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High-Sensitivity Sequence Analysis of Peptides and Proteins by 4-*NN*-Dimethylaminoazobenzene 4′-Isothiocyanate

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A manual high-sensitivity sequencing method is described, in which 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate is used for the stepwise degradation of amino acid residues from the peptides. The 4-*NN*-dimethylaminoazobenzene 4′-thiazolinones of amino acids that were released, after conversion into their thiohydantoin derivatives, were identified by t.l.c. on polyamide sheets. This new method is simple and sensitive, and requires only 2–10nmol of peptides or proteins for extended sequence analysis. The method was tested on the sequence analysis of a hexapeptide (Leu-Trp-Met-Arg-Phe-Ala), bradykinin, glucagon and native lysozyme. Results show that the proposed procedure is a sensitive method for the sequence determination of short peptides as well as for the partial sequence determination of intact proteins.

The use of phenylisothiocyanate (Edman reagent) as a stepwise-degradation reagent for amino acids from peptides or proteins has been one of the most useful methods of amino acid-sequence determination (Edman, 1950; Edman & Begg, 1967; Laursen, 1971). Although several new reagents (Barrett & Leigh, 1975; Previero *et al.*, 1975) and new techniques (Morris *et al.*, 1971; Bradbury *et al.*, 1974; Priddle *et al.*, 1976) have been developed, none is in widespread use to date.

The introduction of the modified Edman reagent, 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate, is an attractive one (Chang *et al.*, 1976; Chang & Creaser, 1976). The highly coloured 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate, and the colour change between its thiocarbamoyl and thiohydantoin derivatives would enable the identification of the released *N*-terminal amino acids (as 4-*NN*-dimethylaminoazobenzene 4′-thiohydantoins) to be carried out in a more sensitive and simple way than that of the conventional phenylisothiocyanate method.

The present paper therefore describes the application of 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate as a possible stepwise-degradation reagent for the sequence determination of peptides and proteins. A manual micro-sequencing method based on the chosen optimum conditions is proposed and tested on the sequence analysis of 10nmol each of a hexapeptide (Leu-Trp-Met-Arg-Phe-Ala), bradykinin, glucagon and native lysozyme.

Materials and Methods

The hexapeptide (Leu-Trp-Met-Arg-Phe-Ala) was from Schwarz/Mann, Orangeburg, NY, U.S.A. Bradykinin, glucagon and egg-white lysozyme were from Sigma, St. Louis, MO, U.S.A. Trifluoroacetic acid and pyridine were sequential grade from Pierce Co., Rockford, IL, U.S.A. All other chemicals and solvents used in this report were commercial analytical grade and the solvents were redistilled before use. Polyamide sheets were from Chen-Ching Chemical Company, Taipei, Taiwan.

Synthesis of 4-*NN-dimethylaminoazobenzene 4′-isothiocyanate*

Our previous method (Chang *et al.*, 1976) was modified. 4-*NN*-Dimethylamino-4′-aminoazobenzene (1g), which was obtained by the alkaline hydrolysis of 4-*NN*-dimethylamino-4′-acetamidoazobenzene (Chang & Creaser, 1977), was dissolved in 30ml of benzene and mixed with 1ml of thiocarbonyl chloride. The mixture was refluxed for 1h, when all the amino compound was converted into the isothiocyanate (this could be followed on silica-gel t.l.c. with benzene as solvent). The mixture was then dried under reduced pressure and dissolved in 200–150ml of acetone. The acetone solution was filtered to remove insoluble materials and then mixed with an excess of water to precipitate crude 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate. This product was collected, dried *in vacuo* and then recrystallized twice from the boiling acetone. This procedure avoids the use of silica-gel-column purification and produces a much higher yield.

Sequence analysis of peptides and proteins

Peptides (10nmol), placed in an acid-washed tube (0.6cm internal diam. × 5cm) fitted with a Quickfit

glass stopper, were dissolved in 60 μl of aq. 50% (v/v) pyridine and treated with 30 μl of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate solution [10 nmol/μl of pyridine (2.82mg/ml), freshly prepared every day]. The tube was flushed with N_2 for 30s, sealed tightly with the glass stopper and the join was wrapped with paraffin film. The tube was placed in a water bath at 75°C for 1.5h and was shaken occasionally. After the reaction, the excess of reagent and hydrolysed by-products were removed by mixing the reaction mixture with two portions of 0.5ml of heptane/ethyl acetate (2:1, v/v) on a vortex mixer and centrifuging. The organic phase was removed with a fine pipette and discarded. After the removal of the second extracts, the mixture was evaporated in a high vacuum. The dried residue was dissolved in 50 μl of anhydrous trifluoroacetic acid, flushed with N_2, sealed with a glass stopper and heated in an oven at 50°C for 15min. The sample was evaporated in a vacuum desiccator and dissolved in 50 μl of water. Extraction of the cleaved 4-*NN*-dimethylaminoazobenzene-4'-thiazolinones of the amino acids was performed by mixing with two portions of 300 μl of diethyl ether on a vortex mixer and centrifuging. A third volume of 500 μl of ether was used to wash the aqueous phase and discarded. The sample was evaporated in a desiccator and subjected to the next degradation cycle. One cycle takes about 3.5h.

