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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY IN *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; cyclic nucleotide phosphodiesterase; cyclic AMP; phosphodiesterase activator; phosphodiesterase inhibitor.

Abstract—Centrifugal fractionation showed that 70% of the cyclic nucleotide phosphodiesterase activity of *Phaseolus vulgaris* seedlings is recovered in the 100000 g pellet with most of the remaining activity in the supernatant. With cyclic AMP as substrate, the activity has a pH optimum of 6.2, a K_m of 0.125 mM, and caffeine and theophylline are competitive inhibitors. Fractional precipitation with $(NH_4)_2SO_4$, followed by gel filtration on Sephadex G-200 gave two peaks of phosphodiesterase activity (apparent MWs 3.4×10^5 and 7.6×10^3). Both hydrolysed cyclic AMP and to a lesser extent other cyclic 3′:5′-nucleotides to a mixture of the corresponding nucleoside monophosphates. No significant activity was exhibited towards cyclic 2′:3′-AMP. An endogenous activator was present in *P. vulgaris* seedlings; its effect was potentiated by $CaCl_2$ and by abscisic acid. Isoelectric focusing of either of the two peaks of phosphodiesterase activity separated a major and a minor component. Preincubation of either peak with the activator caused all the activity to appear in a single band on isoelectric focusing. The two peaks of activity may represent either different aggregation states of the same enzyme or different multienzyme complexes containing the same enzyme. The significance of these findings is discussed in the context of a possible regulatory role for cyclic AMP in plants.

INTRODUCTION

Although the role of 3′:5′-cyclic AMP as a mediator of hormone action in mammals [1] and as a 'primary' messenger in lower organisms [2] is now well established, its possible regulatory function in higher plants is controversial [3–5]. Cyclic AMP has been demonstrated in higher plants by a variety of techniques [6–10]. Adenylate cyclase has been reported in Jerusalem artichoke tubers [11] and there are several reports of cyclic nucleotide phosphodiesterase activity in plant tissues [12–15]. Observations on the plant phosphodiesterases [12, 13] indicate that they differ in a number of respects from their mammalian and bacterial counterparts. These differences concern pH optima, substrate specificity and susceptibility to inhibition by methylxanthines.

The present report describes partial purification of the cyclic nucleotide phosphodiesterase activity from *Phaseolus vulgaris*, tissues of which have been previously shown to contain cyclic AMP [6]. The main objective was to see if the properties of the enzyme are compatible with participation in a regulatory system or whether they are more likely those of an enzyme concerned with RNA catabolism, as has been suggested for the enzyme from pea seedlings studied by Lin and Varner [12]. A secondary objective was to seek possible endogenous inhibitors of cyclic nucleotide phosphodiesterase activity with the hope that such compounds could be used to prevent cyclic AMP destruction during associated studies of its production *in vitro*.

RESULTS

Preliminary extraction of phosphodiesterase activity and examination of its properties

Examination of the distribution of cyclic nucleotide phosphodiesterase activity among various centrifugal fractions gave the results shown in Table 1. About 70% of the activity originally present in the non-diffusible fraction of the crude homogenate after dialysis was recovered in the 100000 *g* pellet. A significant part of the remainder was located in the 100000 *g* supernatant. The increase in activity of the crude homogenate following dialysis (Table 1) is attributable to removal of a diffusible inhibitor [15, 16].

Table 1. Centrifugal fractionation of cyclic AMP phosphodiesterase activity from *Phaseolus vulgaris*

Fraction	Total activity (enzyme units)
Crude homogenate	161
Non-diffusible fraction from crude homogenate	229
900 *g* Pellet	0
3000 *g* Pellet	16
10000 *g* Pellet	15
100000 *g* Pellet	166
100000 *g* Supernatant	56

Total phosphodiesterase activity was determined by the standard procedure as indicated in the Experimental section. One enzyme unit releases 1 nmol of P_i per min.

Table 2. Effect of various factors on the activity of the cyclic AMP phosphodiesterase activity of the 100000 *g* pellet

Incubation conditions	Enzymic activity (units/ml)	Activity relative to control (%)
Standard incubate (control)	16.7	100
$(NH_4)_2SO_4$ omitted	12.8	76
$MgCl_2$ omitted	12.1	73
GA_3 (5 mM) added	16.6	100
Kinetin (5 mM) added	16.7	100
Imidazole (40 mM) added	17.9	107
Caffeine (5 mM) added	11.3	68
Theophylline (5 mM) added	12.9	78

Activities were determined in 100 mM cacodylate buffer (pH 6.2). Incubations were at 31° for 60 min. Details of the composition of the standard incubation mixture are given in the Experimental section. The enzyme unit is defined in Table 1.

