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Note

Assay of 5′-nucleotidase and simultaneous detection of interfering enzymes by anion-exchange column chromatography

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Determination of the activity of 5′-nucleotidase (5′-NTase) in the presence of non-specific phosphatases has often relied on inhibition of either phosphatases or 5′-NTase. In the presence of alkaline phosphatase (AlPase), 5′-NTase is measured by using an inhibitor, such as β-glycerophosphate [1], phenylphosphate [2] or L-cysteine [3]. Conversely, by using an inhibitor of 5′-NTase, such as nickel ion [4], α,β-methyleneadenosine 5′-diphosphate [5] or concanavalin A [6], the activity of 5′-NTase can be determined from the difference between activities measured in the absence and presence of the inhibitor, and discriminated from the activity of non-specific phosphatases.

This paper describes the use of an anion-exchange chromatographic method for monitoring the rate of hydrolysis of 5′-AMP and 3′-AMP demonstrating the possibility of utilizing chromatography as a tool to determine more specifically the activity of 5′-NTase in the presence of AlPases, since AlPases hydrolyze 5′-AMP and 3′-AMP at a similar rate. In addition, the method can simultaneously detect the presence of other interfering enzymes such as acid phosphatase (AcPase) and adenylate deaminase.

EXPERIMENTAL

Materials

The materials used were as described previously [7]. The following enzyme preparations were used: AlPase from bovine intestine was obtained from Sigma

0378-4347/87/$03.50

(St. Louis, MO, U.S.A., Product No. P3877). A 1-mg amount of the powder was dissolved in 1 ml of distilled water.

Placental-type AlPase was prepared from 1 g of normal-term human placenta. The tissue was washed with distilled water to remove as much blood as possible, cut into small pieces and then extracted with 10 ml of 20% (v/v) butanol at room temperature for 1 h with occasional vortexing. The water solution was separated from the residue and the butanol layer by centrifugation and filtration. To the water solution, 2 ml of butanol were added and vortexed vigorously. The bottom clear solution was withdrawn and centrifuged at 10 000 *g* for 30 min at 4°C. The AlPase in this preparation was stable for several years at 4°C.

Prostatic AcPase was prepared from human urine. A 1-ml of urine sample from a male subject was passed through a column packed with Sephadex G-25 (Column PD-10, Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) with distilled water. A 2-ml volume containing the enzymes was collected at the void volume.

Various tissue extracts were prepared by homogenizing the individual tissue in distilled water (1:5, w/v) and by centrifugation at 600 *g* for 15 min to remove cell debris.

Column chromatography

The chromatographic method was similar to that described previously [8]. A longer column (220×6 mm) packed with AG MP-1 resin (Bio-Rad Labs., Richmond, CA, U.S.A.) was used. The separation of adenosine, inosine, 5′-AMP and 3′-AMP was accomplished in 11 min by gradient elution. Reservoir A contained distilled water and reservoir B 0.192 *M* hydrochloric acid. Gradient: 0–2% B in 3 min, 2% B for 2 min, 6% B for 2 min and 10% B for 4 min. Flow-rate: 2.5 ml/min. After each analysis the column was washed with 0.192 *M* hydrochloric acid for 0.5 min and then with distilled water for 3 min at a flow-rate of 10 ml/min.

Assay procedure

The enzyme reaction was carried out at 37°C in a reaction mixture containing 50 m*M* buffer at three different pH values, 10 m*M* magnesium chloride and 1m*M* each of 5′-AMP and 3′-AMP. The buffers used were diethylamine–HCl at pH 10.6, imidazole–HCl at pH 7.4 and sodium acetate at pH 4.8. The reaction was started by addition of either an enzyme preparation or the mixture of substrates. Immediately after mixing and thereafter at every 15 min, 100-μl aliquots of the reaction mixture were analyzed by the chromatographic method.

The reaction rate was measured by following the decrease in the amounts of 5′-AMP and/or 3′-AMP, or the increase in the amount of adenosine produced. The amount of each compound was calculated from its respective peak according to the equation described previously [8]. The unit of enzyme activity is expressed as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of substrate or the production of 1 μmol of product in 1 min.

RESULTS

Resolution of substrates and products

As shown in Fig. 1, adenosine, inosine, 5′-AMP and 3′-AMP are well separated by chromatography.

