A NEW ASSAY METHOD FOR CELL-FREE POLYPEPTIDE SYNTHESIS USING 1-FLUORO-2,4-DINITROBENZENE

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Received 1 August 1969

1. Introduction

The solubility of peptides in aqueous solution varies considerably depending on the amino acid composition and peptide chain length. These variations in solubility lead to a great deal of difficulty in the quantitative study of cell-free polypeptide synthesis, particularly in assaying the efficiency of amino acid incorporation into shorter peptides. At present, no reliable and universally applicable assay method is available to permit the examination of cell-free polypeptide synthesis on the same basis regardless of the acid-solubility of the products [1,2]. In order to circumvent this problem, a new assay method was devised to synthesize phenylthiohydantoins of amino acids in the cell-free system, and to extract them with organic solvents from the peptides which are to be assayed [3]. This method, however, suffers from the disadvantage that N-terminal residues of all newly synthesized peptide chains are extracted along with the free amino acids in the reaction mixture. Thus this method gives a good parameter of peptide synthesis but it does not indicate the exact amount of peptide bond formation in a cell-free peptide synthesizing system. We therefore have been trying to improve this method by changing the blocking group. Of several aromatic blocking groups tested, dinitrophenyl derivatives of amino acids, extracted with organic solvents, have been shown to give quantitative results in the assay of peptide formation in a cell-free system directed by various messenger RNA's.

2. Materials and methods

[^{14}C]-labelled L-amino acids were purchased from Schwarz BioResearch. Specific activity of each amino acid was 50 mC/mmole, except for [^{14}C]-L-methylmethionine (49 mC/mmole). Polyribonucleic acids were also from Schwarz BioResearch. 1-Fluoro-2,4-dinitrobenzene was purchased from Eastman Organic Chemicals. The other chemicals were from Fisher Scientific Co.

The amino acid incorporating cell-free system, and the hot trichloroacetic acid (TCA) assay method were described in an earlier report [4]. Bray's scintillation fluid (60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol, and dioxane to bring the volume to 1 litre) was used in radioactivity measurements with a Beckman 200B liquid scintillation counter.

3. Results

3.1. *Liquid state method*

The well established amino terminal blocking procedure of Fraenkel-Conrat et al. [5] using 1-fluoro-2,4-dinitrobenzene (DNFB) was applied directly to the assay of the peptide synthesizing system. During the cell-free peptide synthesizing reaction, 0.1 ml of sample reaction mixture was withdrawn at intervals, and placed in a test tube containing 0.4 ml of 1N NaOH. An excess amount of sodium bicarbonate was then added to this mixture so that sodium bicarbonate crystals were visible. Thereafter, one ml of 5% DNFB in ethanol was added to the mixture. The test tubes were sealed with silicone stoppers and placed

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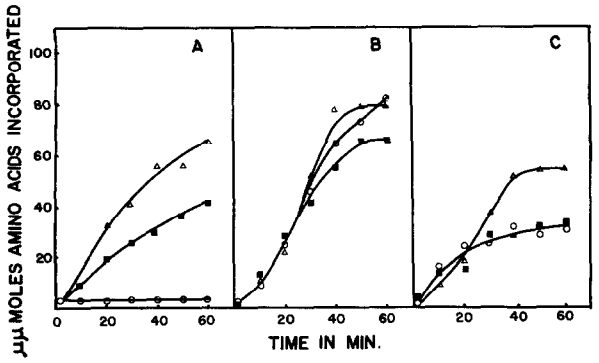

Fig. 1. Comparison of the DNP-assay method and others for cell-free peptide synthesis (liquid state method). The cell-free peptide synthesizing system has been described as a crude system in an earlier communication [6]. The reaction was at 37°C for 60 min. At intervals, three 0.1 ml samples were withdrawn. One was assayed by the DNP-liquid state method and the others by the hot TCA [1] or the phosphotungstate methods [2]. A: Poly-A directed lysine incorporation at 12 mM $MgCl_2$. B: Poly-U directed phenylalanine incorporation at 12 mM $MgCl_2$. C: R17 RNA directed mixed amino acid incorporation at 15 mM $MgCl_2$. ○: hot TCA method; ■: phosphotungstate method; △: DNP-liquid state method.

