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CYCLIC AMP PHOSPHODIESTERASE FROM DORMANT TUBERS OF JERUSALEM ARTICHOKE

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Abstract—An enzyme, which hydrolyzes 3',5'-cyclic AMP to 3'-AMP and 5'-AMP, has been isolated from dormant tubers of Jerusalem artichoke and purified 850× with a recovery of 15% of total activity The partially purified enzyme differs greatly from both animal and bacterial phosphodiesterases in terms of pH optimum, substrate specificity, cation dependence and sensitivity to methylxanthines The plant hormones are without effect, whereas ATP, 5'-AMP, 3'-AMP, inorganic phosphate and pyrophophosphate are inhibitors. The enzyme seems to be greatly inhibited *in vivo* by inorganic phosphate during dormancy

INTRODUCTION

ADENOSINE 3', 5' cyclic monophosphate (cyclic AMP) has been shown to be an intracellular mediator of the response to a number of animal hormones[1] and to control some aspects of bacterial metabolism.[2] The level of cyclic AMP depends upon two enzyme activities; adenylate cyclase, which catalyzes the formation of cyclic AMP from ATP; and phosphodiesterase, which hydrolyzes cyclic AMP to 5'-AMP. The animal hormones induce a change in the concentration of cyclic AMP within the cells of their target tissues by activating a membrane-bound adenylate cyclase.[1]

Cyclic AMP has recently been detected in higher plant tissues by different assay techniques[3–6] and its possible roles as mediator in plant hormone effects has been envisaged.[7–16] Some information on the metabolism of cyclic AMP in higher plant tissues is also avail-

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able enzymatic synthesis of cyclic AMP from ATP has been detected in extracts from *Vinca rosea* tumor cells[17] and Jerusalem artichoke tubers;[16] cyclic AMP degrading phosphodiesterases have been purified from potato tubers,[18,19] pea seedlings,[20] soybean callus,[21] barley seeds[22] and *Phaseolus*.[23]

After the discovery of an enzymatic system forming and degrading cyclic AMP in dormant tubers of Jerusalem artichoke,[16] we have investigated its possible role in the metabolism of cyclic AMP. We observed[4] that the rapid decline of cyclic AMP level, occurring at the dormancy break of Jerusalem artichoke tubers, was accompanied by a corresponding increase in phosphodiesterase activity toward cyclic AMP and by a dramatic degradation of cyclic AMP *in vivo*. Since these observations suggested a role for phosphodiesterase(s) in the control of the levels of cyclic AMP in Jerusalem artichoke tubers, it seemed interesting to study this enzyme in more detail. This paper deals with the purification and characterization of a soluble cyclic AMP phosphodiesterase from dormant tubers of Jerusalem artichoke. The effect of different factors, including the plant hormones, on the activity of partially purified enzyme has also been investigated in order to elucidate the mechanism regulating its activity.

RESULTS

Purification

The results of a typical purification of the enzyme are summarized in Table 1. A purification of 850× was obtained with a recovery of 15% of total activity. As shown in Table 1, dialysis of crude extract is the purification step giving the greatest increase in enzyme specific activity. Crude extract contains 2 mM inorganic phosphate (Pi). The dialysis of the crude extract completely removes the Pi and increases the enzyme activity 10-fold. The addition of 2 mM Pi to the dialyzed extract reduces the phosphodiesterase activity to the pre-dialysis level.

TABLE 1. PURIFICATION OF JERUSALEM ARTICHOKE PHOSPHODIESTERASE

Fraction	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg)	Relative purity	Recovery of activity
Supernatant 105000 g	1284	25.6	0.02	1.0	
Supernatant after dialysis	708	141.6	0.20	10.0	100
Ammonium sulphate (25–45% satd)	132	67.2	0.51	25.5	47
Sephadex G_{200}	10	24.0	2.40	120.0	17
DEAE-cellulose	1.3	22.0	17.0	850	15

