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PARTIAL PURIFICATION AND PROPERTIES OF A MULTIFUNCTIONAL 3′,5′-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *LACTUCA* COTYLEDONS

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Key Word Index—*Lactuca sativa*; Compositae; lettuce; cotyledons; cyclic nucleotides; phosphodiesterase; 3′,5′-cyclic AMP; 3′,5′-cyclic GMP; 3′,5′-cyclic CMP.

Abstract—The extraction, partial purification and properties of a 3′,5′-cyclic nucleotide phosphodiesterase from lettuce cotyledons is described. Purification involved fractional precipation with $(NH_4)_2SO_4$, chromatography on Sephadex G-200, affinity chromatograpy on Affi-Gel Blue and non-denaturing polyacrylamide gel electrophoresis. The behaviour of the final enzyme preparation on SDS-polyacrylamide gel electrophoresis was examined and indicated an M_r of *ca* 62 000. The enzyme differs from 3′,5′-cyclic nucleotide phosphodiesterases previously isolated from plant tissues in that it exhibits activity towards pyrimidine as well as purine cyclic nucleotides. Furthermore, it hydrolyses cyclic CMP at a comparable rate to that with which it hydrolyses cyclic AMP and cyclic GMP. Both 3′- and 5′-AMP were released, with the 5′-nucleotide being the major product. Whereas the K_m with all three substrates remained constant during the purification procedure, V_{max} with cyclic AMP was lower than that for cyclic CMP but increased as purification proceeded. The effects were examined of a range of di- and trivalent metal ions on the enzymic activity. Fe^{3+} significantly stimulated the activity, more so when cyclic GMP was the substrate. Cu^{2+} inhibited the activity.

INTRODUCTION

The presence of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP in higher plants has been firmly established [1, 2] but their functions remain to be elucidated [3–6]. It has been suggested [7] that in order to determine the role of these two compounds, a clearer understanding should first be sought of the regulation, properties and subcellular location of the cyclic nucleotide phosphodiesterases.

Cyclic nucleotide phosphodiesterases hydrolyse cyclic nucleotides to yield corresponding mononucleotides. In mammalian tissues and microorganisms they constitute the only known enzymatic mechanism for terminating the signalling effect of cyclic nucleotide pulses. One potent effect of this phosphodiesterase activity in mammalian tissue is to control the rate at which cells progress through the cell cycle [8]. Most mammalian cells contain more than one form of cyclic nucleotide phosphodiesterase. Often, these forms differ in size, substrate specificity, subcellular location [9] and susceptibility to activation by the calcium binding protein calmodulin [10]. The majority of these enzymes are capable of the hydrolysis of either cyclic AMP, cyclic GMP or both. Two novel enzymes recently isolated are capable of hydrolysing cyclic nucleotides containing a pyrimidine base. One showed a broad substrate specificity for hydrolysing purine and pyrimidine cyclic nucleotides at a comparable rate [11–13]. The second showed absolute specificity for a pyrimidine cyclic nucleotide, cyclic CMP, as substrate [14, 15]. The significance of these multiple forms of cyclic nucleotide phosphodiesterase and their biochemical interrelationship is however not clear at this time.

Although early reports suggested that the natural substrates of higher plant phosphodiesterases are 2′,3′-cyclic nucleotides and that the enzymes constitute part of the RNA catabolic system [16, 17], an enzyme from *Phaseolus* was found to hydrolyse the 3′,5′-cyclic nucleotide substrate but not the 2′,3′-isomer [18, 19]. Later investigations indicated that there are at least two types of cyclic nucleotide phosphodiesterase in spinach, one primarily responsible for hydrolysing 3′,5′-cyclic AMP and GMP, and the other more active with the 2′,3′-isomer. These enzymes exhibit various other differences in properties in addition to specificity [20, 21]. These differences include molecular size, pH optima, K_m and other kinetic parameters, and subcellular distribution. Although calmodulin has also been shown to occur in higher plants as well as in mammalian tissues [22], no direct regulatory role for it on the activity of a higher plant phosphodiesterase has yet been demonstrated. Nevertheless, the occurrence in *Phaseolus* of an endogenous activator of cyclic nucleotide phosphodiesterase, similar to mammalian calmodulin, has been reported [19]. All these findings concerning plant phosphodiesterase suggest that this system could be as complex in higher plants as it is in mammalian tissues.

