Phytochemistry 1974 Vol 13 pp 2735 to 2740 Pergamon Press Printed in England

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM CARROT

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(*Revised Received* 25 *April* 1974)

Key Word Index—*Daucus carota*, Umbelliferae, carrot, phosphodiesterase; cyclic nucleotides, inhibitor

Abstract—A cyclic nucleotide phosphodiesterase has been isolated and partially purified from carrot tap-root tissue The properties of this enzyme are very different from cyclic AMP phosphodiesterases found in mammalian cells A dialyzable inhibitor of carrot cyclic nucleotide phosphodiesterase was also isolated from carrot tissue Because the inhibitor behaved like inorganic phosphate on ion-exchange chromatography and inhibited the enzyme in proportion to its inorganic phosphate content, the inhibitor was tentatively identified as inorganic phosphate

INTRODUCTION

RECENT reports have provided evidence for the incorporation of adenine-[^{3}H] into 3′,5′-cyclic adenosine monophosphate (cAMP) in higher plant tissues.[1,2] Treatment of plant tissue (*Avena* coleoptiles) with indole acetic acid (IAA) gave rise to a large incorporation of adenine-[^{3}H] into cAMP,[3] similar to the stimulatory effect of hormones in the animal system

cAMP was shown to effect the synthesis of gibberellic acid in embryo-less barley seeds[4] and to stimulate hormone-induced changes in other tissues [5] However, in pea stems and other tissues[6] cAMP failed to elicit a hormone-like response.

Initial attempts to detect basal levels of cAMP and adenyl cyclase activity in carrot homogenates were not successful. However, we have found an enzyme capable of hydrolyzing 3′,5′-cyclic AMP and this report is concerned with its partial purification and properties.

RESULTS

Initial attempts to detect phosphodiesterase activity were carried out at pH 7·4, near the pH optimum of most mammalian phosphodiesterase. However, since only a small amount of activity was detected a pH study was performed with the crude homogenate. The optimum pH for enzyme activity is 5·5. This is close to the pH optimum for pea cyclic

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[1] POLLARD, C J (1970) *Biochim Biophys Acta* **201,** 511
[2] SOLOMON, D and MASCARENHAS, J P (1971) *Life Science* **10,** 879
[3] SOLOMON, D and MASCARENHAS, J P (1972) *Plant Physiol* **49,** 30 (Abstr)
[4] KESSLER, B and KAPLAN, B (1972) *Physiol Plant* **27,** 424.
[5] KAMISAKA, S (1972) *Plant Growth Substances* (CARR, D J , ed), pp 654–660, Springer, Berlin
[6] WEINTRAUB, R L and LAWSON, V R (1972) *Plant Physiol* **49,** 30 (Abstr)

nucleotide phosphodiesterase isolated by Lin and Varner [7] Subsequently all experiments, unless otherwise indicated, were run at pH 5 5

Experiments with dialyzed crude homogenate showed a dependency on a metal ion for maximal activity (Table 1). Mg^{2+} and Mn^{2+} gave the best stimulation with Ca^{2+}, Co^{2+} and Li^{2+} being less effective The dependency on metal ions increased with more purified enzyme preparations, with the optimum ion concentration being about 30 mM for Mn^{2+}. Metal ions had no effect on the snake venom activity

TABLE 1 EFFECT OF METAL IONS ON CARROT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY*

Metal ion	Units†	% of Control
None	1 92	100
Mg	2 46	128
Mn	2 49	130
Ca	2 40	125
Co	2 31	120
Li	2 07	108

* Dialyzed whole homogenate was used as the enzyme source All metal ions were in the Cl form at a final concentration of 2 mM Enzyme assays were performed as described under Experimental

† A unit is equal to the conversion of 1 nmol of cAMP into 1 nmol of adenosine in 30 min at 30

The enzyme seems to be fairly stable, at least in crude preparations, as judged by its high activity at 45° and its almost linear increase in activity up to 120 min incubation

pH has a profound effect on the solubility of the enzyme. If a whole homogenate is centrifuged at 10000 *g* for 20 min in pH 5·5 buffer, 87% of the activity is found in the pellet. However, the same type of centrifugation in pH 7·4 buffer gives 93% of the activity in the supernatant (Table 2).