The first two portions of ether extract (600 μl) were evaporated and the residue was redissolved in water (20 μl) and acetic acid saturated with HCl (40 μl). Conversion of the thiazolinones of the amino acids into thiohydantoins was carried out in a 50°C oven for 45min. The sample was dried and redissolved in a suitable amount of ethanol for identification by t.l.c.

For a single peptide, two degradations (including the t.l.c. identifications) could be achieved in 1 working day. However, one would be able to handle four to six peptides simultaneously.

Identifications by t.l.c.

The technique and procedure for identification of 4-*NN*-dimethylaminoazobenzene 4'-thiohydantoins of amino acids by t.l.c. on the micro-scale were essentially the same as those described in our previous report (Chang & Creaser, 1976).

A synthetic blue marker (20–30pmol), which was prepared by the reaction of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate with diethylamine, was applied on the same side of the polyamide sheet to help the identification of unknown compounds (Fig. 1).

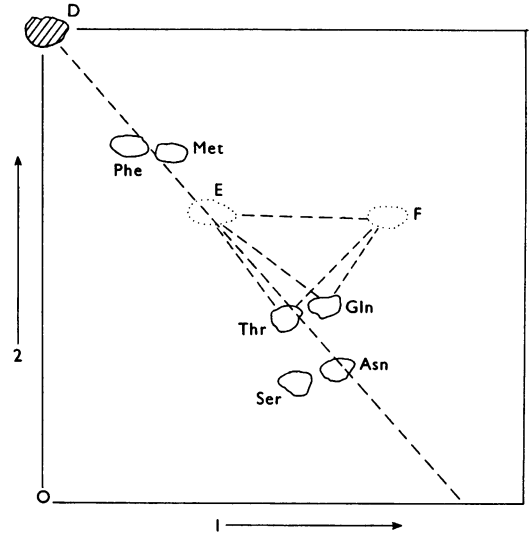

Fig. 1. *T.l.c. identification of 4-NN-dimethylaminoazobenzene 4'-thiohydantoins of amino acids*
The blue marker, 4-*NN*-dimethylaminoazobenzene 4'-thiocarbamoyldiethylamine (E), and the unextracted 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate (D) (purple colour, excess of reagent) and 4-*NN*-dimethylamino-4'-aminoazobenzene (F) (greenish-red colour, hydrolysed product) are three major reference points. A straight line connecting spots D and E (Chang & Creaser, 1977) or the triangular shapes between the spots of the unknowns and spots E and F provide a rapid identification of amino acid residues with close R_F values. Solvent 1 was water/acetic acid (2:1, v/v) and solvent 2 was toluene/n-hexane/acetic acid (2:1:1, by vol.).

Results and Discussion

Rather drastic conditions (i.e. 75°C for 1.5h) were found to be necessary for the quantitative coupling between 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate and *N*-terminal amino acids. Hydrolysis of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate to 4-*NN*-dimethylamino-4'-aminoazobenzene, which proceeds at an extremely high rate in the high pH medium, was nearly 75% under the proposed coupling condition (J. Y. Chang, unpublished work).

The solvents used for the extractions of by-products and released thiazolinone derivatives after coupling and cleavage reactions respectively are the crucial problems for the sequence determination of short non-polar peptides. Benzene, which has been commonly used for the extraction of excess of reagent and by-products after the coupling reaction in the conventional phenylisothiocyanate method, was found to extract at least 90% of the 4-*NN*-dimethylaminoazobenzene 4'-thiocarbamoyl derivatives of most non-polar amino acids and dipeptides from the coupling mixture (i.e. aq. 67% pyridine). However, the solvent heptane/ethyl acetate (2:1; v/v), suggested by Tarr (1975), would extract at most 15–20%

Table 1. *T.l.c. results of the sequence determination of* 10 *nmol each of the hexapeptide, bradykinin, and the first* 15 *residues of glucagon and native lysozyme by the sequencing method described in the text*

The portions of the ethanol extracts applied for t.l.c. identifications are indicated ($1/x$).

