AN ISOTOPE DILUTION PROCEDURE FOR DETECTION AND QUANTITATIVE ESTIMATION OF PHENYLTHIOHYDANTOINS RELEASED IN THE EDMAN DEGRADATION[1]

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A simple, rapid procedure for quantitative estimation of phenylthiohydantoins liberated in the Edman degradation is described. Peptides are degraded using ^{3}H-phenylisothiocyanate. The radioactive thiazolinones released in the acid cleavage step of the degradation are added to a mixture of unlabelled phenylthiohydantoins and are isomerized *in situ* to the corresponding phenylthiohydantoins. The phenylthiohydantoin mixture is separated using thin layer chromatography, ultraviolet-absorbing areas are scraped off, and radioactive areas are located by liquid scintillation counting. This procedure is rapid, sensitive and inexpensive. No specialized apparatus other than a scintillation counter is required. The procedure is especially applicable to automated versions of the Edman degradation.

The Edman degradation (1), particularly in its automated form (2,3), constitutes one of the most useful tools for determining the amino acid sequences of proteins. One of the problems retarding even more widespread use of the Edman degradation has been that of identifying the amino acid phenylthiohydantoins released after each degradative cycle. Published procedures include qualitative identification (2) and quantitative estimation (4), using ^{35}S-labelled phenylthiohydantoins, on thin layer chromatography plates, and gas-liquid chromatography of derivatized phenylthiohydantoins (5,6). A difficulty common to all methods of analysis is the tendency for the small quantities of phenylthiohydantoins involved to be lost before analysis because of air oxidation or solvent impurities, unless special precautions are taken. A second problem, which becomes especially apparent when working with

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very small quantities, is the appearance of traces of artifacts, which are detectable by ultraviolet absorption or gas-liquid chromatography and thus interfere with identification of the phenylthiohydantoins. In connection with our work on automation of the solid-phase Edman degradation (7), we have developed a simple procedure, using isotope dilution, for identifying and estimating quantities of phenylthiohydantoins.

The first step in this procedure involves degrading peptides using tritiated phenyl isothiocyanate. In the second step the radioactive thiazolinone released after each cycle is immediately diluted with a mixture of unlabelled phenylthiohydantoins and is then isomerized. The unlabelled, dilutant phenylthiohydantoins serve as protective agents against decomposition and as internal standards for thin layer chromatography.

EXPERIMENTAL

A sample (0.35 μmole) of the heptapeptide, gly-phe-val-ala-pro-leu-gly, was attached to aminoethylaminomethyl polystyrene (obtained from BioRad Laboratories) and was degraded through eight cycles using an automated version (8) of the solid-phase Edman degradation (7). During each two-hour cycle the peptide was treated for 20 min with about 100 μl of ^{3}H-phenyl isothiocyanate (31,000 dpm per μmole; New England Nuclear) and for 50 min with trifluoroacetic acid-dichloroethane (1:1), with washing steps between. Thiazolinones were collected after each cycle in a fraction collector in tubes containing 0.5-1.0 μmole each of the phenylthiohydantoins derived from glycine, phenylalanine, valine, alanine, proline and leucine, as well as 0.1 ml of water to promote isomerization *in situ*. The tubes were kept at room temperature and were not protected from air. At the end of the degradation (16 hr) each tube contained about 2 ml of trifluoroacetic acid and 4 ml of dichloroethane in addition to the phenylthiohydantoins.

The contents of each tube were evaporated to dryness and the residue was heated in 0.2 ml of 20% trifluoroacetic acid at 70° for 10 min to ensure isomerization. The resulting mixture was diluted with 0.5 ml of methanol and the

solution was passed through a column (5 x 20 mm; made from a plugged disposable pipette) of Dowex-50 (H^+ form) to remove basic salts. The column was washed with 4 ml of methanol, the effluent was evaporated to dryness, and the residue was dissolved in 40 µl of dichloroethane. Alternatively, the extraction procedure of Edman and Begg (2) was used.

A 4-µl aliquot (containing about 0.035 µmole of radioactive phenylthiohydantoin)of each of the eight solutions was applied to a precoated silica gel plate containing a fluorescent indicator (E. Merck and Co.).[3] The plate was developed through 13 cm in chloroform-ethanol (98:2). After location under an ultraviolet lamp, absorbing areas were scraped loose from the plate, and the silica gel was transferred[4] to scintillation vials. Ethanol (0.2 ml) was added to the vial (to extract the phenylthiohydantoin), followed by 15 ml of toluene-scintillator solution.

