A NEW METHOD FOR THE DETERMINATION OF N-TERMINUS OF PEPTIDES CHAIN WITH FLUORESCEIN-ISOTHIOCYANATE

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Received March 12, 1968

Present method describes a new micro-analysis of N-terminus of protein and peptide using fluorescein-isothiocyanate I. The method has advantages over the previously known methods such as dinitrophenylation with 2, 4-dinitrofluorobenzene (Sanger, 1951, Fraenkel-Conrat, 1955) or the DANSYL (with 1-Dimethylaminonaphthalene-5-sulfonyl chloride) method (Gray et al., 1963 a, b and 1967). The present method is capable of detecting very low levels of peptide (at least 1/100 - 1/1000 of dinitrophenylation). At the same time it is suggested that our method will be used for a sequence analysis of peptide chain, as is the case of Edman's phenylisothiocyanate method (Schroeder, 1967) for which the DANSYL method is not applicable by itself.

EXPERIMENTALS AND RESULTS

Fluorescein-isothiocyanate (FITC)-I (Fig. 1) was synthesised in our laboratory using the method of McKinney (McKinney et al., 1962) and Riggs (Riggs et al., 1958) with slight modifications, since many of the commercial

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materials contain the isomer II as well as fluorescein, fluorescein-amine and some other contaminations. The FITC preparation used in the present experiment was proved to be pure by electrophoresis and thin layer chromatography.

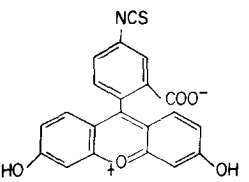

Fig. 1 Structure of Fluorescein-isothiocyanate (FITC), Isomer I. (Corey et al., 1966)

Fluoresceinthiocarbamylation of proteins and N-terminus cleavage.

Protein samples, a) insulin, (Sigma Co., U.S.A.), and b) neocarzinostatin (prepared in our laboratory) (Maeda et al., 1966), each 1.0 mg was dissolved in one ml of 0.5 M carbonate buffer pH 9.0, and 50 molar excess of FITC-I was added to the solution. The coupling reaction mixture of FITC-I and protein was kept for two hours at 25° C in dark. The reaction mixture was passed through Sephadex G-25 column which was equilibrated with 0.01 M phosphate buffer pH 7.4 to remove the unreacted reagent. Since the fluorescein-thiocarbamylated (FTC)-protein exhibits a high extinction at 493 mμ, it was easily detectable as a yellow band at the Sephadex front. The FTC-protein moved faster toward the anode than the original protein upon electrophoresis on polyacrylamide gel or cellulosepolyacetate at either pH 8.0 or 6.0. It was fluorescent under ultraviolet light.

The fractionated FTC-protein after gel filtration was brought to dryness and treated with trifluoroacetic acid saturated with HCl-gas to cleave the N-terminal amino acid as a fluorescein-thiohydantoin (FTH) amino acid (Fig. 2).