In this paper we describe the partial purification and report the properties of a novel cyclic nucleotide phosphodiesterase from *Lactuca*. The enzyme exhibits broad specificity in that it hydrolyses both purine and pyrimidine cyclic nucleotides.

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RESULTS

Purification of the 3',5'-cyclic nucleotide phosphodiesterase

A typical set of data obtained during purification of the phosphodiesterase from *Lactuca* cotyledons is shown in Table 1. The observed increase in activity of the crude homogenate following dialysis is attributable to removal of a diffusible inhibitor [19]. Fractional precipitation with ammonium sulphate resulted in 85% of the total 3',5'-cyclic AMP phosphodiesterase activity being recovered in the 30–45% (w/v) $(NH_4)_2SO_4$ fraction. This fraction also exhibited phosphodiesterase activity towards 3',5'-cyclic GMP and 3',5'-cyclic CMP (Table 2). It was deduced that this relatively crude preparation also contained 3'-nucleotidase and 5'-nucleotidase activities since exogenous nucleotidases could be omitted from the assay incubation mixture without affecting the phosphodiesterase activity measured and examination of the reaction products by TLC confirmed the absence of 5'- and 3'-AMP. This was not the case at the subsequent purification steps.

The results of gel-filtration chromatography of the 30–45% $(NH_4)_2SO_4$ fraction on Sephadex G-200 are shown in Fig. 1. Two peaks of 3',5'-cyclic AMP phosphodiesterase activity were eluted. These had apparent molecular weights of 250000 and 72000 respectively. Insufficient of the first peak (M_r 250000) was available for further study. The major peak (M_r 72000) showed phosphodiesterase activity with all three 3',5'-cyclic nucleotides studied (Table 2).

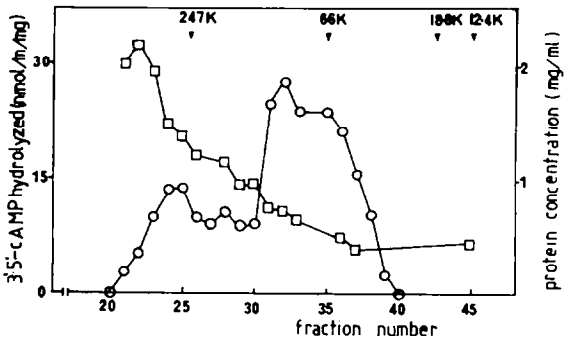

Fig. 1. Chromatography on Sephadex G-200 of the ammonium sulphate fraction (30–45% satn) containing the cyclic nucleotide phosphodiesterase activity. Each fraction was assayed for enzymic activity against 0.125 mM 3',5'-cyclic AMP (—○—○—) and protein content (—□—□—) was determined as described in the Experimental section. The arrows indicate the position in the elution sequence of the calibration proteins, cytochrome *c* (12400), myoglobin (18800), BSA (66000) and catalase (247000).

The fractions comprising the main peak from the gel-filtration step were pooled and concentrated by ultrafiltration. EGTA was added to give a final concentration of 1 mM and the concentrate dialysed overnight at 4° against 40 mM Tris buffer (pH 6.5) containing 1 mM EGTA. The non-diffusible fraction was reapplied to the

Table 1. Purification of *Lactuca* 3',5'-cyclic nucleotide phosphodiesterase

Purification step	Total activity (nmol/min)	Specific activity (nmol/min/mg protein)	Fold purification
Crude homogenate	17	0.3	1
Crude homogenate (non-diffusible fraction)	72	1.5	5.6
30–45% $(NH_4)_2SO_4$ fraction	378	5.4	19.7
Sephadex G-200	285	36.1	132
Affi-Gel Blue	100	83	304
Gel electrophoresis	65	2515	9212

Specific activities were determined with substrates at a final concentration of 1.25 mM.

Table 2. Substrate specificity of the cyclic nucleotide phosphodiesterase at different stages of purification

	Specific activity with different substrates (nmol/min/mg protein)		
Purification step	3',5'-cyclic AMP	3',5'-cyclic GMP	3',5'-cyclic CMP
30–45% $(NH_4)SO_4$ ppt.	9.2	2.3	34
Sephadex G-200	36.1	33.6	119
Affi-Gel Blue	119	65	408.3
Gel electrophoresis	2515	1003	1146

Specific activities were determined with substrates at a final concn of 1.25 mM.

same gel-filtration column. Although the conditions were those used previously, the enzyme eluted at a different position in the elution sequence giving a new apparent molecular weight of 410000. This peak retained the enzymic activity. This observation suggests that variations in the concentration of divalent metal ions present in the extract affect the state of the aggregation of this enzyme without inhibiting its activity.

