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EXTRACELLULAR PHOSPHODIESTERASE FROM THE GROWTH MEDIUM OF THE MYXOMYCETE *PHYSARUM FLAVICOMUM*

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ABSTRACT

The plasmodium of the myxomycete *Physarum flavicomum* secretes cyclic AMP phosphodiesterase into the medium. The extracellular enzyme had a pH optimum between 7 and 8 and a Km of about 500 μM cyclic AMP and was inhibited by theophylline, caffeine and 3-isobutyl-l-methyxanthine (MIX). A marked decrease of enzyme activity was noted in the presence of EDTA, suggesting the requirement of Mg^{++} by the enzyme. Addition of Mg^{++} and Ca^{++} stimulated the enzyme while Zn^{++}, Co^{++}, Pb^{++}, Mn^{++}, Fe^{+++}, Ni^{++}, and Cu^{++} all inhibited phosphodiesterase activity. An interesting feature of this extracellular phosphodiesterase was its ability to retain full catalytic activity after prolonged exposure to elevated temperatures.

INTRODUCTION

Cyclic AMP phosphodiesterase has been extensively studied in both prokaryotic and eukaryotic systems, and is the only known enzyme that hydrolyzes cyclic AMP. Although most phosphodiesterase is intracellular, some organisms release an extracellular phosphodiesterase which apparently acts as a signalling device. One of the best examples of an extracellular phosphodiesterase is released by the cellular slime mold *Dictyostelium discoideum*. Cyclic AMP has long been recognized as the chemotactic agent involved with aggregation in *Dictyostelium* along with an extracellular phosphodiesterase responsible in part for maintaining a gradient of cyclic AMP (Gerisch et al., 1972; Chassy, 1972; Toorchen and Henderson, 1979).

The role of cAMP in growth and differentiation of myxomycetes is not nearly as well understood. This cyclic nucleotide has been shown to be a positive attractant for the plasmodium of *Physarum polycephalum* (Kincaid and Mansour, 1979a) and the dibutyl derivative of cyclic AMP alters glucose metabolism during differentiation of the plasmodium of *P. flavicomum* into the sclerotium (Lynch and Henney, 1973). *P. polycephalum* has also been shown to release an extracellular phosphodiesterase (Murray et al., 1971; Kincaid and Mansour, 1979b) although the physiological significance of the latter is unknown.

This paper represents the first report of an extracellular enzyme from the plasmodium of *P. flavicomum*. One unusual feature of this enzyme is that it appears to be very heat stable.

MATERIALS AND METHODS

The plasmodium of *Physarum flavicomum* was grown in liquid shake cultures as previously described (Lynch and Farrell, 1984). Microplasmodia were harvested at 6-7 days by centrifugation at 2500 xg for 10 min and after decantation, medium were either frozen or used immediately.

The reaction mixture for cAMP phosphodiesterase measurements contained 40 mM Tris•HCl (pH 8.0), 5 mM $MgCl_2$, 50 μM $CaCl_2$, (^{3}H)-cAMP (.01 mM to 1.0 mM) and an appropriate amount of medium containing extracellular phosphodiesterase. The final volume of the reaction mixture was 0.1 ml unless otherwise stated. Incubation was at 30.0°C for 10-20 min.

Phosphodiesterase activity was measured by two independent techniques. One is a well established two-stage assay using an anion exchange resin to separate the substrate from product (Lynch & Cheung, 1975). In this assay (^{3}H) cAMP is converted to (^{3}H)-5'-AMP by phosphodiesterase. This first stage of the assay was terminated with .01 ml of 500 mM HCl. After two min., the solution was neutralized with .01 ml of 500 mM NaOH followed by the addition of .01 ml of 400 mM Tris•HCl pH 8.0. Snake venom (*Crotalus atrox*, 50 μg/tube) was then added as a source of 5'-nucleotidase. This quantitatively converted (^{3}H)-5'-AMP to (^{3}H)-adenosine and inorganic phosphate. An anion exchange resin (AGl-X2) is added and the tubes centrifuged. The resin bound and precipitated any remaining substract (^{3}H-cAMP). Aliquots of the supernatant, containing (^{3}H)-adenosine, were processed by liquid scintillation counting.

