IDENTIFICATION OF THE 3', 5'- CYCLIC AMP PHOSPHODIESTERASE INHIBITOR IN POTATO: FEED-BACK CONTROL BY INORGANIC PHOSPHATE

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Received June 1, 1972

SUMMARY

We attempted to identify the low molecular sized 3', 5'- cyclic AMP phosphodiesterase inhibitor in potato. Finding that the partially purified inhibitor yielded an elution pattern and a phosphodiesterase inhibition identical with those of inorganic phosphate in Sephadex G-25 column chromatography, and finding that the Rf value for this inhibitor agreed with that for inorganic phosphate on paper chromatographic analysis, we are able to identify this inhibitor as inorganic phosphate, a known end-product of cyclic AMP catabolism.

INTRODUCTION

In our preceding paper (1), we reported that a dialysis of potato 105,000 x g supernatant had resulted in a dramatic increase in 3', 5'- cyclic AMP (cyclic AMP) phosphodiesterase activity, the dialysis having brought about the removal of some unknown inhibitor of low molecular size. Furthermore, this inhibitor had been found resistant to boiling at neutral, acidic, or alkaline pH and to charcoal treatment as well. Since cyclic AMP phosphodiesterase plays an important role in the regulation of the intracellular level of cyclic AMP (2, 3), it might be important to identify this potent enzyme inhibitor in potato.

In this communication, it will be seen that the low molecular inhibitor in potato is inorganic phosphate, a known end-product of the cyclic AMP catabolism.

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MATERIALS AND METHODS

Potato (Salanum tuberosum), weighing 200-250 g was used. The ^{3}H-3', 5'-cyclic AMP was obtained from New England Nuclear. The snake venom (Naja naja) was obtained from the Sigma Chemical Company. All the other reagents were purchased from Nakarai Chemicals, Kyoto.

Enzyme assay

The assay for phosphodiesterase activity was carried out by the two stage isotopic procedure previously described (1). All assays were carried out a 20 % or less total reaction appearing in the linear portion of the enzyme assay. One unit of enzyme activity was defined as the amount cleavage nmole cyclic AMP per hour.

Phosphodiesterase preparation

250 g of peeled potato was ground for 5 min in a Waring blender with 125 ml of a 0.01 M Tris-Cl buffer, pH 7.5 containing 5 mM $MgCl_2$ (designed as Mg^{++}-buffer) and pressed through cheesecloth; then the extract was centrifuged at 105,000 x g for 60 min and the supernatant solution was dialyzed overnight at 4° against a large volume of Mg^{++}-buffer. The precipitate yielded during dialysis was removed by centrifugation, and the pH of the supernatant solution was adjusted to 5.5 with 0.1 N acetic acid. After removal of the precipitate by centrifugation at 9,000 x g for 20 min, the pH of the supernatant was adjusted to 7.5 by the addition of 0.1 N NaOH. The ammonium sulfate fraction (35-55 %) of this preparation was dissolved with a minimum volume of Mg^{++}-buffer. Further purification of the enzyme was carried out by means of gel filtration. The ammonium sulfate fraction was applied to a column (2.5 x 75 cm) of Agarose-A 5m which had been equilibrated with Mg^{++}- buffer. The column was eluted with the same buffer. The flow rates for the separation did not exceed 20 ml per hour. Then, the two distinct fractions (F_1 and F_2) presenting phosphodiesterase activity were isolated from the Agarose-A 5m column. The overall purification was about 250 fold in the F_1 and 500 fold in the F_2 preparations. The details of the purification process as well as the properties of