To purify further the enzyme from the gel filtration peak, it was applied to a column of Affi-Gel Blue. The column was washed with Tris buffer (pH 6.5, 40 mM) and eluted with 2 M sodium chloride. The elution sequence is shown in Fig. 2. Although part of the 3',5'-cyclic phosphodiesterase activity was not retained by the column, 74% of the total activity applied was eluted as a single peak at 20–30 ml. A final wash (30 ml) with buffer did not elute any other proteins from the column. The enzyme activity constituting the peak was able to hydrolyse all three 3',5'-cyclic nucleotide substrates examined (Table 2). The unretained activity showed substrate specificity and metal-ion sensitivity similar to that of the retained peak. That this 'unretained activity' was not due to having overloaded the column was shown by increasing the bed volume of Affi-Gel Blue and decreasing the flow rate. A similar amount of activity was still lost from the column. Furthermore, according to the supplier's information sheet, the volume of Affi-Gel Blue used has a capacity for 55 mg of bovine serum albumin, whereas the plant extract applied to the column contained 5–6 mg of protein.

After chromatography on Affi-Gel Blue, the enzyme was further purified by non-denaturing polyacrylamide gel electrophoresis. The protein standards used for molecular weight calibration were bovine serum albumin (66000). phosphorylase *b* (97400), β-galactosidase (116000) and myosin (205000). The enzyme preparation showed a band of cyclic nucleotide phosphodiesterase in the region of the gel to which proteins with molecular weights of 50000–63000 migrate. This activity hydrolysed all three cyclic nucleotide substrates (Table 2) but there was insufficient of it to attempt further purification. Nevertheless, by this stage in the procedure described, a 9000-fold purification had been achieved in respect of the hydrolysis of 3',5'-cyclic AMP (Table 1). The full purification procedure was repeated on four separate occasions with closely similar results.

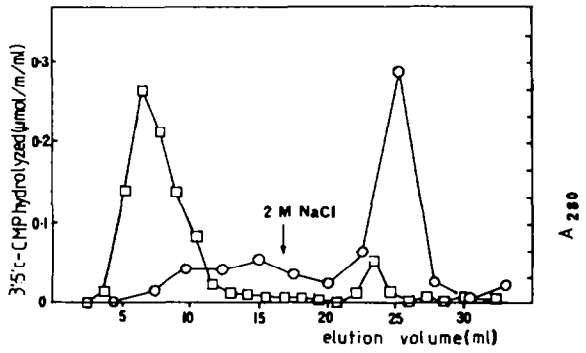

Fig. 2. Affinity chromatography of the *Lactuca* 3',5'-cyclic nucleotide phosphodiesterase on Affi-Gel Blue. Fractions were monitored at 280 nm (—□—□—) and assayed for enzymic activity against 1.25 mM 3',5'-cyclic CMP (—○—○—).

Electrophoretic, kinetic and other properties of the enzyme

The results of SDS-polyacrylamide gel electrophoresis of the enzyme preparation at the Affi-Gel Blue step, and at the non-denaturing gel electrophoresis step, are shown in Fig. 3. The enzyme activity from the Affi-Gel Blue step gave three major bands corresponding to M_r 84000, 61000 and 38000, respectively. The enzymic activity obtained from the non-denaturing gel electrophoresis step represented a very low protein concentration and an over-staining procedure was therefore necessary to make visible the major band at M_r 62300.

Taking samples of the enzyme at various stages in the purification procedure, double reciprocal plots of the enzyme-catalysed hydrolysis of 3',5'-cyclic AMP, 3',5'-cyclic GMP and 3',5'-cyclic CMP were prepared (Table 3). With all three substrates studied, the K_m values were similar and remained constant during purification. This was not true, however, of the V_{max} values which changed 1–2 orders of magnitude during purification (Table 3). In the early stages of the purification, the V_{max} for 3',5'-cyclic AMP was lower than that for 3',5'-cyclic CMP but became higher as purification proceeded. The V_{max} for 3',5'-cyclic GMP remained the lowest through all the purification steps.