An alternate procedure used paper chromatography to separate substrate from product. This procedure is a one-step assay with (^{3}H)-5'-AMP as the product. Aliquots of the reaction mixture were spotted on Whatman #1 filter paper. Descending paper chromatography was done overnight using a solvent of 1M ammonium acetate and 95% ethanol (15/35, v/v). Spots corresponding to (^{3}H)-5'-AMP were visualized under ultraviolet light, cut out and the radioactivity determined by liquid scintillation counting. Cyclic AMP and 5'AMP were well separated by this solvent, with Rf values of .54 and .28 respectively.

RESULTS

Effect of Various pH Values

Extracellular phosphodiesterase from the plasmodium of *Physarum flavicomum* exhibited optimal enzyme activity in the pH range 7-8 (Table 1). All future enzyme assays were done at pH 8.0.

Table 1. The effect of pH on extracellular phosphodiesterase activity.

BUFFER	PH	nMOLE/MIN/ML	% ACTIVITY
CITRATE	4	2.5	11%
CITRATE	5	5.6	25%
PIPES	6	14.3	64%
PIPES	7	26.4	117%
TRIS	8	22.5	100%
NO ADDED BUFFER		5.5	25%

Enzyme activity was determined by the resin assay. The above indicated buffers were used to yield the desired pH. The "No Added Buf-

fer" contained no exogenous buffer other than that in the media. The pH of the media at harvest is usually between 5.0-6.0.

Kinetic Data

The extracellular enzyme followed linear kinetics with an apparent Km of about 500 μM cyclic AMP and a Vmax of about 20 nMole/min/ml of medium (Figure 1).

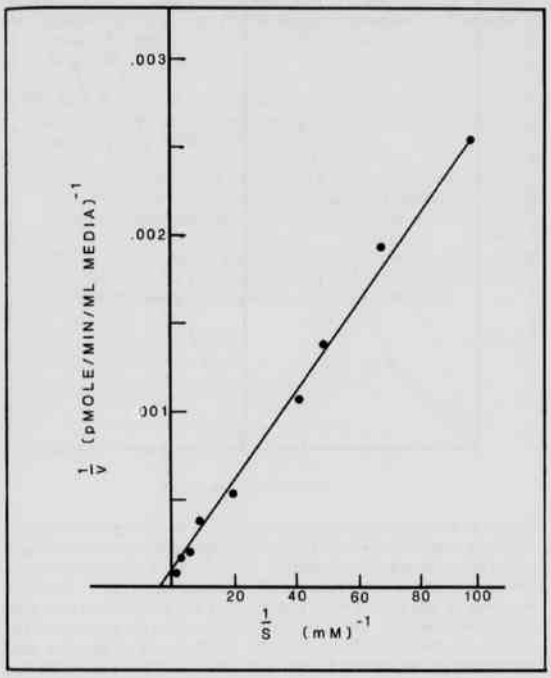

Figure 1. Lineweaver-Burk plot of the hydrolysis of (^{3}H)-cAMP by extracellular phosphodiesterase. The enzyme was assayed with (^{3}H)-cAMP concentrations from 0.01 mM to 1.0 mM for 20 min. Enzyme activity was measured by the resin procedure.

Table 2. The effect of various inhibitors on extracellular phosphodiesterase activity.

ADDITIVE	CONCENTRATION	NMOLE/MIN/ML MEDIA	%
NONE	-	18.3	100%
IMIDAZOLE	10 mM	21.6	118%
THEOPHYLLINE	10 mM	5.6	31%
CAFFEINE	10 mM	4.3	24%
MIX	2 mM	4.0	22%
EDTA	10 mM	7.4	41%
EDTA - Mg^{++}	10 mM	3.1	17%

Enzyme activity was determined by the resin assay. To determine the effect of EDTA without Mg^{++}, $MgCl_2$ was excluded from the reaction mixture.