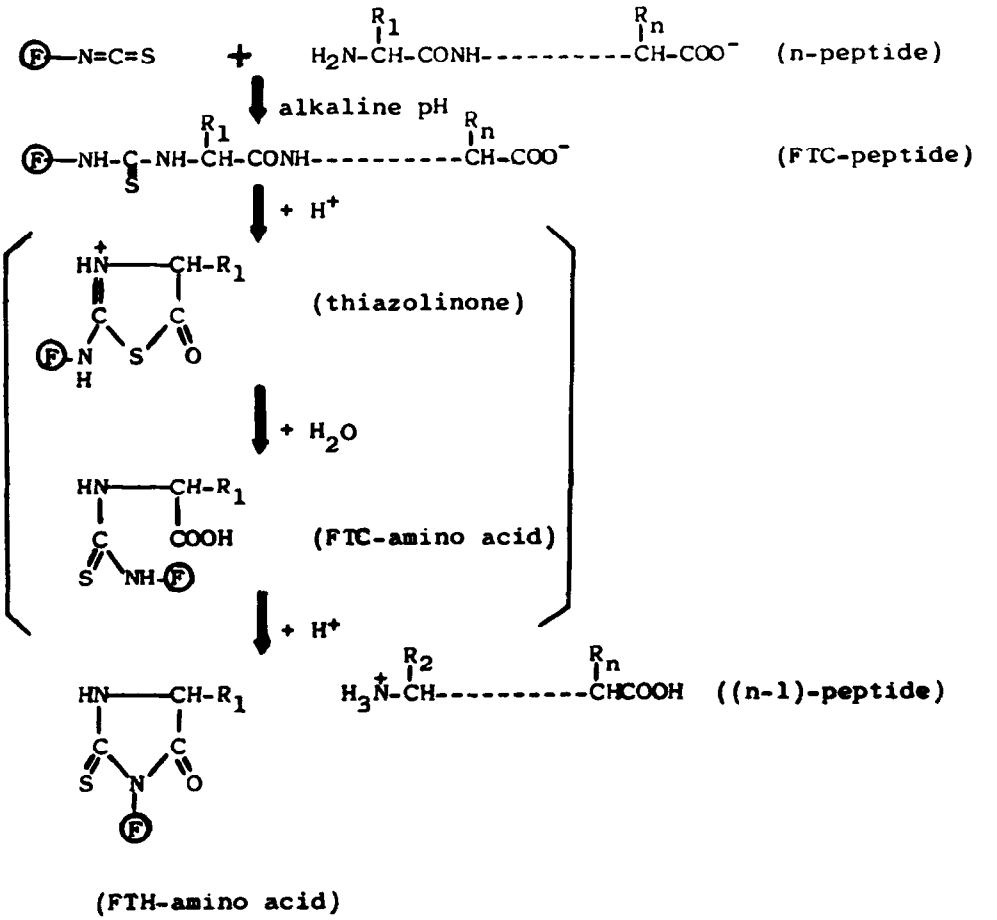

Fig. 2 Proposed mechanism for sequential cleavage of FTC-protein and preparation of FTH-amino acid. (F) indicates fluorescein chromophore.

The FTH-amino acid was extracted with ethylacetate followed by thin layer chromatography and paper electrophoresis. The residue after extraction was used for further sequence analysis. The standard FTH-amino acid was prepared for 18 amino acids under the conditions described for FTC-protein preparation except 5 molar excess amino acid over FITC was used. The FTC-amino acid derivative was cyclized in 2N HCl for 12 hours at room temperature. The FTH-amino acids, thus prepared from standard amino acid, were extracted with ethylacetate from aqueous solution and used as the standard FTH-amino acids. The detailed structural study for each FTH-amino acid will be reported shortly.

Identification of FTH-amino acid by chromatography.

The N-terminus of a) insulin (M. Wt. 6,000) and b) neocarzinostatin (M. Wt. 9,000) (Maeda et al., 1966) have been known for some time, as a) Gly, and Phe, and b) Ala. The FTH-amino acids possess yellowish color, and upon UV radiation give fluorescence just like FTC-protein. Thus their location on the chromatogram are easily identified. The result of the present experiment confirmed those previously known amino acids as shown in Fig. 3 by the silica gel thin layer (Merk, U.S.A.) chromatography using $CHCl_3$-pyridine-acetic acid (10:1:2). Using the other developer system, benzene-ethylacetate-acetic acid (5:5:1), the above results were again confirmed (Rf. of Gly, Ala, Phe = 0.32, 0.55, 0.66). Basic amino acids stayed at the origin(not shown). In both runs free FITC, if present, ran most closely with developer-front and fluorescein-amine-HCl stayed at the origin. The paper electrophoresis also gave satisfactory results in which FITC-coupled amino acids (neutral and acidic amino acid) migrated faster than the parent material toward the anode above pH 4 due to the addition of one more charged carboxyl group by FITC.

By this technique less than 0.1 mμ mole of FTH-amino acid was easily detectable under the ultraviolet light.

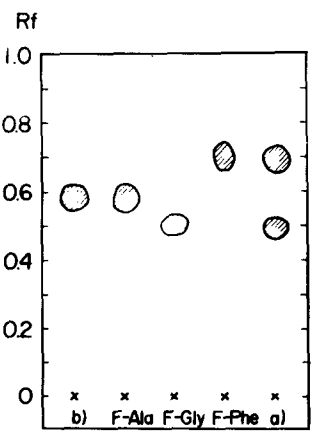

Fig. 3 Thin layer chromatography of FTH-amino acid and N-terminal FTH-amino acids from a) insulin and b) neocarzinostatin. Developer=$CHCl_3$:pyridine: acetic acid (10:1:2). F prefixed to amino acids indicates FTH-, which is yellow spot at high concentration, and exhibits intense greenish-yellow spot upon UV radiation.

DISCUSSION

The stoichiometry indicated that a mole of insulin has coupled with 1.8 mole FITC in 2 hours, while neocarzinostatin which has one N-terminal amino acid coupled 1.06 mole. These data as well as others to be published indicate that FITC attacked the α-amino of N-terminal amino acids, where the pKa values were lower than the ε-amino of lysine which is ionized at this pH, and cannot be attacked. However prolonged reaction (over 10 hours) at higher pH led to further incorporation of FITC (perhaps to the ε-amino of lysine).

In Fig. 2, the mechanism of FITC-coupling reaction and N-terminus cleavage is proposed. All FTC-amino acids should be brought to their stable hydantoin in an acidic condition. The method presented here has both the advantages of the phenylisothiocyanate-method and the DANSYL-method.

Work is under its way to improve further the method described in this report.

ACKNOWLEDGEMENTS

The authors are gratefully indebted to Professor K. Tuzimura, and Professor N. Ishida of Tohoku University, Sendai, in whose laboratories these experiments were conducted. We would like to acknowledge Professor R.E. Feeney of University of California, Davis, for his interest and encouragement, and Mr. Y. Nara of Tohoku University for his excellent technical assistance.

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