CHAPTER 36

The Dansyl-Edman Method for Peptide Sequencing

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1. Introduction

The Edman degradation is a series of chemical reactions that sequentially removes N-terminal amino acids from a peptide or protein. The overall reaction sequence is shown in Fig. 1. In the first step (the coupling reaction) phenylisothocyanate (PITC) reacts with the N-terminal amino group of the peptide or protein. The sample is then dried and treated with an anhydrous acid (e.g., trifluoracetic acid), which results in cleavage of the peptide bond between the first and second amino acid. The N-terminal amino acids is therefore released as a derivative (the thiazolinone). The thiazolinone is extracted into an organic solvent, dried down, and then converted to the more stable phenylthiohydantoin (PTH) derivative (the conversion step). The PTH amino acid is then identified, normally by reverse-phase HPLC. This is known as the direct Edman degradation and is, for example, the method used in an automated sequencing machine. The dansyl-Edman method for peptide sequencing described here is based on the Edman degradation, but with the following modifications. Following the cleavage step the thiazolinone is extracted, but rather than being converted to the PTH derivative it is discarded. Instead, a small fraction (5%) of the remaining peptide is taken and the newly liberated N-terminal amino acid determined in this sample by the dansyl method (*see* Chapter 35). Although the dansyl-Edman method results in successively less peptide being present at each cycle of the Edman deg-

From *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by J M Walker

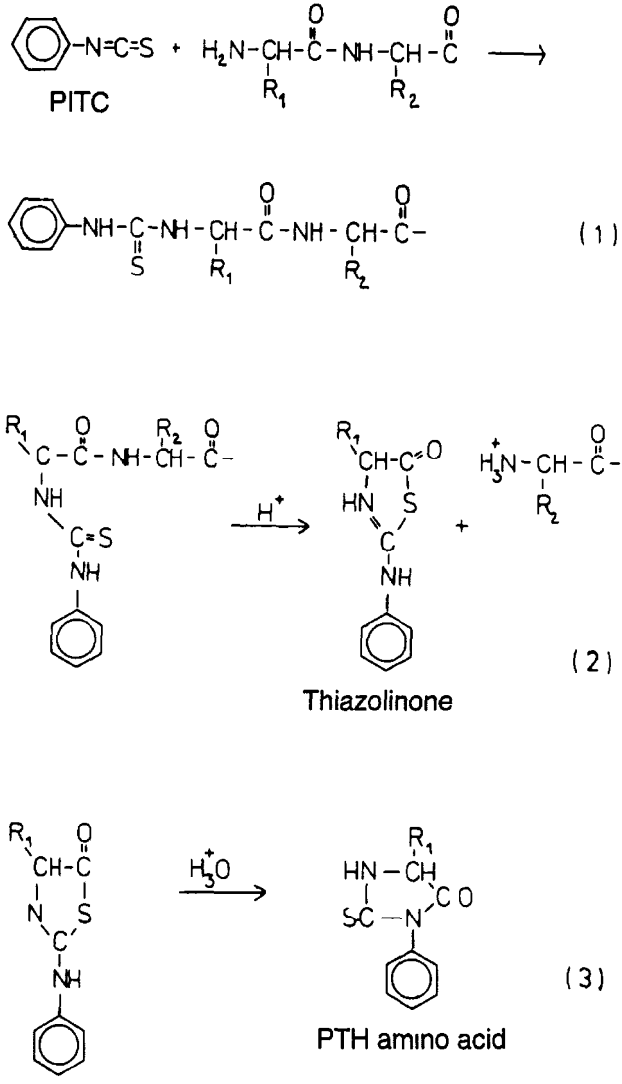

Fig. 1. The Edman degradation reactions and conversion step: (1) the coupling reaction, (2) the cleavage reaction; (3) the conversion step.

radation, this loss of material is compensated for by the considerable sensitivity of the dansyl method for identifying N-terminal amino acids. The dansyl-Edman method described here was originally introduced by Hartley *(1)*.