Effect of Phosphodiesterase Inhibitors

Theophylline, caffeine, and MIX all inhibited phosphodiesterase activity with MIX showing the greatest inhibition. These compounds appeared to alter the activity of the extracellular enzyme in a manner similar to the cytoplasmic enzyme (Lynch and Farrell, 1984). EDTA also inhibited the extracellular enzyme, suggesting the requirements of magnesium for enzyme activity (Table 2).

Response to Metals

The presence of either Ca^{++} or Mg^{++} increased phosphodiesterase activity above the control and both metals together showed the highest phosphodiesterase activity (Table 3).

Table 3. The effect of metals on extracellular phosphodiesterase activity.

METAL	NMOLE/MIN/ML MEDIA	% ACTIVITY
NO ADDITION	22.6	100
Ca^{2+}	27.5	123
Mg^{2+}	24.8	110
Ca^{2+} + Mg^{2+}	29.0	129
Zn^{2+}	20.3	90
Co^{2+}	18.4	82
Pb^{2+}	16.8	75
Mn^{2+}	15.9	71
Fe^{2+}	8.2	37
Ni^{2+}	5.8	26
Cu^{2+}	4.2	19

Enzyme activity was determined by the resin assay. "No addition" indicates basal enzyme activity assayed with all metals excluded from the reaction mixture. The per cent activity in the presence of metals is based on "No addition" as 100%. With the exception of Pb^{2+} at 3 mM, the concentration of all metals is 5 mM.

Heat Stability

Throughout the course of our initial studies, several experiments suggested that the extracellular phosphodiesterase from the plasmodium was a heat stable enzyme. To investigate this possibility, samples of growth media from the plasmodium were heated in a boiling water bath for up to 20 min. and then assayed for enzymatic activity at 30 °C. The plasmodial enzyme retained full catalytic activity even after prolonged heating (Figure 2).

The enzyme activity in the above heating experiment was determined by a two-step ion-exchange resin procedure as described in Materials and Methods. To verify these results, a similar experiment was done using the resin assay and a paper chromatography procedure for determining enzyme activity. The chromatography procedure separates the substrate (^{3}H-cAMP) from the product (^{3}H-5-AMP). Two samples of media were used, one that was heated in a boiling water bath for 10 min. and one that was unheated. Both samples were then assayed for phosphodiesterase activity after 5 min. of incubation and 10 min. of incubation. Both the resin assay and the paper chromatography assay demonstrated that the enzyme was indeed heat stable (Figure 3).

DISCUSSION

The optimal pH requirement of the extracellular phosphodiesterase was in the range of 7-8. This is interesting in that the optimal pH for growth of the plasmodium was much lower. The pH of the medium

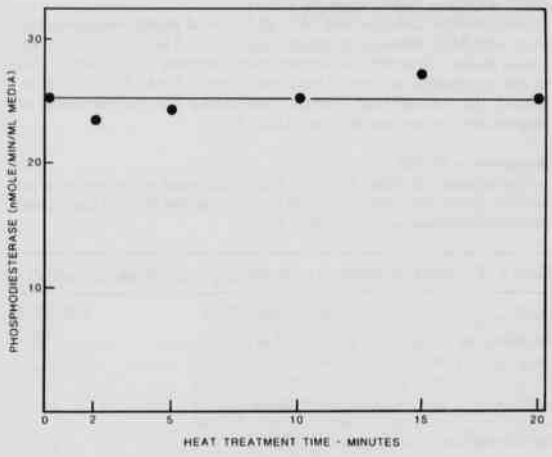

Figure 2. Heat stability of extracellular phosphodiesterase. Before each assay, aliquots of media were heated in a boiling water bath for the times indicated on the x axis. At the end of each heating, the tubes were thermal equilibrated to 30 °C and added to reaction mixture to start the enzyme assay. The enzyme was assayed for 20 min. and measured by the resin procedure.

