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Note

Application of anion-exchange column chromatography for determination of alkaline phosphatase activity using 2′-AMP or 3′-AMP as substrate

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In our previous paper we described a simple automated ion-exchange column chromatography method using AG MP-1 resin for the determination of adenosine [1]. This has been applied to the assay of the activity of 5′-nucleotidase which catalyzes the hydrolysis of 5′-AMP to adenosine and inorganic phosphate. Alkaline phosphatase which catalyzes the hydrolysis of AMP at high pH can therefore be determined by a similar procedure. However, in order to discriminate the activity from that of 5′-nucleotidase, 2′-AMP or 3′-AMP is used as substrate. The optimal conditions for the assay, and a modification of the procedure are described in this communication.

EXPERIMENTAL

Materials

AG MP-1 anion-exchange resin was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Adenosine monophosphates were obtained from Sigma (St. Louis, MO, U.S.A.). The purity and concentration of 2′-AMP and 3′-AMP were determined by a modification of the column chromatography [2, 3] as described below. A normal serum from the Blood Bank at Long Island Jewish—Hillside Medical Center was used.

Column chromatography

The same column packed with AG MP-1 resin (50 × 9 mm) was used for the analysis of adenosine as well as AMP throughout the entire experiment. Adenosine peak was eluted out from the column with distilled water in 8 min,

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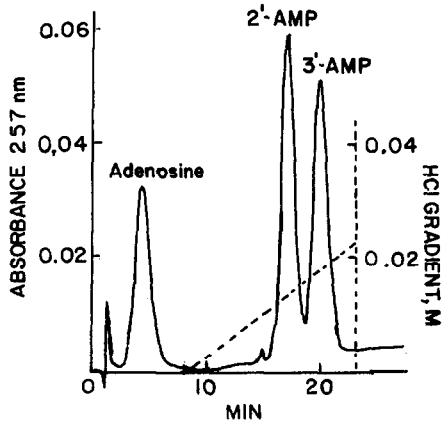


Fig. 1. Chromatogram of separation of adenosine and adenosine monophosphates (AMP). Adenosine is eluted out with distilled water, while AMP's can only be eluted out with hydrochloric acid. A mild acid gradient resolved 2'-AMP and 3'-AMP. For determination of adenosine only, the column is continuously eluted with distilled water while samples are consecutively injected into the column every 8 min. AMP's will remain in the column without interfering with the assay.

then 2'-AMP and 3'-AMP were separated by eluting the column with a linear hydrochloric acid gradient established by using a Gilson Mixograd gradient former from distilled water and 0.024 *M* hydrochloric acid in 15 min. The amounts were calculated from the area of each peak according to the equation previously described [2]. A chromatogram of their separation is shown in Fig. 1.

Assay for serum alkaline phosphatase activity

A general procedure for the assay of serum alkaline phosphatase was as follows: A substrate in 0.9 ml of various alkaline buffer solutions was preincubated to 37°C. The reaction was started by the addition of 100 μl of serum. Aliquots (200 μl) of the reaction mixture were withdrawn at 10-min intervals and injected into the column for the quantitation of adenosine produced according to the automated column chromatography described previously [1]. A unit of the activity is defined as the amount of the enzyme that will catalyze the production of 1 μmol of adenosine per min at 37°C under specified conditions.

RESULTS AND DISCUSSION

Purity and stability of adenosine monophosphates

The commercial 2'-AMP and 3'-AMP contained less than 0.04% of adenosine. 2'-AMP contained no detectable amount of 3'-AMP while 3'-AMP contained 0.28% of 2'-AMP.

Storage of AMP's in various alkaline buffer solutions at pH 10.6 at room temperature revealed that both 2'-AMP and 3'-AMP were stable in diethylamine or triethylamine, while they were slightly hydrolyzed in 2-amino-2-methyl-1-propanol. It is estimated that about 0.17% was hydrolyzed to ade-

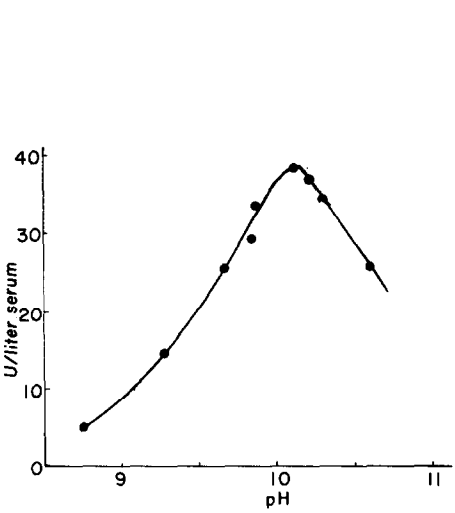
nosine in a week. Presence of magnesium chloride at 10 mmol/l tended to accelerate the non-enzymatic hydrolysis.

Effect of buffers and pH optimum

Serum alkaline phosphatase gave similar reaction rates in the buffers of 2-amino-2-methyl-1-propanol as well as of diethylamine. In triethylamine, the rate was reduced about 20% of the rate in diethylamine. Therefore, diethylamine was used as the buffer in subsequent experiments.