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Properties of the enzyme

In acetate buffer, maximal enzyme activity was obtained between pH 5·2 and 5·4. At a concentration of 1 mM, Mg^{2+}, Mn^{2+} and EDTA slightly (110–115%) stimulated the enzyme activity, whereas 1 mM Ca^{2+} slightly inhibited. Caffeine (8 mM), theophylline (8 mM) and Ro-7-2956 (0·01 mM)[24] were not inhibitory to Jerusalem artichoke phosphodiesterase, although they have been shown to inhibit animal and bacterial enzymes, whereas imidazole (8 mM) slightly stimulated (+15%) enzyme activity Most phosphate esters were strongly inhibitory: at 1 mM, ATP (−65%), 5′-AMP (−90%), 3′-AMP (−92%), 3′5′-cyclic AMP (−58%), 2′,3′-cyclic AMP (−97%), 3′,5′-cyclic GMP (−59%), 2′,3′-cyclic GMP (−98%), 3′,5′-cyclic UMP (−65%), 3′,5′-cyclic IMP (−52%) and 3′,5′-cyclic CMP (−38%). Inorganic phosphate and pyrophosphate at 0·10 mM both showed 70% inhibition. On the other hand indoleacetic acid, gibberellic acid, abscisic acid and fusicoccin had no effect on the enzyme activity at 0·1 or 1 mM. The cytokinin, isopentenyladenosine, inhibited the enzyme activity slightly (115%) at 3 mM. To determine the K_m, purified phosphodiesterase was assayed, as described under experimental, but with a different concentrations of cyclic AMP (9×10^{-5} to 8×10^{-4}). The apparent K_m calculated for 3′,5′ cyclic AMP phosphodiesterase was approximately $6{\cdot}8 \times 10^{-4}$ M.

Chromatographic identification of the reaction products. When the reaction mixture was chromatographed with either Solvent A or Solvent B, radioactivity was always localized in the area of authentic 5′-AMP (R_f = 0·10 in Solvent A and 0·43 in Solvent B) and 3′-AMP (R_f = 0·15 in Solvent A and 0·28 in Solvent B). Cyclic AMP phosphodiesterase also degraded 2′,3′ cyclic AMP. When the reaction mixture for 2′,3′ cyclic AMP phosphodiesterase was chromatographed with Solvent B, only 3′-AMP (R_f = 0·28) but not 2′-AMP (R_f = 0·35) was detected. The R_f of 2′,3′ cyclic AMP was 0·12. Cyclic AMP phosphodiesterase did not contain nucleotidase activity, but showed slight activity toward RNA. However, the ribonuclease could be separated from cyclic AMP phosphodiesterase by disc-gel electrophoresis.

DISCUSSION

The partially purified enzyme greatly differs, in terms of pH optimum, substrate specificity, reaction products, cation dependence and sensitivity to methylxanthines, from both animal[1,25] and bacterial[26,27] phosphodiesterases and seems very similar to that extracted recently from pea seedlings[20] and barley seeds.[22]

The plant hormones tested are ineffective on the activity of partially purified phosphodiesterase. In this respect, Jerusalem artichoke phosphodiesterase is similar to animal phosphodiesterases, for which direct hormonal regulation has not been found.[1] Furthermore, we have observed that ATP,5′-AMP,3′-AMP, inorganic pyrophosphate (PPi) and Pi are inhibitors, suggesting that the enzyme is susceptible to some regulation. Since ATP is a substrate of the reaction catalyzed by adenylate cyclase,[16] in contrast to 5′-AMP and 3′-AMP which have been found to be the products of phosphodiesterase, one might speculate that the regulation occurs during the synthesis and the breakdown of cyclic AMP.

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As for potato phosphodiesterase,[19] Pi is a natural inhibitor of Jerusalem artichoke phosphodiesterase. Pi was found in crude extract of dormant tubers at very high concentration, suggesting that the enzyme may exist during the dormancy period in a greatly inhibited state *in vivo*. This possibility is supported by the findings that, in spite of the presence of phosphodiesterase, the cyclic AMP content in dormant tuber tissues is very high compared with that of activated tissues.[4]

Jerusalem artichoke phosphodiesterase, like pea seedling and barley seed phosphodiesterases,[20,22] hydrolyzes both 3',5' cyclic AMP and 2',3' cyclic AMP. Lin and Varner,[20] assuming that the hydrolysis of both cyclic nucleotide phosphates was due to the same enzyme, suggested that the pea seedling phosphodiesterase, hydrolyzing the 2',3' cyclic nucleotides produced by ribonuclease, could play a crucial role in the degradation of RNA. Our previous observations on the metabolism of cyclic AMP during dormancy break of Jerusalem artichoke tubers[4] suggest that the enzyme investigated in the present study may represent a physiological mechanism of cyclic AMP degradation in higher plant tissues.