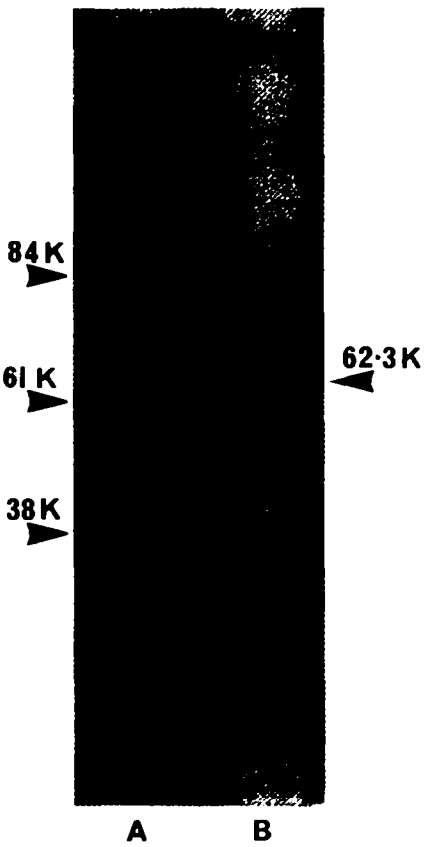

Fig. 3. SDS-polyacrylamide gel electrophoresis of the 3',5'-cyclic nucleotide phosphodiesterase at (A) the Affi-Gel Blue stage of the purification procedure, and at (B) the non-denaturing gel electrophoresis stage. Details of the electrophoresis and staining procedure are given in the Experimental section. The marker proteins used to calibrate the gel were myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), BSA (66000), egg albumin (45000) and carbonic anhydrase (29000).

Table 3. Kinetic properties of the cyclic nucleotide phosphodiesterase at different stages of purification

Substrate	K_m (mM)				V_{max} (μmol/min/mg)				$V_{max}/K_m \times 10^3$			
	$(NH_4)_2SO_4$ ppt	Gel-filtrn	Affi-Gel Blue	Gel-elect.	$(NH_4)_2SO_4$ ppt	Gel-filtrn	Affi-Gel Blue	Gel-elect.	$(NH_4)_2SO_4$ ppt	Gel-filtrn	Affi-gel Blue	Gel-elect.
3',5'-cyclic AMP	1.03	1.5	0.6	1.1	0.01	0.07	0.5	5.7	0.01	0.04	0.8	5.1
3',5'-cyclic GMP	—	1.85	—	0.71	—	0.05	—	2.6	—	0.03	—	3.66
3',5'-cyclic CMP	1.78	2	—	0.64	0.05	0.14	—	2.2	0.03	0.07	—	3.43

—Not determined.

With the purified enzyme, when 3'- and 5'-nucleotidases were omitted from the cyclic nucleotide phosphodiesterase assay (see Experimental) there was a substantial drop in apparent phosphodiesterase activity, and examination of the product by TLC showed that both 5'- and 3'-nucleotides were present. This indicates that at this stage of the purification procedure, endogenous nucleotidases are no longer present. Hydrolysis of [8-^{3}H]-cAMP, catalysed by the phosphodiesterase preparation at the gel filtration stage or at the affinity chromatography stage, yielded [^{3}H]5'-AMP and [^{3}H]3'-AMP in the ratio of 3:1, indicating that the 5'-nucleotide is the major product of this enzymic reaction.

The effect of divalent and trivalent ions on the phosphodiesterase activity of the enzyme preparation obtained at the Affi-Gel Blue purification step is shown in Table 4. With 3',5'-cyclic AMP as substrate, ferric chloride was the most effective of the salts tested in increasing the enzyme activity. Cupric chloride was, conversely, the most effective in inhibiting it. A lower stimulatory effect by $FeCl_3$ was observed when a higher concentration of substrate was used (Table 5). When the stimulatory effect of $FeCl_3$ was examined in conjunction with each of the three substrates separately, in spite of the higher rate of hydrolysis with 3',5'-cyclic CMP, the greatest stimulation was observed during the hydrolysis of 3',5'-cyclic GMP (Table 5). $FeCl_3$ and $FeCl_2$ were equally effective in stimulating the activity of the enzyme preparation obtained at the ammonium sulphate step of the purification procedure.