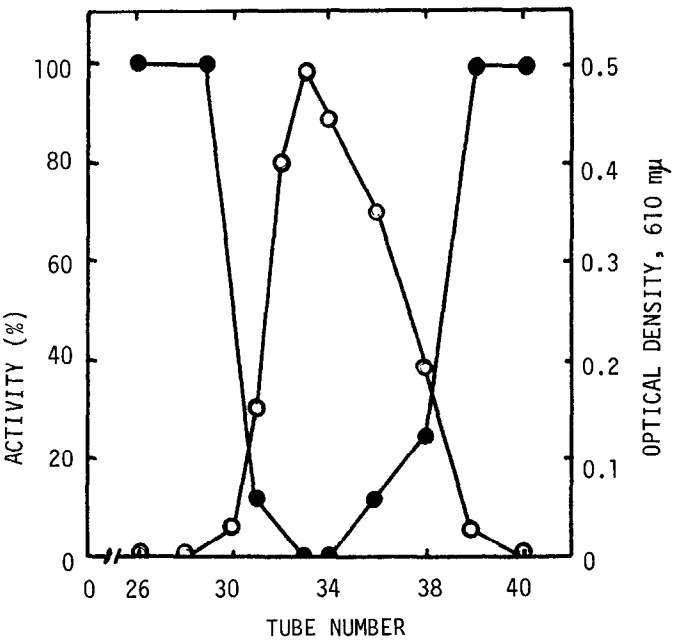

Fig. 1. Sephadex G-25 column chromatogram of the partially purified inhibitor. To determine the inhibitor fraction, 0.1 ml of each fraction taken from a Sephadex G-25 column was added to a reaction mixture containing 11.9 units of the dialyzed enzyme (see ref. 1) in a total volume of 0.5 ml. The enzyme activity in the absence of an inhibitor fraction was settled as 100 %. The determination of inorganic phosphate was carried out by the method of Fiske and SabbaRow (7). The amount of inorganic phosphate was expressed as optical density at 610 mμ. •, enzyme activity; o, amount of inorganic phosphate.

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this enzyme will be presented elsewhere (4).

Inhibitor preparation

This 105,000 x g supernatant of peeled potato was obtained by same method used for the enzyme preparation, except that distilled water was used instead of Mg^{++}- buffer for potato homogenization. The supernatant solution was heated for 2 min in a boiling water bath. After cooling, the samples were centrifuged at 8,500 x g for 15 min; 4.5 g of active charcoal was added to 90 ml of the supernatant solution and the mixture was stirred for 15 min at room temperature; 75 ml of the filtrate were evaporated by means of a rotary evaporator at 50°. The dried residue was dissolved with 7.5 ml of distilled water; 1 ml samples were then applied to Sephadex G-25 column (2.5 x 22.5 cm), and the

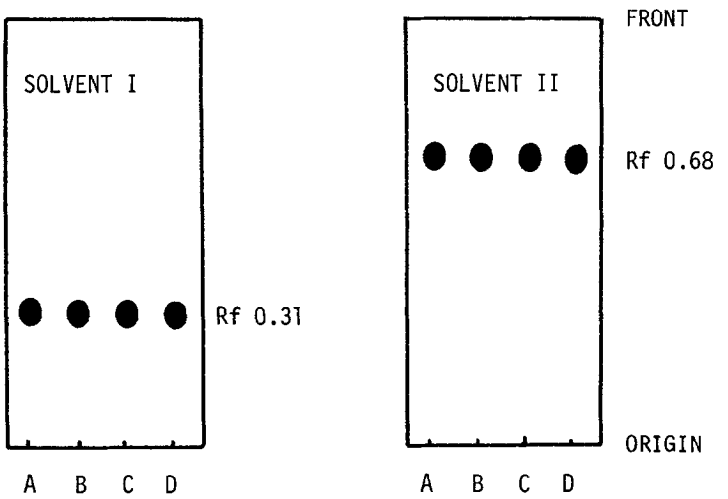

Fig. 2. Paper chromatogram of inhibitor. An aliquot of the inhibitor fraction described in Fig. 1 was chromatographed in solvents I and II. Authentic standards were also chromatographed at the same time. The detection of the Pi spot was carried out essentially by the method of Fiske and SabbaRow(7); a mixture of equal amounts of 5 N sulfuric acid and 2.5 % ammonium molybdate was sprayed on paper followed by spraying with 2 % 1-amino-2-naphthol-4-sulfonic acid containing 12 % of sodium bisulfate and 12 % of sodium sulfite. After 10 min at room temperature, the blue spots would appear. A, inhibitor fraction; B, sodium monophosphate (monobasic); C, potassium monophosphate (dibasic); D, orthophosphate.