Taking the 100000 *g* pellet as a crude preparation of phosphodiesterase, a preliminary examination of its properties was made. Under the standard conditions indicated in the Experimental section, it was found to have a pH optimum of 6.2 and using the Lineweaver–Burk [17] procedure, a K_m value of 0.125 mM was determined with 3′:5′-cyclic AMP as substrate. Both Mg^{2+} and $(NH_4)_2SO_4$ were necessary for full enzymic activity (Table 2), omission of either resulted in a loss of *ca* 25%. Gibberellic acid, kinetin and imidazole (all at 5 mM) had no significant effect on the activity but caffeine and theophylline at this concentration were inhibitory. Lineweaver–Burk plots of this inhibition in the presence of different concentrations of the substrate (0.1–1.0 mM cyclic AMP) showed that both compounds are competitive inhibitors.

Fractionation of the crude extract; properties of phosphodiesterase fractions

Further purification of the phosphodiesterase activity from the 10000 *g* supernatant was effected by fractional precipitation with $(NH_4)_2SO_4$. All activity was recovered in the fraction obtained at 37–48% saturation with $(NH_4)_2SO_4$. This fraction was redissolved in 5 ml Tris–HCl buffer (50 mM; pH 7.4) and further resolved by gel filtration on Sephadex G-200 (see Experimental section). Two peaks of phosphodiesterase activity were obtained, one at 50–90 ml and the other at 145–185 ml. Both peak 1 and peak 2 activities exhibited optima at pH 6.5; their apparent MWs were 3.4×10^5 and 7.6×10^3 respectively. Determination of the respective sp. act. showed that, relative to the original homogenate, a 136-fold purification of peak 1 had been effected together with a 275-fold purification of peak 2 (Table 3). The data presented in Table 3 were obtained using cyclic AMP as substrate but both peaks exhibited phosphodiesterase

Table 3. Partial purification of cyclic AMP phosphodiesterase from *Phaseolus vulgaris*

Fraction	Total activity (enzyme units)	Specific activity (units/mg of protein)	Fold purification	Activity relative to homogenate (%)
Crude homogenate	304	0.02	1	100
Diffusible fraction from crude homogenate	458	0.06	3	151
10000 *g* Supernatant	515	0.11	5.5	169
37–48% $(NH_4)_2SO_4$ ppt.	352	0.65	32.5	115
Sephadex G-200				
Peak 1	308	2.72	136	101 } 159
Peak 2	176	5.50	275	58 }

Details of the fractionation procedures and enzyme assays are given in the Experimental section. Peaks 1 and 2 were eluted from Sephadex G-200 as described in the Results section

Table 4. Relative rates of hydrolysis of various cyclic nucleotides by the phosphodiesterase activities of peaks 1 and 2*

Substrate (2 mM)	Peak 1		Peak 2	
	Enzyme activity (units/ml)	Rate of hydrolysis relative to 3′:5′-cyclic AMP (%)	Enzyme activity (units/ml)	Rate of hydrolysis relative to 3′:5′-cyclic AMP (%)
3′:5′-Cyclic AMP	3.71	100	1.93	100
3′:5′-Cyclic GMP	3.55	95	1.83	94
3′:5′-Cyclic UMP	3.36	91	1.75	91
3′:5′-Cyclic IMP	3.08	83	1.56	81
3′:5′-Cyclic XMP	3.00	81	1.51	78
3′:5′-Cyclic CMP	2.95	79	1.35	70
3′:5′-Cyclic dTMP	2.86	77	1.30	67
3′:5′-N^6,2′-Dibutyryl-cyclic AMP	2.46	66	1.08	56
3′:5′-Cyclic dAMP	1.61	43	0.93	48
2′:3′-Cyclic AMP	0	0	0	0

* Peaks 1 and 2 were obtained by gel filtration of the 37–48% $(NH_4)_2SO_4$ ppt. (Table 3) on Sephadex G-200; details of the elution volumes for the two peaks are given in the text.