At the gel-filtration purification stage, the sensitivity of the enzyme to stimulation by the Ca^{2+}-binding protein calmodulin was tested. Concentrations of calmodulin ranging from 0.09 to 3.4 μg/assay were used in presence of Ca^{2+} concentrations between 1 μM and 2 mM. None of the combinations of calmodulin and Ca^{2+} increased the rate of hydrolysis of 3',5'-cyclic AMP but this may have been because the procedure used would not necessarily have yielded an activator-free enzyme preparation.

DISCUSSION

Although the purification procedure described here does not yield a homogeneous preparation of the *Lactuca* enzyme, it does afford a high degree of purification in a few steps. Its advantage over more comprehensive processes is that it only needs a small amount of plant material for extraction, a prerequisite for the investigation of enzymes in the tissues selected. A key step in the purification described here is the use of Affi-Gel Blue. This affinity chromatography support is particularly useful for retaining calmodulin-dependent phosphodiesterases [12] and protein molecules possessing a dinucleotide fold [23]. The *Lactuca* enzyme was not however stimulated by bovine heart calmodulin in the presence of Ca^{2+} at a range of concentrations, neither was it inhibited by EGTA. These observations suggest that the phosphodiesterase is calmodulin-independent, but the presence of tightly bound endogenous calmodulin in the preparation cannot be excluded. EGTA has been reported to fail in some instances to remove endogenous calmodulin [24].

The molecular weight of the *Lactuca* cyclic nucleotide phosphodiesterase (M_r 61000–63000) is substantially different to those of the enzymes previously found in spinach [20, 21] and *Phaseolus* [19, 25]. It is, however, closely similar to those of cyclic nucleotide phosphodies-

Table 4. Sensitivity of the *Lactuca* 3',5'-cyclic nucleotide phosphodiesterase to metal ions

Compound added	Specific activity (nmol/min/mg protein)	Change relative to control (%)
None (control)	50·4	0
$CoCl_2$	59.7	+18
$MnCl_2$	58.8	+17
$MgSO_4$	59.8	+19
$FeCl_3$	114.9	+128
$ZnCl_2$	39.4	−22
$CuCl_2$	12.1	−76
$Al_2(SO_4)_3$	45.6	−9
$CrCl_3$	79.0	+57
$(NH_4)_2SO_4$	63.8	+26
NaCl	68.3	+35
$CoCl_2 + MnCl_2$	74.6	+48
$CaCl_2 + MgSO_4$	57.9	+15

The substrate throughout was 0.625 mM 3',5'-cyclic AMP. Metal salts were present at a final concentration of 0.5 mM.

Table 5. Effect of $FeCl_3$ (0.5 mM) on the specific activity of the 3',5'-cyclic nucleotide phosphodiesterase

Substrate (1.25 mM)	Specific activity (nmol/min/mg protein)		
	No addition	+ $FeCl_3$	% increment
3',5'-cyclic AMP	108	140	+30
3',5'-cyclic GMP	58	175	+202
3',5'-cyclic CMP	351	478	+36

terases from dog kidney [26] and bovine brain [27]. In properties, it is dissimilar to the earlier reported plant and animal cyclic nucleotide phosphodiesterases but resembles most closely the recently described pig liver enzyme with affinity for both pyrimidine and purine substrates [12, 13, 28]. While most of the other plant and mammalian phosphodiesterases have an absolute requirement for Mg^{2+} or Mn^{2+} [29], the *Lactuca* cotyledon enzyme is similar to the partially purified pig liver enzyme in that it is stimulated to the greatest extent by Fe^{3+}. Although there are a number of differences between these two enzymes, for example K_m, molecular weight and the ability to bind to Affi-Gel Blue, both enzymes have several properties in common, such as yielding 3'- and 5'-nucleotides as products, and the ability to hydrolyse cyclic CMP at a comparable rate to that in which it hydrolyses cyclic GMP and cyclic AMP. As the role of cyclic CMP in mammals has yet to be elucidated, and the natural occurrence in plants of this third cyclic nucleotide has yet to be investigated, the full significance of these observations remains to be elucidated. The potential involvement of cyclic CMP in the regulation of cell proliferation [30] underlines the need for such investigations. The difference between the enzyme described in this report and those previously found in plants (reviewed in ref. [6]) in terms of specificity, molecular weight, K_m and V_{max}, suggest that cyclic nucleotide phosphodiesterase in higher plants exists as a very complex system as it does in mammalian tissues [31].