In order to characterize the enzyme more precisely, it was necessary to attempt partial purification The results of typical preparation are presented in Table 3. A 5 6-fold increase in purification and a 4-fold increase in total units is achieved simply by dialysis of the 20000 *g* supernatant This suggests the presence of a dialyzable inhibitor in the crude

TABLE 2 ACTIVITY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FOLLOWING CENTRIFUGATION IN pH 5 5 AND pH 7 4 BUFFERS*

Fraction	Specific activity†	% of whole homogenate
pH 7 4 Whole homogenate	6 00	100
pH 7 4 Supernatant	5 60	93
pH 7 4 Pellet	0 30	5
pH 5 5 Whole homogenate	6 00	100
pH 5 5 Supernatant	0 51	8
pH 5 5 Pellet	5 24	87

* Centrifugation was performed at 10000 *g* for 20 min in 10 mM sodium acetate buffer, pH 5 5 and 10 mM Tris HCl buffer, pH 7 4 Pellets were resuspended in a volume of appropriate buffer equal to that of the supernatant Assays were performed as described under Experimental

† Specific activity = units/mg protein

[7] LIN, P P and VARNER, J E (1972) *Biochim Biophys Acta* **276**, 454

homogenate. The characterization of this inhibitor is discussed later. The precipitates obtained at 30–60 and 60–90% saturation with $(NH_4)_2SO_4$ contained equal amounts of enzyme activity and were combined and dialyzed. The $(NH_4)_2SO_4$ step yields a 10-fold purified enzyme and another increase in total units

TABLE 3 SUMMARY OF PURIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM 300 g OF CARROT TISSUE

Step	Volume	Total units	Protein (mg)	Specific activity	Recovery (%)	Purification
Whole homogenate	322 ml	2028	860	2·4	100	—
20000 *g* supernatant	320 ml	1886	832	2·7	93	1·1
Dialyzed 20000 *g* supernatant	370 ml	8880	592	15·0	438	6·2
30–90% saturated $(NH_4)_2SO_4$ (dialyzed)	59 ml	9381	336	27·6	462	11·5
Acid treatment (pellet)	59 ml	9086	242	37·5	448	15·6

Since Lin and Varner[7] achieved a large increase in the specific activity of pea cyclic nucleotide phosphodiesterase by a pH 5·0 treatment, their method was followed with the carrot enzyme. A slight increase in purification was achieved (Table 3); however, contrary to their results, 96% of the total activity was recovered in the pellet rather than the supernatant.

An attempt was also made to fractionate the enzyme on DEAE-cellulose. The phosphodiesterase in pH 7·4 buffer remained bound to the anion exchange resin, indicating that its isoelectric point was probably below 7·0. It was eluted from the column as one peak at 0·15 M KCl. Less than 2% of the starting material was recovered from the column and no increase in purification was achieved. Other DEAE fractions were tested for the possible presence of an activating factor[8] which might have been separated during the chromatography. None of the fractions contained such an activity factor.

The inhibitors of mammalian phosphodiesterase, caffeine and theophylline, had no significant effect on the carrot enzyme, neither did the plant growth substances, phenylisobutyladenine, 1-methyl-3-isobutylxanthine, 3-indolylacetic acid, kinetin and gibberellic acid.

Because of the low recovery of active material from the DEAE-column, ultracentrifugation was performed to see if a portion of the enzyme was particulate. Carrot tissue was homogenized in 10 mM Tris–HCl buffer, pH 7·4, containing 1 mM dithiothreitol The homogenate was dialyzed overnight against 100 vol of homogenization buffer to remove endogenous inhibitor Homogenate was then centrifuged at 20000 *g* for 30 min, and the supernatant from this treatment centrifuged at 100000 *g* for 3 hr Although only 11% of the starting material was recovered in the 100000 *g* pellet, it had a higher specific activity than any of the other fractions (Table 4). Rewashing the pellet several times did not result in any further solubilization of the enzyme.

Various properties of the soluble and particulate phosphodiesterases were compared (Table 5). Although the pH optima for the two fractions were similar, consistently different V_{max} and K_m values were observed. Additionally, the two fractions exhibited differential sensitivity to imidazole, inorganic phosphate (P_i) and the dialyzable inhibitor. Compari-

[8] TEO, T. S., WANG, T. H. and WANG, J. H. (1973) *J. Biol. Chem.* **248**, 588.

