AMINO ACID DEPENDENT ATP–$^{32}PP_i$ EXCHANGE MEASUREMENT A FILTER PAPER DISK METHOD

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1. Introduction

Enzymes that activate amino acids in the presence of ATP such as amino acid ligases, have been measured by the ATP–$^{32}PP_i$ exchange in the presence of that particular amino acid. The overall reaction for such an exchange will be as below:

$$E + ATP \rightleftharpoons E\text{–}AMP + PP_i \quad (1)$$

$$E\text{–}AMP + A.A. \rightleftharpoons E\text{–}A.A. + AMP \quad (2)$$

A number of procedures have been described for the measurement of this activity [1–4]. They differ slightly in their experimental conditions, but all require the adsorption of the labelled ATP on activated charcoal after incubation in the presence of labelled PP_i. The labelled ATP was then measured directly [1, 2] on the charcoal or after elution with 1 N HCL [3] or by NH_4OH [4] and measuring an aliquot of the solution in a liquid scintillation counter.

In our studies on the isolation of a bacitracin synthesizing enzyme from *Bacillus licheniformis* [5], the activity of the enzyme was also measured by ATP–$^{32}PP_i$ exchange in the presence of amino acids. Since this enzyme and similar enzymes for the synthesis of gramicidin S and tyrocidine [6] do not activate only one amino acid, but for bacitracin 10 and for gramicidin S and tyrocidine upto 4, during the purification step on column chromatography, each fraction had to be measured for the ATP–$^{32}PP_i$ exchange in presence of each of the ten amino acids. This required considerable time and was not convenient as the enzyme was not stable. In view of this a simple method based on the filter disk method for protein synthesis [7, 8] has been developed for the measurements of this activity.

2. Materials and methods

To test this procedure, the most active fraction of the bacitracin synthesizing enzyme i.e. enzyme II from DEAE-cellulose column chromatography as described by Pfaender et al. [5] was used. This enzyme had the ATP–$^{32}PP_i$ exchange activity dependent on all the ten bacitracin amino acids. Ederol charcoal filter paper disks, 2.5 cm in diameter (No. 69/K) were obtained from J.C. Binzer, Hatzfeld/Eder, Germany. In most experiments, the disks were used as such, but a presoaking in water to remove the loosely adhering particles and then drying the disks before use would be helpful.

2.1. *Assay procedure*

The incubation mixture was similar to that of Calendar and Berg [1] with slight modification. The composition of the incubation mixture was ATP, 4.5 mM; $MgCl_2$, 3.75 mM; KF, 22.5 mM; 2-mercaptoethanol, 7.5 mM; $Na_4P_2O_7$, 4.5 mM (specific activity 0.6 mCi/mmole); bovine serum albumin, 0.23 mg/ml; Tris–HCl buffer, 109 mM; final pH 7.5. 20 μl of the enzyme solution in buffer A [5] were mixed in the cold with 40 μl of incubation mixture and incubated at 37°C for the stated periods. A control was also incubated which contained water in place of amino acids.

After the incubation, the mixture was immediately frozen, to stop the reaction, thawed and 10 μl of a solution of 0.4 M $Na_4P_2O_7$ in 15% perchloric acid was added. After shaking the solution it was centrifuged and 50 μl of the clear solution was then applied

to the charcoal filter disk. Disks were held either on pins or laid flat on a bed of pins and numbered with a green ball-point pen. The filter paper disks were allowed to dry at room temperature. For washing the disks any of the techniques used in filter paper disk methods can be used. However we have found the following procedure equally satisfactory: Filter paper disks were placed in a large Petri dish separated from each other and a cold solution of 0.04 M sodium pyrophosphate in 1.5% perchloric acid was added. Disks were submerged in the solution and with the help of forceps kept from settling to the bottom and adhering to each other. After about 5 min, the wash solution was sucked off and replaced with a fresh cold solution. This was followed by a third wash. Disks were now washed in the same manner with cold distilled water. Finally individual disks were held with forceps and washed in a stream of distilled water and placed on the bed of pins for drying. After partial air drying (30–60 min), the disks were completely dried for 10 min at 110°C. After cooling, the disks were placed under 5 ml of 5% PPO in toluene in scintillation counter vials and the radioactivity was measured in a liquid scintillation counter (LS 150, Beckman Inc., Fullerton, USA) using the channel range of ^{14}C plus ^{32}P.