Table 5. Modification of cyclic nucleotide phosphodiesterase activity from *Phaseolus vulgaris* by endogenous proteins

Protein added	Phosphodiesterase activity: Peak 1 — Total activity (enzyme units)	Peak 1 — Change relative to control (%)	Peak 2 — Total activity (enzyme units)	Peak 2 — Change relative to control (%)
None (control)	161	0	108	0
Non-diffusible fraction of homogenate	59	−1	101	−6
Fraction C (0–37% $(NH_4)_2SO_4$ ppt.)	37	−77	23	−79
Fraction E (48–100% $(NH_4)_2SO_4$ ppt.)	161	0	109	0
Fraction F (pooled pre-peak 1 fractions from Sephadex G-200)	148	−8	107	0
Fraction G (pooled post-peak 1 fractions from Sephadex G-200)	377	+134	235	+118
Fraction H (pooled post-peak 2 fractions from Sephadex G-200)	141	−12	94	−13

Details of gel filtration of the 37–48% $(NH_4)_2SO_4$ ppt. on Sephadex G-200 are given in the text.

activity to a lesser extent against other cyclic 3′:5′-nucleotides (Table 4). Cyclic GMP and cyclic UMP were *ca* 90% as effective as substrates whereas cyclic IMP, cyclic XMP, cyclic CMP and cyclic dTMP were about 80% as effective. N^6,2′-Dibutyryl cyclic AMP and cyclic dAMP gave values of *ca* 50% of that obtained with cyclic AMP. The relative specificity of peak 1 was essentially similar to that of peak 2 (Table 4) and the products of hydrolysis were in both cases a mixture of 3′-AMP and 5′-AMP. The ratio of the amounts of 3′-AMP and 5′-AMP released was *ca* 1:2 but this could not be accurately assessed as the enzymes were associated with endogenous nucleotidases (unpublished observation). Neither peak 1 nor peak 2 exhibited any significant activity towards 2′:3′-cyclic AMP. Comparison of freeze dried with fresh preparations of peaks 1 and 2 showed that the freeze dried phosphodiesterase activity of both peaks could be stored for several weeks with little loss of activity.

Endogenous activator and inhibitors

All phosphodiesterase-inactive fractions obtained during the resolution of peaks 1 and 2 on Sephadex G-200 were examined for possible regulatory effects on the enzymic activity of peaks 1 and 2. The only significant effects were obtained with fraction C (0–37% $(NH_4)_2SO_4$ ppt.) and fraction G (100–140 ml fraction from Sephadex G-200). Fraction C was strongly inhibitory whereas fraction G produced a marked stimulation (Table 5). The inhibitory effects of fraction C were neither alleviated nor potentiated by Ca^{2+}, Mg^{2+} or Mn^{2+}, or by phytohormones. Activation of the phosphodiesterases by fraction G did not appear to be due to non-specific stabilization by protein since the effect could not be

Table 6. Effect of various compounds on the cyclic nucleotide phosphodiesterase activity of peaks 1 and 2

Additions	Phosphodiesterase activity: Peak 1 — Enzymic activity (units/ml)	Peak 1 — Activity relative to control (%)	Peak 2 — Enzyme activity (units/ml)	Peak 2 — Activity relative to control (%)
None (control)	13.4	100	6.8	100
Gibberellic acid (2 mM)	13.4	100	6.9	100
Abscisic acid (2 mM)	14.2	106	7.1	104
$CaCl_2$ (2 mM)	13.5	100	6.8	100
Fraction G (2 mg)	35.5	265	15.2	224
Fraction G (2 mg) + gibberellic acid (2 mM)	35.5	265	15.1	223
Fraction G (2 mg) + abscisic acid (2 mM)	40.3	301	16.7	246
Fraction G (2 mg) + $CaCl_2$ (2 mM)	40.3	301	17.3	254

Phosphodiesterase activity was determined by a minor modification of the method of ref. [29] (see Experimental section).

simulated by various concentrations of bovine serum albumin. Furthermore, no potentiation of the effect of the activator was obtained in the presence of bovine serum albumin. It was observed that fraction G was also able to activate bovine brain phosphodiesterase to a comparable extent.

Neither gibberellic acid nor abscisic acid had any significant effect on the phosphodiesterase activity of peaks 1 and 2 but 5 mM caffeine and theophylline caused a 20% inhibition of the activity of both peaks (Table 6). Results showed that $CaCl_2$ (2 mM) had no effect on peaks 1 and 2 when added alone but when added in the presence of fraction G potentiated the activation giving an additional 30–35% increase (Table 6). A similar effect was seen with abscisic acid.

Isoelectric focusing of phosphodiesterase activity and the effect of activator

Isoelectric focusing of peaks 1 and 2 revealed the presence, in each, of several proteins. With peak 1, only two of these exhibited cyclic AMP phosphodiesterase activity. The minor component (band 1a) had an activity of 3.8 units and a pI value of 4.5 whereas the major component (band 1b) had an activity of 10.2 units and a pI value of 3.5. A similar isoelectric focusing pattern was observed with peak 2. The minor band (band 2a) had an activity of 2.6 units and a pI value of 4.5; the major band (band 2b) had 6.1 units and a pI value of 3.5. Pre-incubation of either peak 1 or peak 2 with fraction G caused all the phosphodiesterase activity to appear in a single band with a pI value of 3.5. The cyclic AMP phosphodiesterase activities of peak 1 and 2 altered after pre-incubation with fraction G both in respect of increased phosphodiesterase activity and modification of pI.