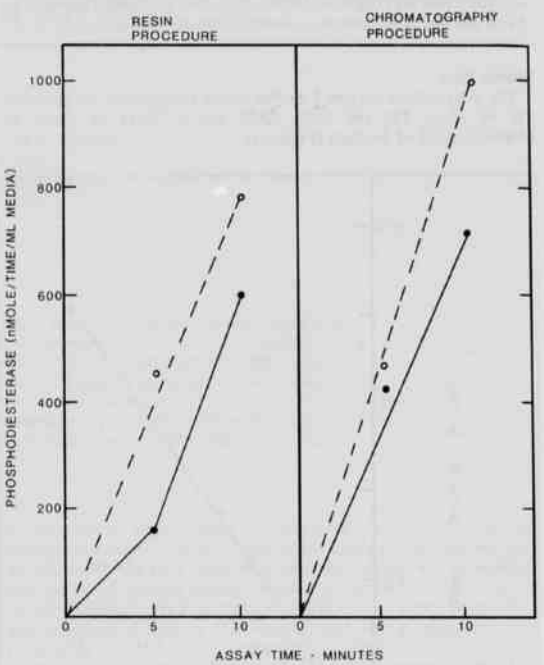

Figure 3. Comparison of enzyme activity of heat-treated (0- -0) and non-heat treated (•—•) extracellular phosphodiesterase by resin and paper chromatography procedures. Aliquots for heat treatment were placed in a boiling water bath for 10 ' and returned to 30 °C. Reaction mixtures of 300 µl each were prepared for the heat-treated and non-heat treated samples. The reaction was initiated by the addition of 100 µl each of the extracellular enzyme. Duplicate 25 µl samples were withdrawn at the indicated times and added to tubes containing 5 µl of 500 mM HCl to terminate the reaction. End product was determined using either the resin or paper chromatography procedure.

before inoculation was 4.3 and during growth the pH rose to about pH 6.0 at stationary phase. Although the enzyme did retain activity in this physiological pH range, it was far below the optimum. The role of an extracellular phosphodiesterase in plasmodial growth and differentiation is unknown. Any statements concerning the optimum growth pH versus the optimum enzyme activity pH can only be speculative at best.

The Km of the extracellular enzyme and the activity of the enzyme in the presence of various inhibitors used in Table 2 appeared to be very similar to that previously reported for the cytosolic enzyme from the same organism. Inhibition of the enzyme in the presence of EDTA suggested the requirement of magnesium by the enzyme. Phosphodiesterase from most organisms to date demonstrates increased activity in the presence of magnesium.

The effect of enzyme activity in the presence or absence of Mg^{++} (Table 3) would appear to be somewhat inconsistent with the EDTA data from Table 2. However, this is an enzyme that was released into the medium and aliquots of the medium were used for each assay. Mg^{++} was added to the medium as a trace element at a final concentration of about 0.5 mM. This endogenous Mg^{++} can be chelated by EDTA as shown in Table 2, but still maintained the enzyme close to maximum activity when no exogenous Mg^{++} was added (Table 3).

The apparent heat stability of the enzyme from the media appeared unusual. A few cases of heat stable phosphodiesterase from other organisms have been reported (Bevers et al., 1974; Sankaran et al., 1978; Shaw and Harding, 1983), but in general most phosphodiesterases were very heat labile. The cytoplasmic phosphodiesterase from *P. flavicomum* also appeared to be heat labile (data not shown). Two independent assay methods were used to verify the authenticity of the heat stability. One was a well established two-step resin assay which isolated adenosine as the end product and the other was a one-step paper chromatography procedure which separates 5'-AMP as the end product. Both techniques gave similar data and supported the concept of a heat stable enzyme.

The significance of a heat stable extracellular phosphodiesterase in this organism is unknown. The plasmodium grew best at room temperature in the laboratory and has not been known to tolerate elevated temperatures in nature. Further studies on this enzyme are in progress.

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