NEW METHOD FOR ISOLATION OF THE *C*-TERMINAL FRAGMENT OF PEPTIDES AND PROTEINS

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In a recent report from this laboratory [1], it was shown that alkylated proteins with their carboxyl groups converted into amide groups are readily soluble in water. Further studies have shown that modification with methylamine provides a technique for isolating the *C*-terminal fragment of the modified protein.

When modified with methylamine, all carboxyl groups of the protein, including the terminal α-carboxyl, are converted into amides; thus, the modified protein (MeA-protein) has no free carboxyl groups. In the course of enzymatic fragmentation of MeA-protein, each cleavage generates one carboxyl group. Therefore, all peptides formed by the enzymatic hydrolysis of the MeA-protein, with the exception of the *C*-terminal fragment, have one free carboxyl group. Any method which utilizes this fact enables the identification of the *C*-terminal fragment. For example, all peptides except the *C*-terminal, will be attacked by a mixture of carboxypeptidase-A and carboxypeptidase-B. Thus an identification procedure has been de-

Table 1[a]

C-terminal sequence of h-ACTH 1–28 [3]	Asp. Ala. Gly. Glu
C-terminal fragment found Analysis	(Ala. Gly. Glu) (85/82/67)
C-terminal sequence of the C-chain of chymotrypsin [4]	Leu.Ala.Ala.Asp
C-terminal fragment found Analysis	(Ala. Ala. Asp) (134/135/136)[b]

[a] Analysis data are expressed in nmoles.
[b] Total alanine found: 269 nmoles.

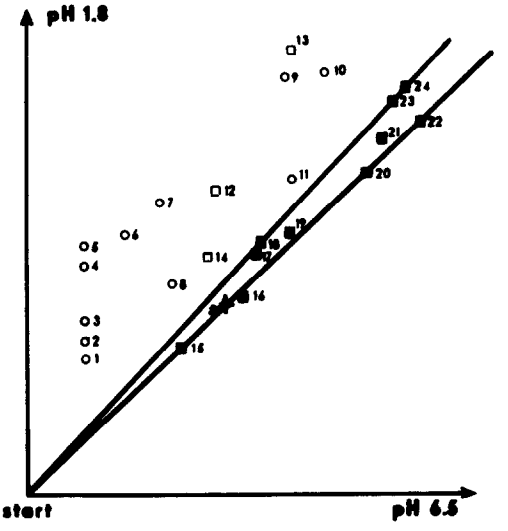

Fig. 1. Two dimensional electrophoresis of model compounds. ○ amino acids and peptides, ■ esters and amides, □ His containing amides and ester. 1) Nε-BOC-Lys.Tyr 2) Phe 3) Leu 4) Ala 5) Gly 6) His.Phe 7) His 8) ACTH 1–28 9) Glu(D) (D = -NH-CH_2-CH_2-N(CH_3)$_2$), 10) Glu(D). Glu(D) 11) Lys, 12) His. Phe-OMe, 13) His-OMe 14) MeA-ACTH 1–28 15) Nε-BOC-Lys. Tyr-OMe 16) Gly. Tyr-OMe 17) Glu(OEt)OEt 18) Phe-OMe 19) Leu-OMe 20) Ala-OMe 21) Glu(D). Glu(D)D 22) Gly-OMe 23) Arg-OMe 24) Glu(D)D. + *C*-terminal tripeptide derived form the C-chain of MeA-carboxymethyl-chymotrypsin-A, ★ *C*-terminal tripeptide of MeA-ACTH 1–28.

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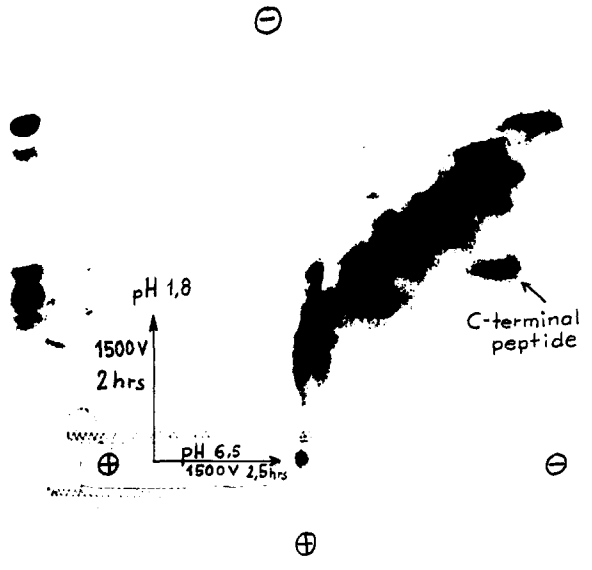

Fig. 2. Two dimensional electrophoresis of the chymotryptic digest of the mixture of B and C chains of reduced, aminoethylated and modified chymotrypsin-A.

veloped (unpublished) similar to that described by Naughton and Hagopian [2]. In this paper a simple electrophoretic method is described for identification of the *C*-terminal of MeA-proteins or MeA-peptides by two dimensional paper electrophoresis.