EXPERIMENTAL

Materials. Cyclic [8-^{3}H]AMP, cyclic [8-^{3}H]GMP and cyclic [8-^{3}H]CMP were obtained from Amersham International plc, Amersham, Bucks. Tris, 3',5'-cyclic nucleotides, 2-mercaptoethanol, 3'-nucleotidase and bovine heart calmodulin were from Sigma. AG-IX8 (Cl^- form, 100–200 mesh) and Affi-Gel Blue (100–200 mesh) were from BioRad Labs. Ltd., Watford, Herts. Sephadex G-200 was from Pharmacia, 5'-nucleotidase, acrylamide and bisacrylamide were from BDH Ltd., Poole, Dorset, and seeds of *Lactuca sativa* cv Arctic King were from C. Sharpe and Co. Ltd., Sleaford, Lincs. After surface sterilization for 30 min with sodium hypochlorite soln (1% w/v available Cl_2) seeds were germinated on moist filter paper at 24° in continuous light (3 klx). Cotyledons were detached from 3-day seedlings and immediately placed in ice-cold Tris–HCl extraction buffer until all the material was ready for homogenization and extraction. Excision of cotyledons was effected at 4°.

Assay of cyclic nucleotide phosphodiesterase activity. The assay procedure routinely used was a modification of the method of ref. [32]. Enzyme samples, each containing 0.8–100 ng of protein, were assayed in a reaction mixture containing 0.025 μCi of the appropriate cyclic nucleotide, 3'-nucleotidase (2×10^{-2} units/assay), 5'-nucleotidase (1.3×10^{-3} units/assay), 40 mM Tris–HCl buffer (pH 6.5) containing Ca^{2+}, Mg^{2+} at a final concentration of 1 mM. The final incubation mixture was made of up 50 μl of the enzyme preparation and 50 μl of a soln containing all the other constituents. After incubation at 30° for 25 min, 1 ml of ice-cold H_2O was added and the incubate passed through a column (10×7.3 mm diam.) of AG1-X8 anion exchange resin (1 ml). A further 1 ml of water was then washed through the column and a total of 2.1 ml collected.

Measurement of radioactivity. The radioactivity of samples (0.5 ml) was measured by scintillation counting in 5 ml of OptiPhase RIA scintillation fluid (LKB, South Croydon). The scintillation counter was programmed for automatic quench control and operated at a pre-set error of 0.2% with a counting efficiency of 35%.

Identification of the reaction products. The standard reaction mixtures in a final volume of 0.1 ml each contained Tris buffer (pH 6.5) 40 mM; $FeCl_3$ 0.1 mM; [8-^{3}H]cyclic AMP 0.25 μCi; and between 500 and 0.5 μg of enzyme protein dependent upon the stage of purification reached. Reactions were started by addition of substrate, incubated at 30° for 120 min and stopped by boiling the mixtures for 1 min. After centrifuging at 11 600 *g* for 4 min, the supernatants were collected, freeze-dried and redissolved in 20 μl of aq. EtOH 50% (v/v). Each of these reaction mixtures (20 μl samples) and 20 μl samples of each standard (1 mM 3',5'-cyclic AMP; 0.5 mM 5'-AMP; 0.5 mM 3'-AMP; and 0.5 mM adenosine) were spotted separately on the same cellulose TLC plate, and then separated by ascending chromatography at room temp. in satd $(NH_4)_2SO_4$–0.5 M NaOAc–*iso*-PrOH (40:9:1). Nucleotides were visualized under UV light and their radioactivity quantified using a Panax TLC-scanning Ratemeter P7973.

Determination of protein concns. For samples containing more than 20 μg of protein per ml, protein concentrations were determined by the method of ref. [33]. For samples containing less than 20 μg/ml, the method of refs [34, 35] was used.