TABLE 4 SUMMARY OF CENTRIFUGATION STUDY ON CARROT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

Step	Total units	Protein (mg)	Specific activity	Recovery (%)
Whole homogenate (dialyzed)	1260	50 4	25 00	100
20000 *g* supernatant	926	41 2	22 47	75
20000 *g* pellet	40	4 0	10 00	3
100000 *g* supernatant	614	27 2	22 57	49
100000 *g* pellet	136	4 2	32 38	11

sons of the enzymes were made immediately following ultracentrifugation since the particulate phosphodiesterase was much more labile than the soluble enzyme.

As mentioned previously, our initial studies revealed a large increase in total units of enzyme activity after dialysis of a whole homogenate After lyophilization of the dialyzate from 5 ml of whole homogenate, the concentrated inhibitor was dissolved in 5 ml of glass distilled water. A 50 μl aliquot of this solution gave 70–80% inhibition when tested against the $(NH_4)_2SO_4$ purified enzyme. The concentrated inhibitor had no effect on snake venom activity Further characterization of the inhibitor was achieved by chromatography on Dowex 1–2 × 400 and Dowex 50 columns. The inhibitor binds to Dowex 1–2 × 400, an anion exchange resin, and is eluted with 0·05 M NaOH However, when the solution is adjusted to 0·05 M HCl and applied to Dowex 50, it is not bound These data indicate that the active principle has a net negative charge and probably is not amphoteric Boiling the inhibitor for 30 min does not destroy its activity. Additional experiments showed that the concentrated inhibitor contained P_i (53 μg/ml) and that P_i in similar concentrations added to enzyme reaction mixtures inhibited the enzyme to almost the same degree as the inhibitor

DISCUSSION

An enzyme that hydrolyzes cAMP has been found in carrot root tissue Based on the following evidence, we believe this enzyme to be distinct from the phosphodiesterase from carrots reported by Harvey *et al*[9,10] The pH optimum for their enzyme using *p*-nitro-

TABLE 5 PROPERTIES OF PARTICULATE AND SOLUBLE PHOSPHODIESTERASES*

Property	Particulate phosphodiesterase	Soluble phosphodiesterase
V_{max}	140	196
K_m	$3\,4 \times 10^{-4}$ M	$9\,1 \times 10^{-4}$ M
pH optimum	5 5	5 5
Effect of imidazole (40 mM)†	46% inhibition	20% inhibition
Effect of 2 mM P_i	70% inhibition	39% inhibition
Effect of dialyzable inhibitor	80% inhibition	46% inhibition

* Particulate and soluble activities refer to the pellet and supernatant respectively from a 3 hr 100000 *g* centrifugation When compounds were added to the reaction mixture in 50 μl aliquots, the enzyme fraction was also reduced to 50 μl to keep the total volume constant

† Optimum ion concentration

[9] HARVEY, C., MALSMAN, L and NUSSBAUM, A L (1967) *Biochemistry* **6**, 3689

[10] HARVEY C L OLSON K C and WRIGHT, R (1970) *Biochemistry* **9**, 921

phenyl-5′-thymidylic acid (PNP-pT) as the substrate was 9·5 while cyclic nucleotide phosphodiesterase has a pH optimum of 5·5. The PNP-pT enzyme is slightly activated by NaF, while our phosphodiesterase is inhibited. Finally, Lerch and Wolf[11] have isolated a phosphodiesterase from sugar beet which has properties very similar to the PNP-pT enzyme from carrot. The former enzyme had no activity when cyclic nucleotides were tested as substrates.

Although the carrot cyclic nucleotide phosphodiesterase is similar in many properties to the pea enzyme (pH optimum, K_m for cAMP, inhibition by P_i and NaF) isolated by Lin and Varner,[7] it is different in that it is inhibited by imidazole, stimulated by divalent metal ions, and is precipitated by a pH 5·0 treatment. The last property may be explained by a contaminant in the carrot homogenate which is co-precipitating the enzyme or perhaps the enzyme is at its isoelectric point and therefore precipitates out of solution.

Because the concentrated dialyzable inhibitor contained a significant amount of P_i, behaved like P_i in anion-cation exchange chromatography and inhibited the enzyme in proportion to its P_i content, we conclude that the dialyzable inhibitor is probably P_i. This is in agreement with other investigators[12,13] who have identified a cAMP phosphodiesterase inhibitor from potato tubers as being P_i.