EXPERIMENTAL

Dormant tubers of Jerusalem artichoke (*Helianthus tuberosus*) were harvested in November and stored at 4° for 4–6 months before experimental use. Cyclic AMP (8-H^3) (spec. act. 2·65 Ci/mmole) was purchased from Amersham (England) and purified according to Lin and Varner.[20]

3',5' cyclic AMP phosphodiesterase assay. Each reaction mixture contained in a total vol. of 0·4 ml: 16 μmol of acetate buffer (pH 5·4), 40 nmol of 3',5' cyclic AMP and cyclic AMP (-8-^{3}H) (2×10^4 cpm). The reactions were started by adding 1 μg of enzyme protein except where indicated. After 1 hr at 37° the tubes were heated to 100° for 3 min. 1 ml of 10 mM Tris·HCl buffer, pH 7·4 was added and the tubes vortex-mixed and then centrifuged for 10 min at 2500 rev/min. The supernatant from each tube was applied onto an alumina column prepared as described by White and Zenser[28] except that the alumina was equilibrated with 10 mM Tris·HCl, pH 7·4. The first 3 ml eluted from the columns, containing 85–90% cyclic AMP, were collected and the radioactivity counted. The amount of the cyclic nucleotide hydrolyzed was calculated on the basis of the difference between the amount added to the reaction mixture and that recovered from the column. 1 unit of phosphodiesterase activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol of cyclic AMP per 60 min in a standard reaction mixture.

Chromatographic analysis of the reaction products. 3',5' cyclic AMP phosphodiesterase was also determined by chromatographic analysis. Aliquots of the reaction mixtures were chromatographed on a PEI-cellulose precoated TLC plates by using unlabeled cyclic AMP, 5'-AMP, 3'-AMP, adenosine and 3',5' cyclic IMP as incorporated standards. The chromatograms were developed using *iso*-PrOH–H_2O–NH_4OH (7:2:1) (Solvent A) or saturated $(NH_4)_2SO_4$–1 M NaOAc *iso*-PrOH (20:4:5) (Solvent B). The spots detected under UV light, were scraped from the plates, placed in vials and counted in Instagel. The radioactivity was determined in a liquid scintillation counter. To detect the products of the hydrolysis of 2',3' cyclic AMP, the chromatograms were developed with the solvent B along with 2',3' cyclic AMP, 3'-AMP, 2'-AMP and adenosine as carriers. The compounds were detected under UV light.

Determination of protein and Pi content. Protein was determined according to the method of Lowry *et al.*,[29] with crystalline bovine albumin as standard. Pi was determined according to Chen *et al.*[30] with KH_2PO_4 as standard.

Polyacrylamide disc-gel electrophoresis was performed according to Ornstein[31] and to Davis[32] in Tris-glycine buffer (pH 8·5) at 4° with a constant current of 2 mA/tube. 3 tubes were run at the same time. After 2 hr of electrophoresis, one gel was stained with 0·05% Coomassie brilliant blue in 12·5% TCA and destained by diffusion in H_2O. The other two gels were cut in segments of 0·5 mm each and each segment was tested for cyclic AMP phosphodiesterase or for ribonuclease activity. The cyclic AMP phosphodiesterase and ribonuclease activities were assayed by adding 1 segment of gel to each reaction mixture. Ribonuclease activity was assayed according to Udvardy *et al.*[33]

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Enzyme purification All procedures were carried out at 2°–4° *Step* 1 In a typical experiment, 200 g of peeled tubers were cut into small pieces and homogenized with 200 ml of 10 mM Tris–HCl, pH 7 4, in an OmniMixer set at high speed for five 30-sec periods with cooling between each homogenization The homogenate was then filtered through 3 layers of cheesecloth and the resulting filtrate was centrifuged 2 hr at 105 000 *g* The supernatant was taken as the crude extract for purification of the enzyme *Step* 2 The supernatant was dialyzed against 20 vol of 10 mM Tris–HCl, pH 7 4, with constant agitation for 24 hr, with 4 changes Then the soln was centrifuged at 10 000 *g* for 20 min to remove the ppt which formed during dialysis *Step* 3 The clear supernatant was then fractionated with $(NH_4)_2SO_4$ The precipitate between 25–45% saturation was dissolved in 10 ml mM Tris-HCl, pH 7 4 *Step* 4 A column 2 5 × 89 cm, containing Sephadex G_{200}, was washed with 10 mM Tris–HCl, pH 7 4 30 mg of protein enzyme obtained by $(NH_4)_2SO_4$ precipitation was applied to the column Fractions (4 ml) were collected (at a flow rate of 8 ml/hr) and analyzed for protein content and phosphodiesterase activity Phosphodiesterase activity was eluted between 120–170 ml *Step* 5 The pooled Sephadex fractions (30–42), containing 2 2 mg of protein, were applied to a DEAE-cellulose column (1 × 10 cm) equilibrated with 10 mM Tris–HCl, pH 7 4 The column was washed with 10 mM Tris–HCl, pH 7 4, and the proteins were eluted stepwise in 1 ml fractions with 0 125, 0 250, 0 375 and 0 5 M NaCl in 10 mM Tris–HCl adjusted to pH 7 4 The phosphodiesterase was eluted with 0 125 M NaCl The active fractions were collected and used in all experiments described hereafter At this stage the phosphodiesterase was unstable The enzyme activity was completely lost after 48 hr at 2–4°

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