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Note

Determination of 5′-nucleotidase by automated ion-exchange column chromatography

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A simple anion-exchange chromatographic separation of adenine nucleotides using G MP-1 resin has been described[1,2]. The resin has a strong affinity for adenosine phosphates but not for adenosine. Adenosine is eluted out with water at near void volume while AMP remains absorbed on the column.

This paper describes a simplified chromatographic procedure for automated quantitation of adenosine produced by 5′-nucleotidase reaction.

EXPERIMENTAL

Materials

AG MP-1 resin was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.); and AMP from Sigma (St. Louis, MO, U.S.A.).

Preparation of reaction mixture

A 0.5-g amount of the human cervix was homogenized in 2 ml of 0.25 *M* sucrose for 2 min at 900 rpm using a motor-driven tissue grinder. The homogenate was then centrifuged at 300 *g* for 10 min and 1 to 20 μl of the supernatant solution were used for the assay.

The reaction mixture in a total volume of 1 ml contained 50 m*M* glycylglycine (pH 7.4), 10 m*M* $MgCl_2$, 2 m*M* AMP and the tissue extract. The reaction was initiated by the addition of AMP at 37°C and terminated after 10 min by addition of 1 ml ice-cold 6% trichloroacetic acid. A blank for each sample was prepared by adding AMP after the enzyme was inactivated by the addition of trichloroacetic acid. The precipitate was removed by centrifugation and an aliquot was neutralized with 2 *M* Tris–base (5:1) for chromatography.

Automated column chromatography

In addition to the previous setup[2] for column chromatography, an automatic sample injector was attached to the column. Samples in a volume of 200 μl were injected at 10-min intervals while the column was eluted with water at a flow-rate of 2 ml/min. The effluent was monitored at 257 nm. The adenosine peaks appeared one after another, were recorded and then areas were integrated using an extinction

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coefficient of $15.4 \cdot 10^3$ l·mol^{-1}·cm^{-1}. After 10 injections the column was washed with 1 *M* hydrochloric acid for 5 min then with water for 10 min and the process was repeated.

RESULTS AND DISCUSSION

A 200-μl aliquot of a reaction blank containing 166 nmoles of AMP were injected at 10-min intervals. The adenosine peaks of ten injections were very constant. They gave an average value of 1.22 $\pm$ 0.22 (S.D.) nmoles. When 10 nmoles of adenosine were added to the above solution, the experimental error was minimized to less than 5%. Continuous injection of the blanks over 15 times tended to increase the value slightly. Therefore, alternate injection of a sample and its blank would avoid any experimental error.

The enzyme activity as reflected by the amount of adenosine produced was linearly proportional to the incubation time. The activity levelled off after one-half of AMP was dephosphorylated.

Fig. 1 showed the results of 5′-nucleotide activity *versus* the amount of enzyme present in the crude extract. It was linearly related up to 50 mU/ml and approximately 25% of AMP was consumed.

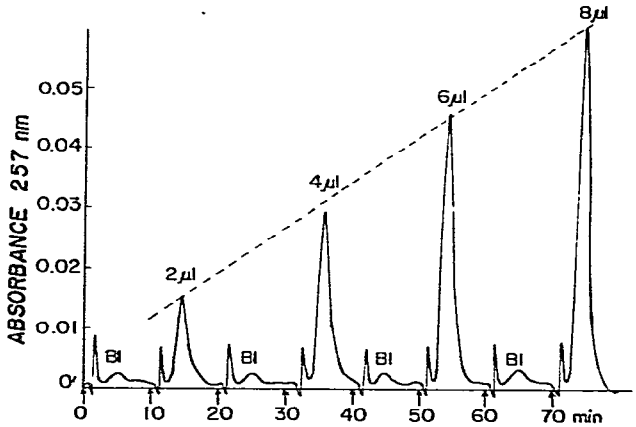

Fig. 1. Amount of adenosine produced *versus* the enzyme concentration. Four reaction mixtures containing different amounts of the enzyme extract (2–8 μl), and respective blanks (Bl) were injected to a column at the points marked by an arrow. The small sharp peaks appeared at the void volume and were from UV-absorbing substances such as unprecipitated proteins. The amount of adenosine and peaks of UV absorption at 257 nm were linearly proportional.

We estimated from the previous study[2] that the column bed (5 × 1.1 cm) has a total exchange capacity of 3 mmoles AMP. AMP was strongly bound to the resin and could not be eluted out with water. As far as the sample solution is neutralized to slightly basic, there is no leakage of AMP by repeating injection of 20 samples to a similar column. To determine the enzyme activity at lower limit, it is suggested to regenerate the column after every 10 injections.

REFERENCES

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