DISCUSSION

Cyclic nucleotide phosphodiesterase activity has been reported in pea seedlings [12, 18], potato [14, 19], soybean callus [15], Jerusalem artichoke [16], barley seedlings [20], carrot leaves [21], tobacco pith [22], *Phaseolus* seedlings [23], and various other higher plants and algae [13]. Several of these observations are interpreted by their authors as indicative of a regulatory role by cyclic nucleotides in plants. Others [12, 13] suggest that the properties of the plant enzymes differ so markedly from their animal and bacterial counterparts that they are more likely concerned with the hydrolysis of cyclic 2′:3′-AMP during RNA catabolism. For example, in contrast to animal phosphodiesterases, the enzyme obtained from pea seedlings by Lin and Varner [12] has been reported to have an acidic pH optimum (5.4), greater activity towards cyclic 2′:3′-AMP than the 3′:5′-isomer, and to produce 3′-AMP as the main hydrolysis product. It was also found to be insensitive to methylxanthines. The cyclic nucleotide phosphodiesterase preparations obtained in the present study with *P. vulgaris* have a pH optimum which is significantly closer to that of the corresponding mammalian enzymes [24] than that described by Lin and Varner [12] for the enzymes from pea seedlings. The *Phaseolus* enzyme hydrolyses cyclic AMP to a mixture of 3′-AMP and 5′-AMP with the latter predominating. The cyclic nucleotide phosphodiesterase activities from *Phaseolus* are competitively inhibited by methylxanthines and have a K_m within the range of values observed for the mammalian enzymes [24]. A major difference between the properties of the enzyme from pea seedlings and the *Phaseolus* enzyme is that whereas the latter is active against a range of 3′:5′-cyclic nucleotides, it is inactive towards cyclic 2′:3′-AMP (Table 4). These latter observations suggest that the *Phaseolus* cyclic nucleotide phosphodiesterase is specifically involved in metabolism of cyclic 3′:5′-AMP.

The apparent MWs of the two peaks of cyclic nucleotide phosphodiesterase activity obtained by gel filtration, taken with the almost identical properties of the two activities, suggests that they represent either different aggregation states of the same enzyme or multienzyme complexes containing the same enzyme. There are indications of a similar situation in soybean callus where phosphodiesterase is associated with acid phosphatase [15].

In mammalian tissues, cyclic AMP concentrations are regulated by the action of hormones on the adenylate cyclase of the cell membranes. Since phytohormones, unlike their animal counterparts, appear to penetrate the target cells [4], control of cyclic AMP concentrations in plant cells may be effected via regulation of the phosphodiesterase. The phytohormones examined in the present work did not provide evidence for direct control of the enzyme (Tables 2 and 6) but the observation that abscisic acid potentiates the effect of an endogenous activator of phosphodiesterase (fraction G; Tables 5 and 6) does afford an effective indirect means of regulation. In view of the reported activation of adenylate cyclase by gibberellic acid [11], the indirect activation of phosphodiesterase by abscisic acid may provide a control mechanism comparable to that in mammalian liver [25]. In the latter, glucagon activates adenylate cyclase while the antagonistic effect of insulin is mediated by a stimulation of phosphodiesterase. The occurrence of an endogenous inhibitor of phosphodiesterase in *Phaseolus* seedlings (fraction C; Table 5) may also be significant in this context.

The effects of the activator on the *Phaseolus* phosphodiesterase are similar to those elicited from the mammalian enzyme by the endogenous activator from bovine brain [26] in that both systems require Ca^{2+} for maximum activity. It is of interest that the plant activator will also activate cyclic nucleotide phosphodiesterase from bovine brain. The effect of the activator on the behaviour of peaks 1 and 2 phosphodiesterase activity during isoelectric focusing is to produce a single fraction from two original fractions in each peak. The bovine enzyme did not respond in this way, indicating that the mechanism of activation is not the same in plants and animals.

EXPERIMENTAL

Materials. Seeds of *Phaseolus vulgaris* L. cv. The Prince were surface sterilized by immersion for 1 hr in a soln of 5% (w/v) Ca hypochlorite containing 0.2% v/v) Stergene, thoroughly washed in H_2O for 24 hr and germinated in Levington compost at 25°. Seedlings were grown in a light cycle of 18 hr light (5.5 klx) and 6 hr dark. For the ensuing experiments 6- to 7-day-old seedlings were used.