in a wrist action shaker for shaking at 37°C for 10 to 15 hours in the dark. Meanwhile, the solution was inspected for sodium bicarbonate crystals. If there were no crystals remaining, excess bicarbonate was added. At the end of the reaction, excess DNFB was extracted six times with an equal volume of peroxide-free ether. The water phase was then neutralized with 6N HCl until CO_2 gas emission ceased. Then the water phase was adjusted to an HCl concentration of about 3N, and extracted four times with ether. The aqueous phase was neutralized with 6N NaOH and extracted again with ether. The final water layer (pH 6–7.5) was placed in scintillation vials containing Bray's scintillation fluid. The result is illustrated in fig. 1, where it may be seen that the amount of amino acid incorporation detected by the DNP-method was greater than that detected by either the TCA or the phosphotungstic acid methods in three different mRNA-directed protein synthesizing systems. The reason for the greater sensitivity of the DNP-method stems from the fact that this method allows for the detection of all peptides formed, including even di- and tri-peptides. The solubility of various peptides in TCA or phosphotungstic acid, however, is variable, so that the amount of amino acid incorporation detectable by these methods is dependent on the acid-solubility characteristics of the particular peptides being formed in these systems.

3.2. *Application of the DNP-method using filter discs (solid state method)*

The liquid state method just described is very time-consuming, making it impossible to handle more than 50 samples a day. We therefore tried to extend the procedure to solid state reactions using the filter disc method originally devised by Mans and Novelli [1] for the hot TCA method. The use of discs reduces individual treatment of the samples during the extraction procedure.

A mixture consisting of 0.1 μC of [^{14}C]-labelled amino acid and 10 μg of nonlabelled amino acid in 0.1 ml of water was placed on a Whatman No. 3 filter paper disc, and each filter was completely dried and placed on a glass plate. 5% DNFB in ethanol was poured directly onto each filter disc. Filter discs were placed in a sealed chamber containing 10 ml each of ethanol and NH_4OH. Incubation was continued at 37°C for 15 hours in the dark. Filter discs were dried in a fume hood and were then extracted five times with acetone. The discs were dried

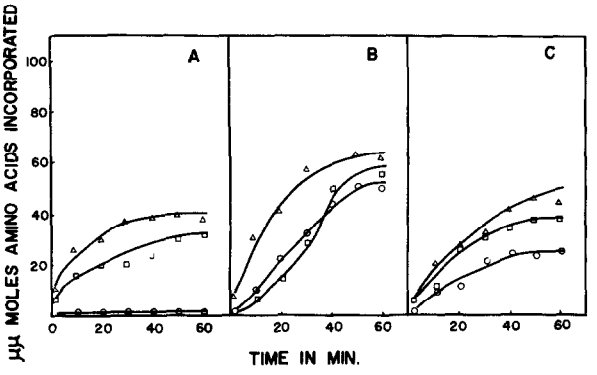

Fig. 2. Comparison of the DNP-solid state method and other assay methods for cell-free peptide synthesis. Reaction conditions were the same as in fig. 1. DNP-assay was carried out using the filter disc method as described in the text.
A: Poly-A directed lysine incorporation at 12 mM $MgCl_2$.
B: Poly-U directed phenylalanine incorporation at 12 mM $MgCl_2$. C: R17 RNA directed mixed amino acid incorporation at 15 mM $MgCl_2$. ○: hot TCA method. □: phosphotungstate method. △: DNP-solid state method.

again and to each was added 0.1 ml of a 4N acetic acid-6N HCl mixture. The filter discs were dried in a fume hood and then desiccated at 80°C for one hour. Then the discs were extracted with acetone four more times and dried. The efficiency of extraction of amino acids with the method just described was excellent. For example, 99.9% of the free glycine was extracted, but 99% of the diglycine peptide remained on the filter disc.

The above method was applied directly to the assay of peptide synthesis. Aliquots (0.05 ml) of peptide synthesizing reaction mixture were withdrawn at intervals and placed on filter discs. The reaction was stopped by adding 0.15 ml of NH_4OH. After drying the filter discs at 80°C for 30 min, DNP-amino acid synthesis and extraction were carried out as above. The result is illustrated in fig. 2. It is clear that the DNP-method with the aid of filter discs is adequate for assaying peptide synthesis directed by various mRNA's.

4. Discussion

By means of the DNP-method described in this manuscript, 99.9% of the free non-incorporated amino acids in a reaction mixture are extracted, while 99% of the peptides synthesized remain in the aqueous phase (or on the filter). The method gives quantitative results for overall peptide bond formation in a cell-free peptide synthesizing system. In this respect, the DNP-method is superior to the PTC-method reported earlier [3]. However, constant exposure of the investigator to DNFB during routine assays can be hazardous, since it can lead to allergic reactions. Therefore, this DNP-method should not be used as a routine assay procedure, but only as a procedure for special purposes.

Acknowledgements

This research was supported by a grant from the Medical Research Council of Canada, MT-3022.

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