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Note

New sensitive technique for the quantitative analysis of initiation peptides

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Initiation of eukaryotic proteins begins with a methionyl residue incorporated in the N-terminal position and which is not removed until the nascent chain is at least 20–30 residues long[1]. When antibiotics such as sparsomycin[2] or pactamycine[3] are added to a cell-free protein synthesis system with [^{35}S]methionine as label, the products are dipeptides and oligopeptides having an N-terminal methionine residue[4].

In this paper, we describe a quantitative, sensitive chromatographic method for analysis of initiation dipeptides using an amino acid analyzer (Chromaspek–Rank Hilger).

MATERIALS AND METHODS

Preparation of the cell-free protein synthesis system

Polysomes and supernatant from rat heart and rabbit reticulocytes were prepared and incubated as described previously, in presence of 70 μM sparsomycin[5], with some slight modifications that will be reported elsewhere.

Isolation of initiation peptides

Initiation peptides were isolated according to the first method of Smith and Wigle[4]: peptidyl tRNAs were prepared by phenol extraction of polysomes after cell-free synthesis, then tRNAs were removed by enzymatic degradation (ribonuclease ase A). Methionine (5 nmoles) and eleven different methionyl dipeptides (20 nmoles of each, listed in legend to Fig. 1) were added as carrier.

The peptides were further purified by gel chromatography on a Bio-Gel P-4 column (20 × 0.8 cm), eluting with water at a flow-rate of 12 ml/h. Monitoring at 280 nm was continuous. Thirty-five fractions of 0.8 ml were collected and 0.01-ml aliquots were counted for radioactivity in a 10-ml Unisolve (Koch-light) scintillator using an Intertechnique SL 32 scintillation counter. The radioactive fractions were pooled, freeze dried and then redissolved in 0.3 ml of 0.025 N HCl prior to chromatography.

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Dipeptide analyses

The dipeptides were analyzed by high-performance ion-exchange chromatography on a Chromaspek J 180 amino acid analyzer using a program specially designed for this purpose. Integration of absorbance peak areas at 570 nm after ninhydrin staining, by a Digico Micro 16 computer linked to the recorder, demonstrated that recovery of carrier dipeptides throughout the experiment was reproducible within $\pm$ 10%.

A fraction collector was connected to the outlet of the photometer cell, and 33 fractions of 1 ml each were counted for radioactivity as described above.

The time required for one analysis was 90 min.

RESULTS

Preparation of initiation peptides

In order to remove RNAase and other high-molecular-weight materials from the digested phenol extract, the sample was subjected to gel chromatography on a Bio-gel P-4 column. RNAase activity was eluted with the excluded volume. The radioactive material was found as a sharp peak in the leading edge of the absorbance peak which contains methionine, peptides and nucleotides resulting from enzymatic degradation of RNAs.

Analyses of initiation peptides

For standard amino acid analysis, a pH gradient generated by the continuous mixing of two 0.2 *M* Na^+ buffers at pH 2.2 and pH 11.5 (Chromaspek program CH 11), was employed. The proportion of acidic to basic buffer is determined by the ratio of black to silver areas read by a photoelectric cell on a rotating drum. The buffer mixing program developed by us for analysis of methionyl dipeptides and the resulting pH gradient are represented in Fig. 1.

The elution pattern of a calibration mixture of eleven methionyl dipeptides and methionine (Fig. 1) was not modified when AMP, CMP, UMP and GMP were all added, up to 60 nmoles of each. Neutral peptides, except Met-Val (peak 7) and Met-Met (peak 8), were well separated. Met-Glu and Met-Pro (peak 4) could not be separated. The methionyl dipeptides were eluted in the same order as the individual amino acids corresponding to the second residue of the dipeptide. Therefore we can predict approximately where methionyl dipeptides, that were not used in the calibration mixture, should be eluted.