Extraction and purification of the cyclic nucleotide phosphodiesterase. Cotyledons were homogenized in 30 ml of ice-cold Tris–HCl buffer (40 mM, pH 6.5) containing $CaCl_2$, $MgSO_4$ (2 mM) and 2-mercaptoethanol (15 mM). The homogenate was

filtered through four layers of Miracloth (Calbiochem-Behring) and the filtrate was designated 'crude extract'. This was subjected to fractional precipitation with $(NH_4)_2SO_4$. Protein ppts were collected, by centrifuging, at 0–30% satn, 30–45% satn and 45–100% satn. Each fraction was redissolved in 1.5 ml of 40 mM Tris–HCl buffer (pH 6.5) and dialysed overnight against the same buffer. Following this, a 1 ml sample of the non-diffusible fraction from the 30–45% $(NH_4)_2SO_4$ ppt was applied to a column (30 cm × 1 cm diam.) of Sephadex G-200 previously equilibrated with 40 mM Tris–HCl buffer (pH 6.5). Elution was effected with the same buffer at a flow rate of 3 ml/hr. Fractions (0.5 ml) were collected and examined for cyclic nucleotide phosphodiesterase activity and protein content. The apparent M_rs of the proteins were determined by the method of ref. [36]. Cytochrome *c*, bovine serum albumin, catalase, myoglobin and aldolase were used as standards. The fractions eluting at 15–19 ml were pooled to give the main peak of activity and concd to *ca* 1 ml by ultrafiltration under N_2 (50 MPa). An Amicon membrane (YM 10) was used for this purpose in conjunction with either a Model 12 or 52 stirred cell (10 ml or 65 ml capacity, respectively). The concentrate was applied directly to a 5 ml column of Affi-Gel Blue equilibrated with 40 mM Tris–HCl buffer (pH 6.5) and washed with several vols of the equilibrating buffer at a flow rate of 3 ml/hr. When all the proteins not retained by the column had been washed through, a soln of 2 M NaCl in the same buffer was applied and 0.5 ml fractions were collected. The peak of protein that eluted was pooled and concd by ultrafiltration as before. In some separations, after overnight dialysis in the equilibrating buffer, each single fraction was tested for phosphodiesterase activity. Alternatively, the fractions comprising the peak were pooled, concd by ultrafiltration, and dialysed in an appropriate non-dissociating buffer for gel electrophoresis.

Samples of the partially purified cyclic nucleotide phosphodiesterase were tested for homogeneity under non-denaturing conditions in a 7% polyacrylamide slab and using (400 mM) Tris–acetate buffer pH 7.7. The slab was prepared as in ref. [37]. Samples containing 20–30 μg of protein in 9 μl of 30% (w/v) sucrose, were run at 25 mA for 15 hr at 4°. The running buffer was changed after 7.5 hr to prevent excessive lowering of pH. As described in the next step, one gel containing standard proteins was also run and then stained with the silver stain described by Merril *et al.* [38]. Gels were cut into 2 cm slices and each was separately homogenized and incubated overnight at 4° in 40 mM Tris–HCl buffer (pH 6.5). After centrifugation at 31 000 *g* for 30 min, the supernatant from each slice was separately concd by ultrafiltration to *ca* 1 ml and tested for cyclic nucleotide phosphodiesterase activity.

SDS-polyacrylamide gel electrophoresis. The procedure used for molecular weight determination by SDS polyacrylamide gel electrophoresis was essentially based on the methods of Weber and Osborn [39] and Davies and Stark [40] and was carried out as described in Sigma Technical Bulletin No. MWS-877. A 7% polyacrylamide gel slab (15 × 19 × 0.5 cm) was prepared and molecular weight markers were run simultaneously with the sample. Phosphate buffer (0.2 M, pH 7) was used at room temp. and a current of 35 mA was employed until the tracking dye (bromophenol blue) had reached the bottom of the gel (17 hr). Slabs were stained, as before, with silver stain [38] and the relative mobility of bands measured.