2. Materials

1. Ground glass stoppered test tubes (approx 65 × 10 mm, e.g., Quickfit MF 24/0). All reactions are carried out in this "sequencing" tube.
2. 50% Pyridine (aqueous, made with AR pyridine). Store under nitrogen at 4°C in the dark. Some discoloration will occur with time, but this will not affect results.
3. Phenylisothiocyanate (5% [v/v]) in pyridine (AR). Store under nitrogen at 4°C in the dark. Some discoloration will occur with time, but this will not affect results. The phenylisothiocyanate should be of high purity and is best purchased as "sequenator grade." Make up fresh about once a month.
4. Water-saturated *n*-butyl acetate. Store at room temperature.
5. Anhydrous trifluoracetic acid (TFA). Store at room temperature under nitrogen.

3. Method

1. Dissolve the peptide to be sequenced in an appropriate volume of water, transfer to a sequencing tube, and dry *in vacuo* to leave a film of peptide in the bottom of the tube (*see* Notes 1 and 2).
2. Dissolve the peptide in 50% pyridine (200 µL) and remove an aliquot (5 µL) for N-terminal analysis by the dansyl method (*see* Chapter 35 and Note 3).
3. Add 5% phenylisothiocyanate (100 µL) to the sequencing tube, mix gently, flush with nitrogen and incubate the stoppered tube at 50°C for 45 min.
4. Following this incubation, unstopper the tube and place it *in vacuo* for 30–40 min. The desiccator should contain a beaker of phosphorus pentoxide to act a drying agent, and if possible the desiccator should be placed in a water bath at 50–60°C. When dry, a white "crust" will be seen in the bottom of the tube. This completes the coupling reaction (*see* Notes 4–6).
5. Add TFA (200 µL) to the test-tube, flush with nitrogen, and incubate the stoppered tube at 50°C for 15 min.
6. Following incubation, place the test tube *in vacuo* for 5 min. TFA is a very volatile acid and evaporates rapidly. This completes the cleavage reaction.
7. Dissolve the contents of the tube in water (200 µL). Do not worry if the material in the tube does not all appear to dissolve. Many of the side-products produced in the previous reactions will not in fact be soluble.
8. Add *n*-butyl acetate (1.5 mL) to the tube, mix vigorously for 10 s, and then centrifuge in a bench centrifuge for 3 min.
9. Taking care not to disturb the lower aqueous layer, carefully remove the upper organic layer and discard (*see* Note 7).

10. Repeat this butyl acetate extraction procedure once more and then place the test-tube containing the aqueous layer *in vacuo* (with the desiccator standing in a 60°C water bath if possible) until dry (30–40 min).
11. Redissolve the dried material in the test tube in 50% pyridine (200 µL) and remove an aliquot (5 µL) to determine the newly liberated N-terminal amino acid (the second one in the peptide sequence) by the dansyl method (*see* Chapter 35).
12. A further cycle of the Edman degradation can now be carried out by returning to step 3. Proceed in this manner until the peptide has been completely sequenced (*see* Notes 8 and 9).
13. The identification of N-terminal amino acids by the dansyl method is essentially as described in Chapter 35. However, certain observations peculiar to the dansyl-Edman method are described in Notes 10–15.

4. Notes

1. Manual sequencing is normally carried out on peptides between 2 and 30 residues in length and requires 1–5 nmol of peptide.
2. Since the manipulative procedures are relatively simple it is quite normal to carry out the sequencing procedure on 8 or 12 peptides at one time.
3. As sequencing proceeds, it will generally be necessary to increase the amount of aliquot taken for dansylation at the beginning of each cycle, since the amount of peptide being sequenced is reduced at each cycle by this method. The amount to be taken should be determined by examination of the intensity of spots being seen on the dansyl plates.
4. It is most important that the sample is completely dry following the coupling reaction. Any traces of water present at the cleavage reaction step will introduce hydrolytic conditions that will cause internal cleavages in the peptide and a corresponding increase in the background of N-terminal amino acids.
5. Very occasionally it will prove difficult to completely dry the peptide following the coupling reaction and the peptide appears oily. If this happens, add ethanol (100 µL) to the sample, mix, and place under vacuum. This should result in a dry sample.
6. Vacuum pumps used for this work should be protected by cold traps. Considerable quantities of volatile organic compounds and acids will be drawn into the pump if suitable precautions are not taken.
7. When removing butyl acetate at the organic extraction step, take great care not to remove any of the aqueous layer as this will considerably reduce the amount of peptide available for sequencing. Leave a small layer of butyl acetate above the aqueous phase. This will quickly evaporate at the drying step.