Fractional centrifugation. Seedlings (100–300 g fr. wt) were ground in ice-cold Tris–HCl buffer (50 mM; pH 7.4) containing 2 mM $MgCl_2$ and 2 mM $(NH_4)_2SO_4$ using a chilled mortar and pestle. Acid-washed sand was used to facilitate grinding. The slurry was squeezed through a double layer of gauze and coarse debris removed by centrifuging at 200 *g* for 1 min at 0°. The

supernatant was used as crude homogenate. For fractional centrifugation, the crude homogenate was first made 0.25 M with respect to sucrose and then centrifuged at 900 *g* for 10 min. The pellet was resuspended in buffered sucrose soln and retained after being recentrifuged at 900 *g* for 10 min. The combined supernatants were centrifuged at 3000 *g* for 5 min and the pellet washed by resuspending in buffered sucrose soln and centrifuging at 3000 *g* for 5 min. The combined 3000 *g* supernatants were recentrifuged at 10000 *g* for 15 min and the pellet washed by resuspension and recentrifuging as previously. The pooled 10000 *g* supernatants were centrifuged at 100000 *g* for 1 hr and both the pellet and final supernatant retained. Each sediment was resuspended in 20 ml of Tris–HCl buffer (50 mM; pH 7.4) and dialysed 18 hr against the same buffer. The 100000 *g* supernatant was dialysed against a 60% (w/v) soln of sucrose containing Tris–HCl buffer (50 mM; pH 7.4).

Fractionation of enzymic activity. The crude homogenate was centrifuged at 10000 *g* for 10 min and the supernatant dialysed against Tris–HCl (50 mM; pH 7.4). The non-diffusible fraction was subjected to fractional pptn with $(NH_4)_2SO_4$. Ppts were collected at 0–37% satn, 37–48% satn and 48–100% satn. Each fraction was dissolved in 40 ml of the Tris–HCl buffer and dialysed against the same buffer. Following dialysis, a 2 ml sample of the non-diffusible fraction from the 37–48% ppt. was applied to a column (55 × 4 cm diam.) of Sephadex G-200 previously equilibrated with 50 mM Tris–HCl buffer (50 mM; pH 7.4). Elution was effected with the same buffer at a flow rate of 15 ml/hr at 4°; 10 ml fractions were collected. The apparent MWs were determined by the procedure of ref. [27]; cytochrome *c*, BSA, alcohol dehydrogenase and urease were used as standards.

Isoelectric focusing. The following aq. solns were prepared and mixed; 10 ml of acrylamide (29.1% w/v), 10 ml of *N,N'*-methylenebisacrylamide (0.9% w/v), 36 ml of sucrose (20% w/v), 3.1 ml of Ampholine (pH 3.5–10; LKB Ltd) and 0.5 ml of Ampholine (pH 5–8). De-aeration was effected by aspirating for 1–2 min. Finally 0.4 ml of riboflavin soln (0.004% w/v) was added and the soln thoroughly mixed and spread onto a glass plate where it was allowed to polymerize at 4°. Sample solns (10 μl), containing 50–350 μg of protein, were introduced onto the polyacrylamide gel with the aid of filter paper strips. The anode soln was M H_3PO_4 and the cathode soln M NaOH; the current was initially 50 mA but fell rapidly as the V was gradually increased from 210 to 1000. Separation was allowed to proceed for 1.5–2.5 hr. After focusing, the gel was removed and cut into two equal portions. One of these was sliced into squares *ca* 10 × 10 mm. For examination of enzymic activity, each square was eluted with 1 ml of dist. H_2O and the eluate dialysed. The other half of the focusing plate was stained for protein with Coomassie brilliant blue R 250, prepared according to ref. [28]. Protein bands were evident after 15 min but staining was allowed to continue for 45 min; the stained gels were stored in H_2O. The location of each protein band was compared with phosphodiesterase activity found in the sliced gel.

Determination of phosphodiesterase activity and protein. Phosphodiesterase activity was determined by a minor modification of the method of ref. [29]. The incubation mixture included 100 μg each of 3'- and 5'-nucleotidase. For determination of the ratio, 3'-AMP and 5'-AMP were separated chromatographically [12] alongside authentic samples. One unit ef enzyme activity was defined as the amount of enzyme releasing 1 nmol of P_i per min. Protein content was estimated by the method of ref. [30] using BSA to construct a calibration curve over the range 50–300 μg/ml.

pH optima. The activity of the enzyme was determined as above using Na cacodylate buffer (100 mM; pH 5.2–7.3) and Tris–HCl buffer (50 mM; pH 7.2–9).

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