The carrot phosphodiesterase is quite distinct from mammalian cAMP phosphodiesterase in that the former has an acid pH optimum, is not inhibited by methylxanthines, but is inhibited by imidazole, P_i and NaF. Lin and Varner[7] have shown that the highly purified cyclic nucleotide phosphodiesterase from pea has a high activity with 2′,3′-cyclic AMP.* From a number of different criteria, they judged that the hydrolysis of 2′,3′-cAMP and 3′,5′-cAMP was due to a single enzyme. They further propose that the enzyme plays a role in RNA degradation, since most plant RNases yield 2′,3′-cyclic nucleotides. In view of the fact that physiologically significant amounts of 3′,5′-cAMP, adenyl cyclase activity, or cAMP-dependent protein kinase have not by use of rigid criteria been detected in carrot homogenates, the role of a 3′,5′-cAMP phosphodiesterase in carrot tissue remains unknown.

EXPERIMENTAL

All carrots (*Daucus carota* L.) used in these experiments were purchased from Shafter Vegetable Growers, Shafter, California, and stored in a cold room until used

Adenosine [8-^{3}H]-3′,5′-cyclic monophosphate (4·4 Ci/mmol from New England Nuclear) was taken to dryness, resuspended in 1·5 ml of 10 mM Tris–HCl buffer, pH 7·4, layered on a small Dowex 1-2 × 400 column, washed with 3 ml of Tris–HCl buffer and eluted with 2 ml of 0·05 M HCl. It was then diluted to 6 μM with 10 mM Tris–HCl buffer, pH 7·4. Adenosine[8-^{14}C] (30 mCi/mmol from New England Nuclear) was used without further purification. Adenosine-3′,5′-cyclic monophosphate from Boehringer Mannheim and snake venom (*Crotalus atrox*) from Ross Allen Reptile Institute, Inc., Silver Springs, Florida, were both used directly. Dowex 1-2 × 400 anion exchange resin was prepared by washing with 10 vol. of 0·5 M NaOH, glass distilled water (gd H_2O), 0·5 M HCl and gd H_2O, respectively. It was stored as a 3X slurry in gd H_2O. DEAE-cellulose, type 20, 0·8 meq/g was prepared in a manner similar to Dowex 1-2 × 400 except that buffer was substituted for gd H_2O and the resin titrated to pH 7·4. Dowex 50 was prepared by washing with 10 vol. of gd H_2O, 2 M NaOH, gd H_2O, 2 M HCl, gd H_2O, 0·1 M NaOH, gd H_2O, 0·1 M HCl, and gd H_2O, respectively. The last wash was continued until the resin was free of Cl^-.

* Note added in proof. Recent experiments in our laboratory have demonstrated that both soluble and particulate fractions of carrot tissue have high cyclic nucleotide phosphodiesterase activities with 2′,3′-cyclic AMP as a substrate.

[11] LERCH, B. and WOLF, G. (1972) *Biochim. Biophys. Acta* **258**, 206

[12] SHIMOYAMA, M., KAWAI, M., TANIGAWA, Y. and UEDA, I. (1972) *Biochem. Biophys. Res. Commun.* **47**, 59

[13] SHIMOYAMA, M., SAKAMOTO, M., NASU, S., SHIGEHISA, S. and UEDA, I. (1972) *Biochem. Biophys. Res. Commun.* **48**, 235

Assay of cyclic nucleotide phosphodiesterase Detection of enzyme activity was accomplished by the coupled reaction method of Thompson and Appleman,[14] with some modifications The standard reaction mixture contained 40 mM NaOAc buffer, pH 5 5, 30 mM $MgCl_2$ or $MnCl_2$ 1 μM cAMP[^{3}H] and 100 μM cAMP in a final vol of 300 μl The reaction was initiated by addition of 100 μl of enzyme and incubated for 30 min at 30° After incubation, the samples were chilled and the pH adjusted to 8 with 0 05 M NaOH 100 μl of snake venom (1 mg/ml) was added and the tubes incubated for an additional 10 min at 30 The reaction was stopped by placing the tubes on ice and pipetting 0 5 ml of a soln containing 60 mM EDTA 1 mM adenosine and 0 02 μCi adenosine-[^{14}C] After centrifugation (1000 *g*, 10 min) the supernatants were placed on small columns containing 1 ml of Dowex 1-2 × 400 which had been thoroughly washed with 10 mM Tris HCl buffer, pH 7 4 An additional 4 1 ml of this buffer was then added to the columns and the effluent was collected (total 5 ml) 1 ml of each sample was placed in plastic scintillation vials and 15 ml of Bray's soln added The samples were counted in a liquid scintillation counter with an automatic activity analyzer which corrects for spillover of ^{3}H into the ^{14}C channel Inclusion of adenosine-[^{14}C] is necessary to correct for variable adenosine recoveries from the Dowex columns A unit of enzyme activity is equal to the conversion of 1 nmol of cAMP to 1 nmol of adenosine in 30 min at 30° Specific activity is defined as units/mg protein