8. A repetitive yield of 90–95% is generally obtained for the dansyl-Edman degradation. Such repetitive yields usually allow the determination of sequences up to 15 residues in length, but in favorable circumstances somewhat longer sequences can be determined.
9. A single cycle takes approx 2.5 h to complete. When this method is being used routinely, it is quite easy to carry out three or four cycles on eight or more peptides in a normal day's work. During the incubation and drying steps the dansyl samples from the previous days sequencing can be identified.
10. Tryptophan cannot be identified by the dansyl method as it is destroyed at the acid hydrolysis step. However, where there is tryptophan present in the sequence, an intense purple color is seen at the cleavage (TFA) step involving the tryptophan residue that unambiguously identifies the tryptophan residue.
11. If there is a lysine residue present in the peptide, a strong ε-DNS-lysine spot will be seen when the dansyl derivative of the N-terminal amino acid is studied. However, when later residues are investigated the ε-DNS-lysine will be dramatically reduced in intensity or absent. This is because the amino groups on the lysine side chains are progressively blocked by reaction with phenylisothiocyanate at each coupling step of the Edman degradation.
12. The reactions of the lysine side chains with phenylisothiocyanate causes some confusion when identifying lysine residues. With a lysine residue as the N-terminal residue of the peptide it will be identified as *bis*-DNS-lysine. However, lysine residues further down the chain will be identified as the α-DNS-ε–phenylthiocarbamyl derivative because of the side chain reaction with phenylisothiocyanate. This derivative runs in the same position as DNS-phenylalanine in the second solvent, but moves to between DNS-leucine and DNS-isoleucine in the third solvent. Care must therefore be taken not to misidentify a lysine residue as a phenylalanine residue.
13. When glutamine is exposed as the new N-terminal amino acid during the Edman degradation, this residue will sometimes cyclize to form the pyroglutamyl derivative. This does not have a free amino group, and therefore effectively blocks the Edman degradation. If this happens, a weak DNS-glutamic acid residue is usually seen at this step, and then no other residues are detected on further cycles. There is little one can do to overcome this problem once it has occurred, although the enzyme that cleaves off pyroglutamyl derivatives (pyroglutamate aminopeptidase) is commercially available (Boehringer, Lewes, UK).

14. The amino acid sequence of the peptide is easily determined by identifying the new N-terminal amino acid produced after each cycle of the Edman degradation. However, because the Edman degradation does not result in 100% cleavage at each step, a background of N-terminal amino acids builds up as the number of cycles increases. Also, as sequencing proceeds, some fluorescent spots reflecting an accumulation of side products can be seen toward the top of the plates. For longer runs (10–20 cycles) this can cause some difficulty in identifying the newly liberated N-terminal amino acid. This problem is best overcome by placing the dansyl plates from consecutive cycles adjacent to one another and viewing them at the same time. By comparison with the previous plate, the increase of the new residue at each cycle, over and above the background spots, should be apparent.
15. The main disadvantage of the dansyl-Edman method compared to the direct Edman method is the fact that the dansyl method cannot differentiate acid and amide residues. Sequences determined by the dansyl-Edman method therefore usually include residues identified as Asx and Glx. This is most unsatisfactory since it means the residue has not been unambiguously identified, but often the acid or amide nature of an Asx or Glx residue can be deduced from the electrophoretic mobility of the peptide *(3)*.
16. Having identified any given residue it can prove particularly useful to carry out the procedure referred to as "double dansylation" on this sample (*see* Chapter 35). This double-dansylated sample will identify the amino acids remaining beyond this residue. Double dansylation at each step should reveal a progressive decrease in the residues remaining in the peptide, and give an excellent indication of the amount of residues remaining to be sequenced at any given cycle.

References

1. Hartley, B. S. (1970) Strategy and tactics in protein chemistry. *Biochem. J* **119,** 805–822.
2. Offord, R E. (1966) Electrophoretic mobilities of peptides on paper and their use in the determination of amide groups. *Nature* **211,** 591–593.