Hexapeptide

	Expected	$1/x$	Found
1	Leu	1/150	Leu/Ile(only)
2	Trp	1/100	Trp(only)
3	Met	1/100	Met(only)
4	Arg	1/100	Arg(only)
5	Phe	1/100	Phe(only)
6	Ala	1/50	Ala(only)
7	None	1/50	None

Bradykinin

	Expected	$1/x$	Found
1	Arg	1/100	Arg(major), Pro(trace)
2	Pro	1/100	Pro(major), Gly(trace)
3	Pro	1/100	Pro(only)
4	Gly	1/100	Gly(only)
5	Phe	1/100	Phe(major), Gly(trace)
6	Ser	1/50	Ser(major), Phe(minor)
7	Pro	1/50	Pro(only)
8	Phe	1/50	Phe(major), Pro(minor)
9	Arg	1/50	Arg(major), Phe(minor)
10	None	1/50	Arg(trace)

Glucagon

	Expected	$1/x$	Found
1	His	1/75	His(only)
2	Ser	1/50	Ser(only)
3	Gln	1/50	Gln(only)
4	Gly	1/50	Gly(only)
5	Thr	1/50	Thr(major), Gly(minor)
6	Phe	1/50	Phe(only)
7	Thr	1/50	Thr and Phe(equal)
8	Ser*	1/25	Ser, Thr and Phe
9	Asp	1/50	Asp(only)
10	Tyr	1/25	Tyr and Asp(equal)
11	Ser†	1/25	Tyr(trace)
12	Lys†	1/25	None
13	Tyr*	1/15	Tyr(only)
14	Leu*	1/15	Leu/Ile(major), Tyr and Asp(minor)
15	Asp*	1/15	Asp and Leu/Ile(equal)

Lysozyme

	Expected	$1/x$	Found
1	Lys	1/50	Bis-Lys‡(only)
2	Val	1/50	Val(only)
3	Phe	1/50	Phe(major), Val(trace)
4	Gly	1/50	Gly(only)
5	Arg	1/50	Arg and Gly(equal)
6	Cys	1/50	Arg and Gly(trace)
7	Glu	1/25	Glu(major), Gly(minor)
8	Leu	1/25	Leu/Ile and Glu(equal)
9	Ala	1/25	Ala(major), Leu/Ile(trace)
10	Ala	1/25	Ala(only)
11	Ala	1/25	Ala(only)
12	Met	1/25	Met and Ala(equal)
13	Lys†	1/25	None
14	Arg*	1/15	Arg and Ala(equal)
15	His	1/15	His(major), Arg(minor)

* Appeared in comparably weak intensities.

† Undetectable.

‡ 4-*NN*-dimethylaminoazobenzene 4′-thiohydantoin-(ε-4-*NN*-dimethylaminoazobenzene 4′-thiocarbamoyl)-L-lysine.

of the same thiocarbamoyl derivatives from the aqueous phase. Diethyl ether was also found to be a better solvent than ethyl acetate to retain the non-polar peptides in the aqueous phase during the extraction of 4-*NN*-dimethylaminoazobenzene 4′-thiazolinones after the cleavage reactions.

The skill of running the small-size polyamide sheet is a critical procedure for the fast and accurate identification of 4-*NN*-dimethylaminoazobenzene 4′-thiohydantoins of amino acids. Application of the blue standard, 4-*NN*-dimethylaminoazobenzene 4′-thiocarbamoyldiethylamine (E, Fig. 1), in addition to the appearance of trace amount of unextracted 4-*NN*-dimethylaminoazobenzene 4′-isothiocyanate (excess of reagent) and 4-*NN*-dimethylamino-4′-aminoazobenzene (hydrolysed product, F) form three major markers on the thin-layer sheet. A fast and accurate confirmation of each amino acid 4-*NN*-dimethylaminoazobenzene - 4′ - thiohydantoin could be achieved by relating their positions to those three different coloured markers, especially for distinguishing between methionine and phenylalanine, between glutamine and threonine, and between asparagine and serine (see Fig. 1). However, distinction between isoleucine and leucine is still not possible at this stage.

Table 1 shows the results of sequence analysis of some peptides and proteins with known structures by the proposed sequencing method. The amino acid sequence of the hexapeptide and bradykinin was unambiguously determined, although there was some overlapping of residue 6, 8 and 9 of bradykinin by the preceding residues. The amino acid sequence of the first 15 residues of glucagon and lysozyme were analysed and some difficulties were encountered. The recovery of residue 8 of glucagon (serine) was extremely small, and residue 11 of glucagon (serine) was actually undetectable. The gaps also appeared at residue 12 of glucagon (lysine) and residue 13 of lysozyme (lysine). Only the first ten residues of

glucagon (except residue 8) and the first 12 residues of lysozyme (except residue 6) could be adequately confirmed. The recoveries of residues beyond these were too low to be established confidently when starting with only 10nmol of materials. From the portions of ethanol extracts used for the t.l.c. identification (see Table 1), it is reasonable to predict that the whole scale of the described sequencing method could be decreased to one-tenth of the original volume. In fact, I have tried the method on the sequence analysis of only 2nmol of the hexapeptide and bradykinin and the same results as those described in Table 1 were obtained.

As the need for a high-sensitivity sequencing method is growing, the proposed method would offer a simple and inexpensive alternative for the sequence determination of micro-amounts of peptides in addition to the conventional techniques (Weiner *et al.*, 1972; Tarr, 1975; Bridgen, 1976).

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