react with dansyl chloride. When these residues are present in a peptide (or protein), the chromatogram will show the additional spots, o-DNS-Tyr and \( \epsilon \)-DNS-Lys, which can be regarded as additional internal marker spots. The positions of these residues are shown in Fig. 2. These spots should not be confused with \( \text{bis-DNS-Lys} \) and \( \text{bis-DNS-Tyr} \), which are produced when either lysine or tyrosine is the N-terminal amino acid.

At the overnight hydrolysis step, dansyl derivatives of asparagine or glutamine are hydrolyzed to the corresponding aspartic or glutamic acid derivatives. Residues identified as DNS-Asp or DNS-Glu are therefore generally referred to as Asx or Glx, since the original nature of this residue (acid or amide) is not known. This is of little consequence if one is looking for a single N-terminal residue to confirm the purity of a peptide or protein. It does, however, cause difficulties in the dansyl-Edman method for peptide sequencing (see Chapter 36) where the residue has to be identified unambiguously.

When the first two residues in the peptide or protein are hydrophobic residues, a complication can occur. The peptide bond between these two residues is particularly (although not totally) resistant to acid hydrolysis. Under normal conditions, therefore, some dansyl derivative of the first amino acid is produced, together with some dansyl derivative of the N-terminal dipeptide. Such dipeptide derivatives generally run on chromatograms in the region of phenylalanine and valine. However, their behavior in solvents 2 and 3, and their positions relative to the marker derivatives should prevent misidentification as phenylalanine or valine. Such dipeptide spots are also produced when the first residue is hydrophobic and the second residue is proline, and these dipeptide derivatives run in the region of proline. However, since some of the N-terminal derivative is always produced, there is no problem in identifying the N-terminal residue when this situation arises. A comprehensive description of the chromatographic behavior of dansyl-dipeptide derivatives has been produced (2).

Three residues are difficult to identify in the three solvent system described in the methods section; DNS-Arg and DNS-His because they are masked by the \( \epsilon \)-DNS-Lys spot, and DNS-Cys because it is masked by DNS-hydroxide. If these residues are suspected, a fourth solvent is
used. For arginine and histidine, the solvent is 0.05M trisodium phosphate:ethanol (3:1 [v/v]). For cysteine the solvent is 1M ammonia:ethanol (1:1 [v/v]) Both solvents are run in the same direction as solvents 2 and 3, and the residues are identified by comparison with relevant standards loaded on the reverse side of the plate.

11. When working with small peptides it is often of use also to carry out the procedure known as “double dansylation.” Having identified the N-terminal residue, the remaining material in the dansyl tube is dried down and the dansylation process (steps 2–4) repeated. The sample is then redissolved in 50% pyridine (10 μL) and a 1-μL aliquot is examined chromatographically. The chromatogram will now reveal the dansyl derivative of each amino acid present in the peptide. Therefore for relatively small peptides (<10 residues) a quantitative estimation of the amino acid composition of the peptide can be obtained. This method is not suitable for larger peptides or proteins since most residues will be present more than once in this case, and it is not possible to quantitatively differentiate spots of differing intensity.

12. Although the side chain DNS-derivative will be formed during dansylation if histidine is present in the peptide or protein sequence, this derivative is unstable to acid and is not seen during N-terminal analysis. Consequently, N-terminal histidine yields only the α-DNS derivative and not the bis-DNS compound as might be expected. The bis-DNS derivative is observed, however, if the mixture of free amino acids formed by acid hydrolysis of a histidine-containing peptide is dansylated and subsequently analyzed chromatographically (i.e., during “double dansylation”).

References