column was eluted with distilled water. The flow rates for the filtration did not exceed 10 ml per hour; the fraction volumes were normally 3 ml. Under these conditions, the inhibitor was eluted in tubes no.33 and 34 (Fig. 1). The inhibitor was pooled for experimental use. Inhibitor potency was assayed by its ability to inhibit phosphodiesterase, as described in the legend to Fig. 1.

Inhibitor identification

An aliquot of the inhibitor fraction obtained by Sephadex G-25 column chromatography was placed on Toyo no. 51 filter paper with authentic samples. The paper was developed in solvent system I (isobutyric acid, 0.5 N ammonia; 10:3) (5) and solvent system II (isoamylalcohol, isopropylalcohol, 5 % trichloroacetic acid, 75 % lactic acid; 5:15:10:0.5) (6) and the Pi was detected by the method of Fiske and SabbaRow (7). The details are described in the legend to Fig. 2.

RESULTS AND DISCUSSION

During the course of inhibitor preparation, we stumbled onto the fact that the fractions presenting the potent inhibitor eluted by gel filtration gave a blue color in the Fiske and SabbaRow procedure (7) (Fig. 1). This result indicates that the inhibitor fraction involves inorganic phosphate. To determine whether inorganic phosphate would bring about phosphodiesterase inhibition, another gel filtration of sodium phosphate (dibasic) was carried out under the same conditions. The result was that both the Pi elution patterns and the enzyme inhibition were quite similar to those shown in Fig. 1 (data not shown).

To confirm out identification of the inhibitor, paper chromatography was carried out. As depicted in Fig. 2, the Rf values for blue spots in both the inhibitor fraction and in inorganic phosphate were found quite consistent in the two solvent systems. Furthermore, the extract of the area corresponding to the Pi spot in the inhibitor fraction represented a potent inhibition of enzyme activity (data not shown).

The inhibitory potency of several inorganic phosphates including the potato inhibitor on F_1 enzyme activity was also tested. As shown in Fig. 3, the potato enzyme was inhibited by sodium phosphate (monobasic), potassium phosphate (dibasic), orthophosphate, and the potato inhibitor fraction at similar rates. Furthermore, similar results were obtained with an F_2 enzyme preparation (data not shown). From these results, we concluded the cyclic AMP phosphodiesterase inhibitor in potato to be inorganic phosphate.

Since potato phosphodiesterase activity ceases through Na_4EDTA addition (4) as mammalian enzyme activity does, it is necessary to demonstrate that this Pi induced decrease in enzyme activity is not due to the removal of magnesium-forming magnesium phosphate. As illustrated in Fig. 4, increasing concentrations of magnessium had no affect on enzyme activity inhibited to 60 % by inorganic phosphate.