Application to biological problems

The method described has been used to analyze the dipeptides produced in rabbit reticulocyte cell-free systems with endogenous globin 9S mRNA. Under these conditions the only expected dipeptide is Met-Val. The results were compared with those obtained by the high-voltage paper electrophoresis technique of Smith and Wigle[4] (Figs. 2 and 3). Both methods gave similar results for Met-Val.

Nevertheless, with the present technique, complete separation of Met-Val (peak 4) is achieved and, in addition, some minor peaks are also detected. Peak 5, eluted in the same position as the Met-His marker, probably contains tripeptides. This is supported by the fact that no major reticulocyte protein has an N-terminal histidine.

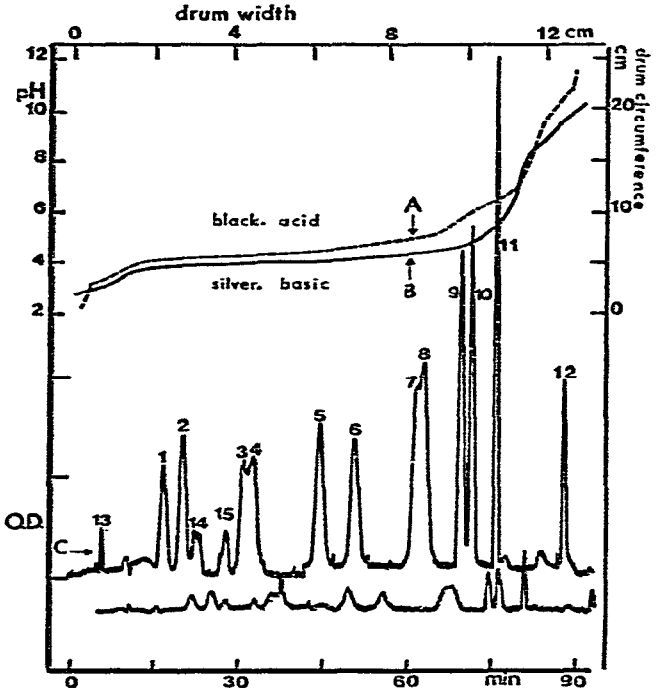

Fig. 1. A, Buffer mixing program for separation of methionyl dipeptides. The proportion of drum circumference occupied by the black profile determines the proportions of acidic to basic buffer to be mixed. B, pH gradient obtained with the program above. The program was run without sample, and ninhydrin inlet was disconnected. The pH was measured in 0.8-ml fractions. The slope is very flat between pH 4 and pH 5 where neutral peptides are eluted. C, Analysis of a calibration mixture of eleven methionyl dipeptides (20 nmoles each) and methionine (5 nmoles). Peaks: 1 = methionine; 2 = Met-Asp; 3 = Met-Ser; 4 = Met-Glu and Met-Pro; 5 = Met-Gly; 6 = Met-Ala; 7 = Met-Val; 8 = Met-Met; 9 = Met-Leu; 10 = Met-Tyr; 11 = Met-His; 12 = NH_3; 13 = methionine sulphone; 14 and 15 = impurities associated with Met-Ser. Upper curve, recording at 470 nm; lower curve, recording at 440 nm. Addition of four nucleotides (60 nmoles each) to the calibration mixture did not change the elution profile.

Moreover, similar proportions of that material are found by both methods. It increased while the amount of Met-Val decreased when sparsomycin was omitted from the incubation medium.

The sharp slope of the gradient between pH 7 and pH 9 could explain the elution of neutral or slightly basic oligopeptides in this region.

DISCUSSION

One-dimensional high-voltage paper electrophoresis[4] and paper chromatography[6] were the two methods previously used for analysis of initiation dipeptides. In both cases quantitation is carried out by counting the radioactivity of paper strips.