Partial purification of cyclic nucleotide phosphodiesterase In a typical preparation 300 g of tap-root tissue were homogenized with 300 ml of 100 mM Tris HCl pH 7 4, containing 1 mM dithiothreitol (buffer A) in a blender for 1 min at high speed Following filtration through 2 layers of cheesecloth the homogenate was centrifuged at 20000 *g* for 30 min The pellet was discarded and the supernatant dialyzed 18 hr against 100 vol of buffer A with 3 changes Solid $(NH_4)_2SO_4$ was added to the dialyzed preparation to a final concentration of 30% After stirring 30 min the soln was centrifuged at 10000 *g* for 20 min The pellet was discarded and sufficient solid $(NH_4)_2SO_4$ added to bring the soln to 90% saturation The suspension was stirred for 2 hr, centrifuged as above, and the supernatant dialyzed to remove the $(NH_4)_2SO_4$ All procedures were performed at 0-4

DEAE-cellulose chromatography Thoroughly washed DEAE-cellulose was equilibrated with buffer A and 326 units of enzyme applied to a 1 × 12 cm column with a flow rate of 0 5 ml/min The column was washed with buffer A until the A_{280} of the effluent was less than 0 02 The enzyme was eluted with a linear gradient of 0 0-0 5 M KCl in buffer A 20 45-drop fractions were collected

Ultracentrifugation, K_m and pH studies To determine solubility of the enzyme, 10 ml of the 30-90% $(NH_4)_2SO_4$ purified enzyme was subjected to centrifugation at 100000 *g* for 3 hr The pellet was resuspended in a minimal amount of buffer A by use of a glass homogenizer and the protein content and enzyme activity of both fractions determined All K_m and V_{max} values were obtained by placing the data points for an Eadie-Hofstee plot into a linear regression analysis computer program In order to obtain a wide range of pH values three separate buffer systems were used in the pH studied NaOAc was used over the range 3 0-5 5, imidazole 6 0-7 4, Tris HCl 7 6-9 4 Except for different buffers, assay conditions were exactly as described previously

Preparation of a dialyzable inhibitor of cyclic nucleotide phosphodiesterase 50 g of carrot were homogenized in 50 ml of gd H_2O and dialyzed against 100 vol of the same at 4 The dialyzate was concentrated by lyophilization and resuspended in 5 ml of gd H_2O Activity of the inhibitor was measured by adding 50 μl to the reaction mixture and also reducing the amount of enzyme to 50 μl so that the total vol of the reaction mixture remained the same as previously described

Anion-cation chromatography of phosphodiesterase inhibitor For the anion exchange, 1 ml of Dowex 1-2 × 400 slurry was put into a small column and washed with 10 ml of 10 mM Tris-HCl buffer, pH 7 4 1 ml of concentrated inhibitor was then applied to the column and the column washed with 10 ml of 10 mM Tris-HCl buffer, pH 7 4 Five 2 ml fractions were collected Elution was accomplished with 25 ml of 0 1 M HCl, 5 5 ml fractions being collected

Cation exchange was performed with Dowex 50 in a 0 4 × 6 cm column The column was washed with 0 05 M HCl 1 ml of inhibitor, previously adjusted with 0 05 M HCl was applied to the column and the column washed with 25 ml of 0 05 M HCl 5 5-ml fractions were collected Five 5-ml fractions were collected on elution with 0 01 M NaOH (25 ml) All fractions were concentrated by lyophilization dissolved in 0 5 ml gd H_2O and tested as described above

Determination of protein and P_i content Protein concentration was determined according to the method of Lowry *et al*.[15] with crystalline bovine serum albumin as a standard P_i was determined by the method of Fiske and Subbarow[16] with $NaPHO_4$ as a standard

Acknowledgements The authors wish to thank Dr R W Butcher, Chairman, Biochemistry Department, University of Massachusetts Medical Center, for his guidance and support of the research This work was supported by grant No AM13904-05 NIH awarded to Dr R W Butcher

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