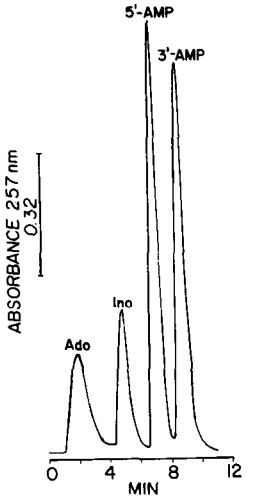

Fig. 1. Resolution of adenosine (Ado), inosine (Ino), 5′-AMP, and 3′-AMP.

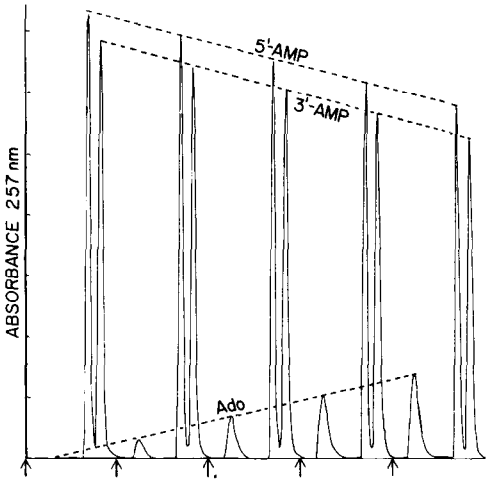

Fig. 2. Reaction of placental AlPase on hydrolysis of 5′-AMP and 3′-AMP carried out at pH 10.2. Immediately after starting the reaction and thereafter at 15-min intervals, 100-μl aliquots of the reaction mixture were analyzed at the points marked by an arrow. 5′-AMP and 3′-AMP are hydrolyzed at an equal rate. Adenosine (Ado) produced is the sum of 5′-AMP and 3′-AMP hydrolyzed, and the amount is linearly proportional to the incubation time.

The detector response was linear between 1 to 200 nmol for 5′-AMP and 1 to 250 nmol for 3′-AMP. The coefficients of variation for five injections of the reaction mixture containing 1175.5 nmol/ml 5′-AMP and 1199.3 nmol/ml 3′-AMP were less than 1%.

Relative reaction rate on hydrolysis of 5′-AMP and 3′-AMP

At pH 10.6, all AlPases from human placenta, bovine intestine and rat liver hydrolyzed 5′-AMP and 3′-AMP at approximately equal rate. Fig. 2 shows a

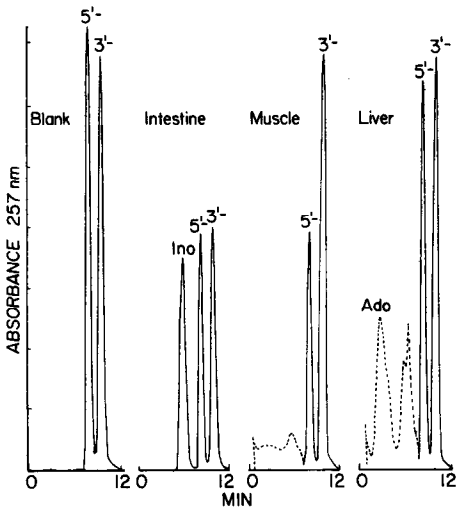

Fig. 3. Reactions of various enzyme preparations on 5′-AMP and 3′-AMP carried out at pH 7.4. An 100-μl aliquot of each reaction mixture after a 15-min incubation was injected onto the column. The four chromatograms represent the reaction mixture containing blank, bovine intestinal AlPase, rat muscle extract and rat liver extract, as indicated on the figure. The effluents were monitored at attenuations of 1.6 a.u.f.s. (solid line) and 0.2 a.u.f.s. (broken line).

chromatogram of the action of placental-type AlPase. At pH 7.4 and in the presence of an excess of the enzyme, the placental-type AlPase showed a similar result. The bovine intestinal AlPase showed a rate ratio of 0.91 in favour of 5′-AMP. This was due to the fact that the preparation was contaminated with a small amount of 5′-NTase because incorporation of 0.1 *M* nickel chloride in the reaction mixture showed a rate ratio of 0.97. The relative rate of liver AlPase at pH 7.4 could not be accurately determined because the liver extract contained 5′-NTase as well as AcPase.

AcPase, however, showed varying reaction rates. At pH 4.8, the liver extract catalyzed the hydrolysis of 3′-AMP 1.53 times faster than that of 5′-AMP and prostatic AcPase 5.4 times faster.