After cell-free incubation, the newly synthetized methionyl peptides are copurified with unlabelled carrier peptides. In order to compare several experiments an

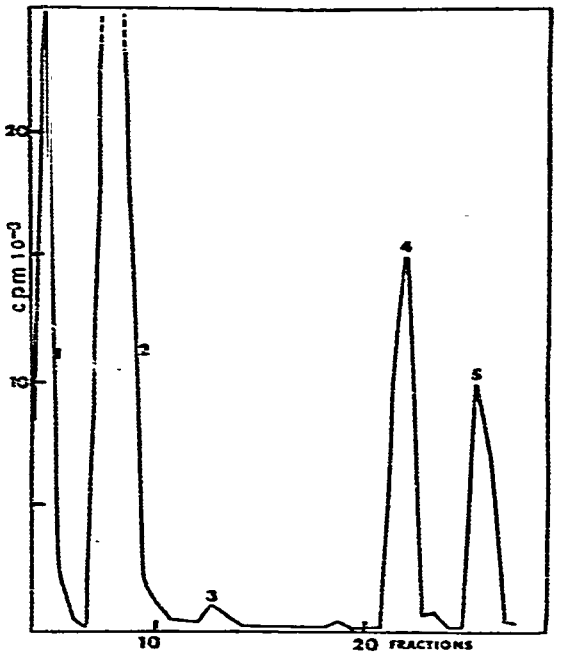

Fig. 2. Analysis of radioactive peptides synthesized in a rabbit reticulocyte cell-free system in presence of sparsomycin. Separation on Chromaspek amino-acid analyzer. Peaks: 1 = methionine sulphone; 2 = methionine; 3 = Met-Ser, Met-Glu or Met-Pro; 4 = Met-Val; 5 = tripeptides eluted with Met-His.

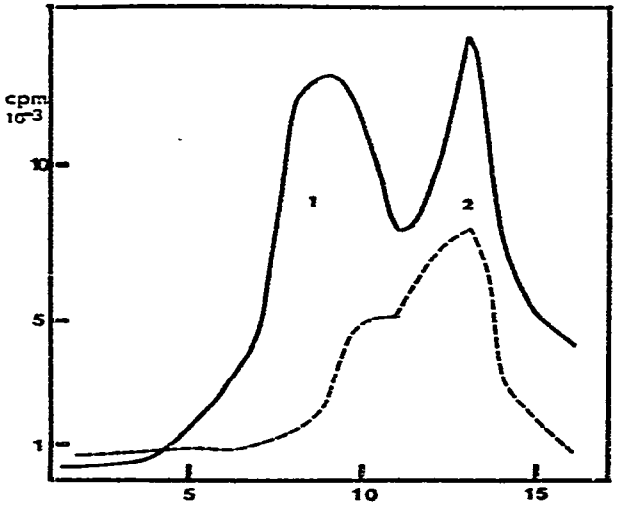

Fig. 3. Analysis as in Fig. 2 but with separation by high-voltage electrophoresis at pH 3.5. Peaks: 1 = Met-Val; 2 = tripeptides. ———, Sparsomycine present in the incubation medium; – – –, without sparsomycin, the proportion of tripeptides compared to Met-Val increases. The small peak (3) in Fig. 2 cannot be detected by high-voltage paper electrophoresis.

estimation of peptide recovery is desirable. This could not be achieved by the above two techniques. In contrast, the present method allows an accurate quantitation by integrating the areas of absorbance peaks after ninhydrin staining.

Moreover this chromatographic technique, discriminating between most of the dipeptides, gives an improved resolution compared to the other methods. This fact is of particular interest when studying initiation in a multi-messenger RNA cell-free system. The resolution could be greatly improved by a much larger modification of the buffer system. This was not done in this work since our Chromaspek is routinely used for amino acid analyses and it was not practical to change the program duration and the buffer composition.

If required, the rather long analysis time necessary for each sample (90 min) could be reduced for specific biological problems by designing appropriate pH gradients.

This method was also successfully applied to a more complex biological system: protein synthesis in a hypertrophied heart cell-free system.

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