Fig. 2 shows the pH optimum of serum alkaline phosphatase using diethylamine as buffer. Since the range of the plateau is less than 0.1 pH, a sufficient buffering capacity at that range is required to yield a constant reaction rate.

Diethylamine at a concentration higher than 0.2 *M* is somewhat inhibitory. However, the variation in the pH is considered as a main factor causing the error in the rate measurement. As shown in Fig. 3, although diethylamine buffer was initially adjusted to pH 10.4, the pH values varied after the addition of serum and during the incubation. In 0.1 *M* diethylamine, a linear reaction rate versus time was obtained. At 0.05 *M* diethylamine, the reaction was reduced due to the lack of the buffering capacity. On the other hand, diethylamine at high concentration is somewhat inhibitory. This is evident from the fact that a non-linear reaction rate was obtained at a concentration higher than 0.2 *M*. Consequently the optimal buffer concentration at 0.1 *M* and pH at 10.2 were used.



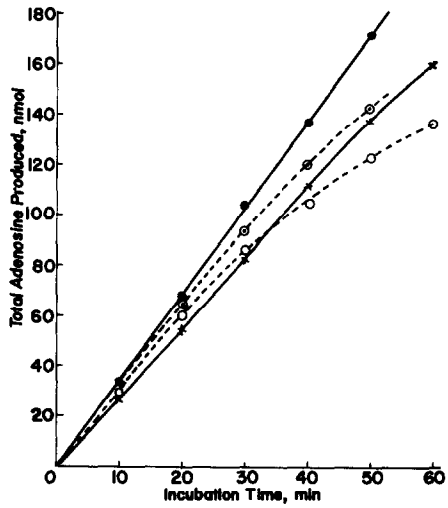


Fig. 2. pH optimum of serum alkaline phosphatase. The activity was measured in the presence of 10 m*M* 2'-AMP and 0.1 *M* of diethylamine at various pH values.

Fig. 3. Effect of buffer concentration on the reaction rate of serum alkaline phosphatase. The total amount of adenosine produced by 100 µl of serum was determined in the reaction mixture containing 100 µl of serum per ml, 10 m*M* 2'-AMP, and various concentrations of the diethylamine as indicated: ×, 0.05 *M* (pH 9.86—9.80); •, 0.1 *M* (pH 10.14—10.11); ⊙, 0.2 *M* (pH 10.31—10.20); ○, 0.3 *M* (pH 10.38—10.34). The values in parentheses are the initial and final pH values.

Optimum substrate concentration

Fig. 4 shows the relationship between the initial reaction rate and the substrate concentration. The reaction was carried out in a mixture containing 0.1 *M* diethylamine, pH 10.2, 100 μl of serum, and various concentrations of substrates. From the curves, it was estimated that K_m and V_{max} for 2′-AMP are 1.46 m*M* and 40.8 units/l, respectively; and for 3′-AMP, 3.32 m*M* and 38.9 units/l. The results indicated that serum alkaline phosphatase has a higher affinity for 2′-AMP than for 3′-AMP; however it catalyzes the hydrolysis of both substrates equally well. Thus, at pH 10.2, serum alkaline phosphatase requires a lower concentration of 2′-AMP than that of 3′-AMP to give a maximum activity. It can be seen from Fig. 4 that a maximum reaction rate was obtained at 8 mmol/l of 2′-AMP. The concentration beyond this did not lead to a significant increase in activity. For practical purposes, 10 mmol/l of 2′-AMP is considered optimum to yield a constant maximal rate. Variation of serum amount from 10 μl to 250 μl in a 1-ml reaction mixture containing 0.1 *M* diethylamine, pH 10.2, and 10 m*M* 2′-AMP showed that the alkaline phosphatase activity was linearly related to the amount of serum.

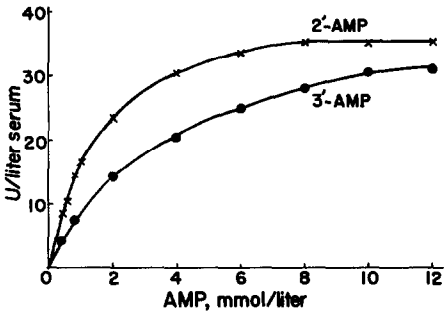


Fig. 4. Effect of substrate concentration on reaction rate of serum alkaline phosphatase. The activity was measured in the presence of 0.1 *M* diethylamine, pH 10.2, and various concentrations of 2′-AMP or 3′-AMP as indicated on the figure.

CONCLUSION

For a routine assay of serum alkaline phosphatase, 50 μl of serum are added into a 200-μl solution containing 12.5 m*M* 2′-AMP and 0.125 *M* diethylamine, pH 10.2. The mixture is incubated at 37°C, and after 10 min, 100 μl of the reaction mixture are injected into the column for chromatography. Since the reaction mixture is injected directly into the column without pretreatment, the whole procedure can be automated.

An assay method using HPLC for acid and alkaline phosphatases in serum has been published [4]. Our system can thus be similarly applied to the assay of acid phosphatase. Using 2′-AMP or 3′-AMP as substrates can eliminate the requirement of employing nickel chloride to inhibit 5′-nucleotidase activity when 5′-AMP is used as substrate. The distinctinve advantage of our method is that it is much simpler.

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