3. Results and discussion

The procedure described here simplifies the method of measuring the amino acid dependent ATP–$^{32}PP_i$ exchange. A large number of disks can be collected and washed simultaneously, thereby reducing the time lag between incubation and the measurement of the radioactivity considerably, which is essential because of the short half life of ^{32}P. When it is not possible to continue with the washings, the samples can be stored as below: a) incubated samples as frozen; b) after addition of the sodium pyrophosphate–perchloric acid solution in a cold room; c) after application of the charcoal filter disks and drying in a cold room, but in any case they cannot be stored too long due to short half life of the isotope.

This procedure gives a linear relationship as shown in fig. 1. Increasing the amount of labelled sodium pyrophosphate in 50 μl of solution when applied to the

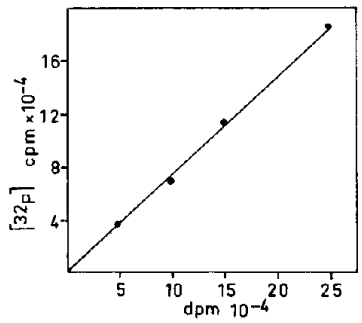

Fig. 1. Counting efficiency of ^{32}P on charcoal filter paper disks in a liquid scintillation counter. The ordinate is the measured counts and the abscissa is the amount of radioactivity placed on the disk.

charcoal filter disks, dried and counted in 5 ml of 5% PPO in toluene showed a linear increase in the measured counts. The straight line passes through the zero point. From this the efficiency of ^{32}P measurement on charcoal filter paper disk was calculated to be 78%, which is high enough for the assay.

Increasing the amount of enzyme also increases the exchange activity as shown in fig. 2. Data were plotted after subtracting the blank values without amino acids. In these experiments a mixture of ten bacitracin amino acids was used in order to obtain higher counts, since this enzyme is capable of catalysing the exchange dependent on all these amino acids.

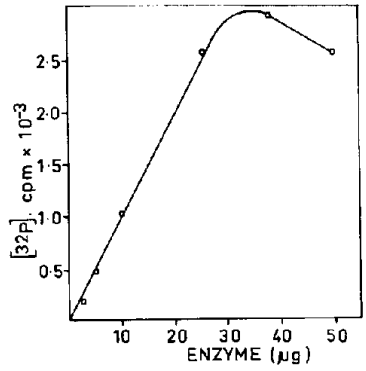

Fig. 2. ATP–$^{32}PP_i$ exchange measurements with increasing amount of enzyme. 20 μl of enzyme solution of varying concentrations were incubated in the presence of L-forms of all 9 amino acids mixed (3 mM each) and L-cysteine (6 mM) for 12 min. Details are given in Materials and methods.

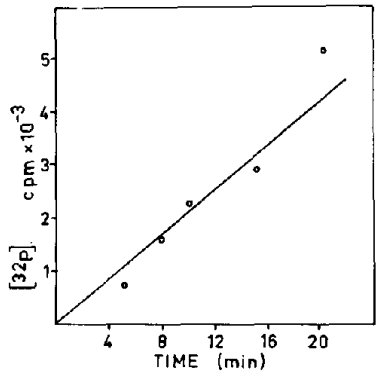

Fig. 3. Kinetics of ATP–$^{32}PP_i$ measurements. 20 μl of the enzyme solution (50 μg) was incubated in presence of all 10 bacitracin amino acids as in fig. 2.

6 mM cysteine was used in the incubation again to activate this enzyme further. However at higher concentration of this enzyme, the activity levels off, perhaps due to a saturation of the enzyme with amino acids. The control values (blanks) obtained in the absence of the amino acids vary with the concentration of the protein and the type of the protein. This is more pronounced when scanning for this activity in effluents from column chromatography, where the type and amount of protein varies in each fraction. Even though the blank values vary, we have never obtained blank values higher than 1% of the total amount of counts applied to the filter disk. This is an indication of the efficient washing procedure. The background of a disk with the same amount of activity, carried through the whole procedure, was about 0.1% of the total load. The replicates were within 5% of each other.

The amount of [^{32}P]ATP formed was proportional to time over a period of 15 min incubation time as shown in fig. 3. The experimental conditions were identical to that given in fig. 2. The reason why a spurt occurred after 15 min in the amount of [^{32}P]ATP formed is not clear. For routine measurement of the activity a 15 min incubation period was satisfactory. In plotting these data, again the blank values without amino acids were subtracted. Zero time incubation gave no exchange activity.

It is clear from the above results that this procedure is simple and fast when a large number of samples have to be treated. The black charcoal filter paper did not quench the counting efficiency considerably. Further advantage of this method is the use of a small volume of the sample. However, for a particular enzyme the concentration range in which the ATP formed is linear should be found. But this is no advantage in quickly scanning the column effluents to locate the activity as the protein solution from the column is dilute enough.

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