The principle of the method is as follows: since the *C*-terminal fragment has no free carboxyl group, its electric charge, and therefore its electrophoretic mobility, are almost the same at both pH 6.5 and 1.8 while the mobilities of the other peptides are increased by changing the pH of the buffer from 6.5 to 1.8.

Fig. 1 shows that, after two dimensional paper electrophoresis (first dimension at pH 6.5, the second at pH 1.8), the amides or esterified derivatives of amino acids and peptides, are located on a diagonal, while the free amino acids and peptides are found above this diagonal; this does not apply for peptide derivatives containing histidine.

A mixture of B- and C-chains of reduced and aminoethylated chymotrypsin-A was treated with methylamine and hydrolyzed by chymotrypsin. The peptide map derived from the digest by two dimensional electrophoresis is shown in fig. 2. The *C*-terminal fragment, indicated by an arrow can easily be identified. There is one spot, not two as expected, since methylamine is probably removed from the *C*-terminal tyrosine of the B chain during digestion.

In fig. 3 the fingerprints are compared of chymotryptic hydrolyzates of synthetic fragment 1–28 of human ACTH, untreated (a) and treated (b) with methylamine.

It will be noticed that on the second fingerprint (fig. 3b) the acidic spots are missing and an easily distinguishable spot appears on the diagonal.

The isolation of the *C*-terminal fragment was performed as follows (see fig. 3b): The hydrolyzate was first submitted to electrophoresis on a wide paper

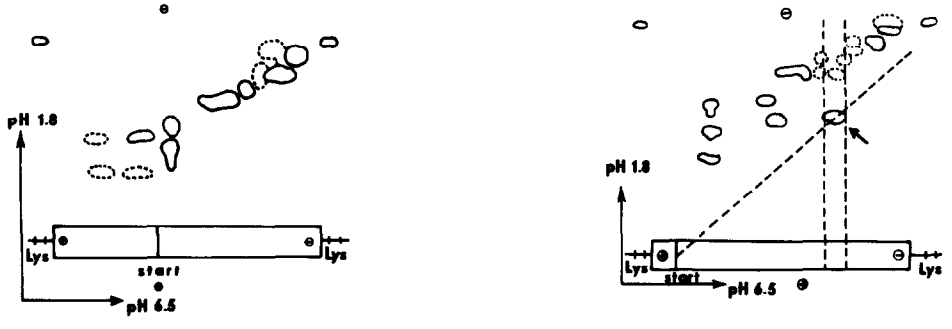

Fig. 3. Two dimensional electrophoresis of chymotryptic digest of synthetic fragment of human ACTH (a) and of the peptide modified with methylamine (b).

sheet at pH 6.5 and a strip was cut out and run at pH 1.8, in a direction at right angles to the first run. The spot of the *C*-terminal fragment was then identified on the fingerprint (drawing the diagonal can be facilitated by running an amino acid ester standard, for example phenylalanine methylester, with the mixture) and a strip was cut from the pH 6.5 sheet containing the *C*-terminal fragment and impurities. After rerunning at pH 1.8, the slowest moving band represented the *C*-terminal fragment.

By this method, the *C*-terminal fragment was isolated from the methylamine derivative of synthetic fragment 1–28 of human ACTH as well as from the C-chain of the reduced and carboxymethylated chymotrypsin-A. Data of amino acid analysis are summarized in table 1. It can be seen that analysis of the isolated tripeptides is consistent with the *C*-terminal sequences of the peptide chains from which they were derived.

The method described cannot be applied when the *C*-terminal fragment contains histidine or when the enzyme used for hydrolysis removes the methylamine from the *C*-terminal residue. However, if removal of the methylamine from the *C*-terminal residue during digestion is only partial, or if blockage of the *C*-terminal carboxyl group during reaction with methylamine is incomplete, the isolation of the *C*-terminal fragment will still be successful, since the presence of a *C*-terminal fragment with a free carboxyl group is not expected to interfere.

Experimental

Synthetic fragment 1–28 of h-ACTH was kindly supplied by Gedeon Richter Chemical Works Ltd. Budapest, α-chymotrypsin was a Calbiochem product. Aminoethylation was performed according to Raftery and Cole [5]. Isolation of the C-chain of the carboxymethylated chymotrypsin-A was performed by the method of Hartley [6]; treatment with methylamine was described earlier [1]. Digestions were conducted in a Radiometer pH-stat, at 37°C, pH 8.0 for 6 hr. After adjusting the pH to 3, the digest of the ACTH derivative was applied directly to Whatman 3 mM paper, while that of chymotrypsin was first centrifuged and the supernatant used for electrophoresis. 0.1 μ-moles digest was applied per 1 cm paper. Electrophoresis was carried out on a cooled flat plate apparatus (Labor, Hungary) at 31 V/cm for 2.5 hr, using volatile buffers. Peptide samples were hydrolyzed in 6 N hydrochloric acid and analyzed on an EEL amino acid analyzer.

Acknowledgement

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