RESULTS AND DISCUSSION

Figure 1 shows the results of the eight-cycle degradation. With the possible exception of valine and phenylalanine, the phenylthiohydantoins of which are poorly resolved, there is no ambiguity in the sequence. The results also show that the degradation, under these conditions, is only about 97% complete in each cycle.

A major advantage of this procedure is its rapidity, as samples from several degradative cycles can be chromatographed on the same plate. Using the technique described in footnote 4, one can transfer 100 spots from thin layer plates to scintillation vials in less than an hour. The most time-

[3]Pre-coated plates obtained from Quantum Industries gave superior resolution of phenylthiohydantoins, but were unusable because of an extremely high counting background, due presumably to luminescence of the phosphor.

[4]Transfer may be accomplished conveniently using a simple vacuum device consisting of a 10 mm diameter glass tube, ground square at one end and attached to a vacuum line. The vacuum is turned on, and a 15 mm filter paper disc (e.g., S and S no. 410 paper) is placed across the squared end of the tube. With the filter disc held in place by air pressure, the device can be used to pick up the loosened silica gel, which adheres to the filter paper. When the vacuum is released, both the disc and the silica gel fall into the scintillation vial.

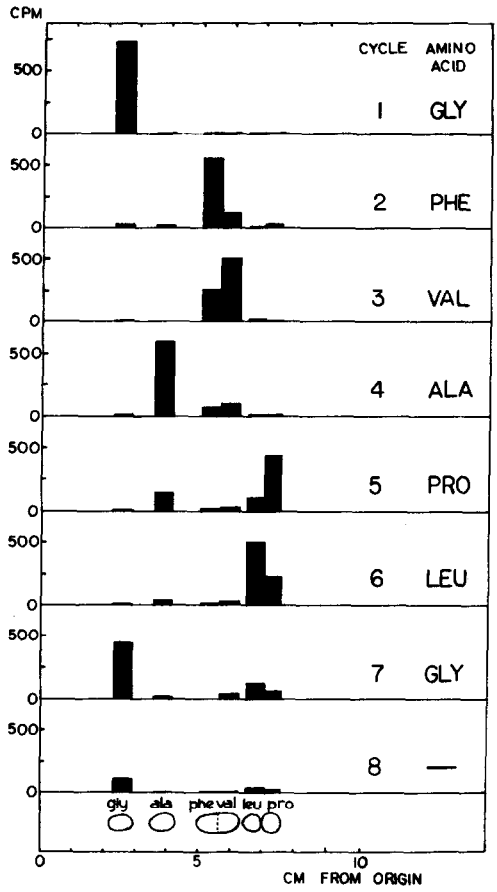

Figure 1. Radioactivity of phenylthiohydantoins separated by thin-layer chromatography. Approximately 0.035 μmole of radioactive phenylthiohydantoin was used for analysis of each cycle.

consuming operations are those involving the isomerization reaction and evaporation of solvents. Substitution of the ion exchange column for the extraction procedure of Edman and Begg (2) also shortens the time.

The low levels of tritium used in these experiments precluded the use of scanning methods for locating radioactive spots on plates. Utilization of ^{14}C-or ^{35}S-labelled phenyl isothiocyanate (4) would overcome this problem, though these reagents suffer from the disadvantages of high cost in the former case and a short half-life in the latter. The sensitivity of the procedure is good, only 0.035 μmole of phenylthiohydantoin being needed for counting in

this experiment. The use of radioactive isotopes has the added advantage that only products derived from phenyl isothiocyanate are detected.

The isotope dilution method is limited by the ability of thin-layer chromatography to separate the phenylthiohydantoins, for example, the pairs valine-phenylalanine and leucine-isoleucine are poorly resolved. When using a large number of phenylthiohydantoins, it is often more convenient to count several spots together to localize the radioactivity, and then to resolve them using another solvent system.

With the solvent system, chloroform-ethanol (98:2), several of the phenylthiohydantoins of polar amino acids remain at the origin along with an unidentified radioactive product(s) having a comparable level of radioactivity as that of the phenylthiohydantoins. This background radioactivity, however, can be separated in other solvent systems, for example, chloroform-ethanol (9:1). Radioactive diphenylthiourea, which chromatographs between proline and leucine phenylthiohydantoins, was not detected in these experiments.

A further practical limitation may be to automated versions of the Edman degradation, where the radioactivity (about 10 μmole per cycle) can be conveniently contained.

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