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REFERENCES

1. Newton, R. P., Gibbs, N., Moyse, C. D., Wiebers, J. L. and Brown, E. G. (1980) *Phytochemistry* **19**, 1909.
2. Newton, R. P., Kingston, E. E., Evans, D. E., Younis, L. M. and Brown, E. G. (1984) *Phytochemistry* **23**, 1367.
3. Levi, M., Chiatante, D. and Sparvoli, E. (1983) *Plant Cell Rep.* **2**, 236.
4. Levi, M., Sparvoli, E. and Galli, M. G. (1981) *Eur. J. Cell Biol.* **25**, 71.
5. Chiatante, D., Levi, M. and Sparvoli, E. (1986) *Caryologia* (in press).
6. Newton, R. P. and Brown, E. G. (1986) in *Receptors in Plants and Slime Moulds* (Chadwick, C. M. and Garrod, D. R., eds) pp. 115–153, Cambridge University Press, Cambridge.
7. Brown, E. G. and Newton, R. P. (1981) *Phytochemistry* **20**, 2453.
8. O'Neill, J. P., Schroder, C. H. and Hsie, A. W. (1975) *J. Biol. Chem.* **250**, 990.
9. Krinks, M. H., Haiech, J., Rhoads, A. and Klee, C. B. (1984) *Adv. Cyclic Nucleotide Res.* **16**, 31.
10. Klee, C. B., Crouch, T. H. and Richman, P. G. (1980) *Ann. Rev. Biochem.* **49**, 489.
11. Cheung, W. Y. (1967) *Biochemistry* **6**, 1079.
12. Helfman, D. M., Shoji, M. and Kwo, J. K. (1981) *J. Biol. Chem.* **256**, 6327.
13. Helfman, D. M., Katoh, N. and Kuo, J. F. (1984) *Adv. Cyclic Nucleotide Res.* **16**, 403.
14. Newton, R. P. and Salih, S. G. (1983) *Biochem. Soc. Trans.* **11**, 355.
15. Newton, R. P. and Salih, S. G. (1984) *Adv. Cyclic Nucleotide Res.* **17a**, 54.
16. Amrhein, N. (1974) *Z. Pflanzenphysiol.* **72**, 249.
17. Lin, P. P. C. and Varner, J. E. (1972) *Biochim. Biophys. Acta* **276**, 454.
18. Brown, E. G., Al-Najafi, T. and Newton, R. P. (1975) *Biochem. Soc. Trans.* **3**, 393.
19. Brown, E. G., Al-Najafi, T. and Newton, R. P. (1977) *Phytochemistry* **16**, 1333.
20. Brown, E. G., Edwards, M. J., Newton, R. P. and Smith, C. J. (1979) *Phytochemistry* **18**, 1943.
21. Brown, E. G., Edwards, M. J., Newton, R. P. and Smith, C. J. (1980) *Phytochemistry* **19**, 23.
22. Anderson, J. A., Charbonneau, H., Jones, H. P., McCann, R. O. and Cormier, M. J. (1980) *Biochemistry* **19**, 3113.
23. Thompson, S. T., Cass, K. H. and Stellwagen, E. (1975) *Proc. Nat. Acad. Sci. U.S.A.* **72**, 669.
24. Strada, S. J., Martin, M. W. and Thompson, W. J. (1984) *Adv. Cyclic Nucleotide Res.* **16**, 13.
25. Brown, E. G., Edwards, M. J., Newton, R. P. and Smith, C. J. (1979) *Phytochemistry* **18**, 1943.
26. Thompson, W. J., Epstein, P. M. and Strada, S. J. (1979) *Biochemistry* **18**, 5228.
27. Morrill, M. E., Thompson, S. T. and Stellwagen, E. (1978) *J. Biol. Chem.* **254**, 4371.
28. Helfman, D. M. and Kuo, J. R. (1982) *Biochem. Pharmacol.* **32**, 43.
29. Londesborough, J. (1985) *Biochem. J.* **225**, 143.
30. Anderson, T. R. (1982) *Mol. Cell Endocrinol.* **28**, 373.
31. Butcher, R. W. (1984) *Adv. Cyclic Nucleotide Res.* **16**, 1.
32. Thompson, W. J. and Appleman, M. M. (1971) *Biochemistry* **10**, 311.
33. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
34. Bradford, M. (1976) *Analyt. Biochem.* **72**, 248.
35. Leach, A. A. C. and O'Shea, P. C. (1965) *J. Chromatogr.* **17**, 245.

36. Andrews, P. (1970) in *Methods of Biochemical Analysis* (Glick, D., ed.) Vol. 18, pp. 1–53. Interscience, New York.
37. Hames, B. D. and Rickwood, D. (1981) in *Gel Electrophoresis of Proteins: A Practical Approach*, pp. 1–91. IRL Press Ltd., London.
38. Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981) *Science* **211**, 1437.
39. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.
40. Davies, G. and Stark, G. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **66**, 651.