Detection of interfering enzymes in tissue extracts

Fig. 3 shows that the presence of other enzymes in addition to AlPase and AcPase can be easily detected simultaneously. With bovine intestinal AlPase, almost all adenosine produced was converted to inosine, indicating the presence of a large amount of adenosine deaminase.

In the muscle extract, 5′-AMP was rapidly hydrolyzed but 3′-AMP was not hydrolyzed at pH 7.4. However, no detectable amount of adenosine and only a small amount of inosine were produced, indicating the presence of a large amount of adenylate deaminase.

In the liver extract, adenosine produced largely from the hydrolysis of 5′-AMP was partially deaminated to inosine and further degraded to hypoxanthine.

Determination of 5′-NTase in human ovarian carcinoma

Since human ovarian carcinomas contain very high activities of both AlPase and AcPase, the possibility of their interference with the assay of 5′-NTase should be considered. The following experimental results illustrate that the chromatographic method described is suitable for both detection of possible interference and actual determination of 5′-NTase.

AlPase in human ovarian carcinomas has a high activity for both 5′-AMP and 3′-AMP. The activity determined by the total amount of 5′-AMP and 3′-AMP hydrolyzed in five tumor samples from different patients gave an average value of 3.41 U/g wet tissue (range 1.12–7.47 U/g). For the determination of 5′-NTase, it is necessary to exclude the activity of the AlPase which hydrolyzes 5′-AMP non-specifically. This can be accomplished by the chromatographic method because it was found that AlPase hydrolyzes 5′-AMP and 3′-AMP at a similar rate.

Conversely, because AcPase in the tumor extract was as high as the activity of AlPase (about two thirds the activity of AlPase determined by colorimetry using *p*-nitrophenyl phosphate as a substrate), interference of this enzyme with the assay for 5′-NTase should be taken into account. However, it was subsequently found that the AcPase hydrolyzes poorly both 5′-AMP and 3′-AMP. At the concentration range of 5′-NTase for an adequate measurement of activity, the activity of AcPase acting on AMP is so low that the possible interference due to the activity of AcPase can be neglected.

From the above findings it is reasonably certain that the activity of 5′-AMP in the tumor extracts can be accurately determined by the described chromatographic method. We found that, contrary to the high activity of AlPase, the tumor contained very low activity of 5′-NTase. The average value of the five tumor extracts was 0.47 ± 0.12 U/g wet tissue. Conversely, normal human ovary had an extremely high activity of 5′-NTase in spite of the fact that the activity of AlPase was extremely low. Five normal ovary extracts had an average 5′-NTase activity of 5.77 ± 1.25 U/g.

DISCUSSION

At pH 10.6, all AlPases from different tissues appear to catalyze the hydrolysis of 5′-AMP and 3′-AMP at a similar rate. At pH 7.4, a small difference in the relative rate was observed for the AlPases obtained from bovine intestine and rat liver. This discrepancy is attributed to the fact that a pure enzyme preparation was not used. Therefore, the described chromatographic method is accurate for the determination of 5′-NTase in the presence of AlPase without using enzyme inhibition.

Since AcPases unlike AlPases catalyze the hydrolysis of 3′-AMP and 5′-AMP at different rates, presence of a large amount of AcPases may thus affect the accuracy of the assay of 5′-NTase. However, the interference may not be significant with the exception of the prostate, because all tissues do not contain prostatic AcPase but small amounts of other types of AcPases which are less active

on both 5′-AMP and 3′-AMP. Any significant interference will be detected by this chromatographic method.

A marked specificity of prostatic AcPase toward the hydrolysis of 3′-AMP suggests a potential utility of the method for the assay of the prostatic AcPase as a marker for prostatic cancer. Goldberg and Ellis [9] have demonstrated higher detectability of prostatic cancer by using 3′-AMP as a substrate. The method was based on the colorimetric determination of ammonia by further coupling to the reaction of adenosine deaminase.

Since the chromatographic method determines the relative reaction rate of the hydrolysis of 5′-AMP and 3′-AMP, and at the same time, adenosine and inosine produced, any enzyme which may interfere with the assay of 5′-NTase can be detected. Therefore, the method is not only unique for the assay of 5′-NTase, but also applicable to the assays of AlPase, AcPase, adenosine deaminase and adenylate deaminase.

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