Feedback control, in which the end-product of a metabolic sequence in-

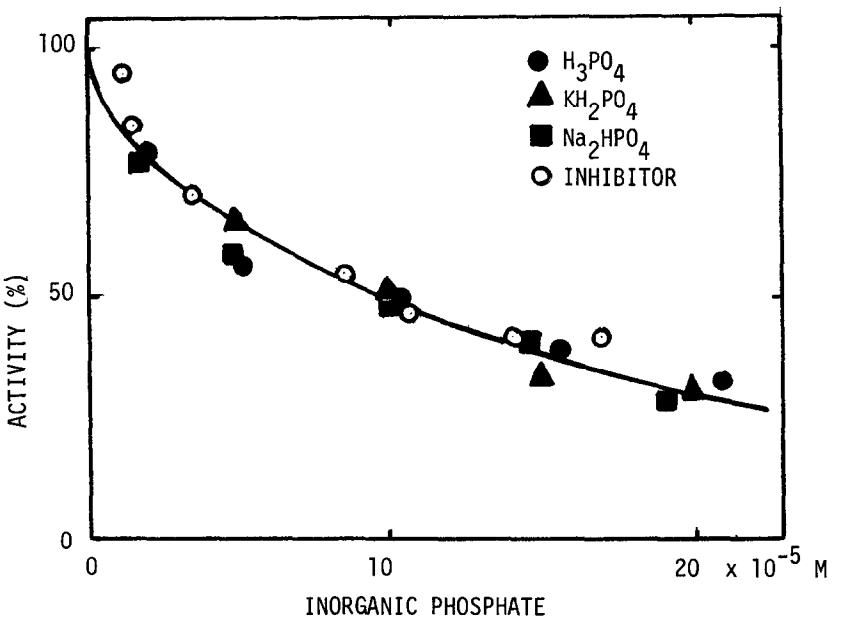

Fig. 3. Effect of inorganic phosphate concentration on enzyme activity. The amount of inorganic phosphate present in the inhibitor fraction described in Fig. 1 was determined by the Fiske and SabbaRow procedure (7) and then adjusted to the authentic inorganic phosphate concentration with an adequate distilled water. The pH of all the compounds was adjusted to 4.5 before use. Activity in the absence of an inhibitor was settled as 100 %. 12.5 units of F_1 enzyme preparation was used in this experiment.

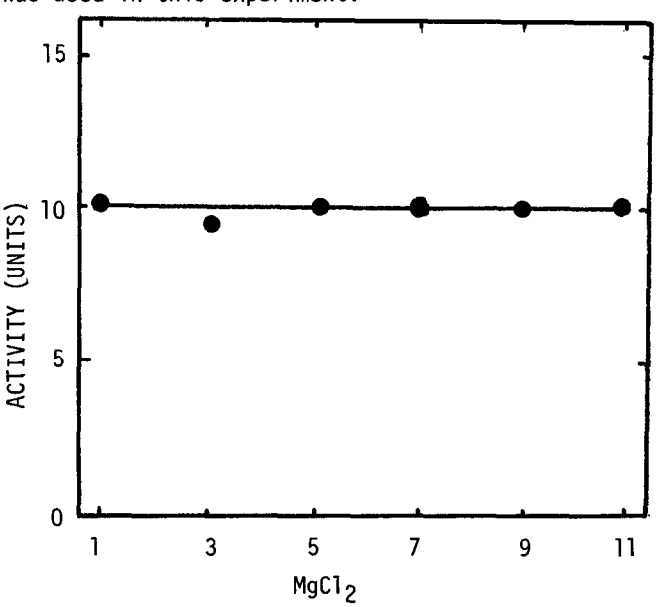

Fig. 4. Effect of increasing concentration of $MgCl_2$ on phosphodiesterase activity in the presence of inorganic phosphate. All assay mixtures contain 17.2 units of F_1 enzyme preparation and KH_2PO_4 at 5 x 10^{-5} M. Except for the $MgCl_2$ concentration, the other assay conditions were the same as those previously described (1).

hibits the enzyme catalyzing the first step in its formation from a precursor, is a well documented phenomenon. It is interesting to speculate that inorganic phosphate, which is one of the end-products found in the catabolic pathway of cyclic AMP, may participate in the control of cyclic AMP levels in potato through the feedback inhibition of phosphodiesterase. In contrast to the Pi effect, we found that adenine, adenosine, and ribose had no affect on potato phosphodiesterase (data not shown).

ACKNOWLEDGEMENT

We wish to thank Miss M. Kawai for her excellent technical assistance.

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