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IMMUNOAFFINITY PURIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *LACTUCA* COTYLEDONS

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IN MEMORY OF TONY SWAIN, 1922–1987

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

Abstract—To facilitate further study of a multifunctional phosphodiesterase, previously partially purified from *Lactuca* cotyledons, a new purification step has been devised. This uses an immunoaffinity column based upon polyclonal antibodies raised against the partially purified enzyme. Preparation of the immunoaffinity column, purification of the enzyme using the new protocol, and analysis of the activity of the purified enzyme are described. The additional step produced an enzyme preparation with a significantly higher specific activity and free of nucleotidase and non-specific phosphatase activity. The observed properties of the enzyme confirm similarities with mammalian multifunctional phosphodiesterase but reaffirm the existence of two types of substrate binding site on the *Lactuca* phosphodiesterase.

INTRODUCTION

The presence of cyclic AMP and cyclic GMP, and of enzymes capable of their biosynthesis and degradation, has been established in higher plants but their functions are not yet understood (for reviews see [1, 2]). In attempts to elucidate these functions, studies of higher plant cyclic nucleotide phosphodiesterases have been carried out [3–12]. With the exception of one enzyme which was specific for purine 3′,5′-cyclic nucleotides [3, 4] and another which was specific for the hydrolysis of 2′,3′-cyclic nucleotides [9], these enzymes exhibit a broad substrate specificity which is independent of the species of base or position of the cyclic monophosphate bond [5–8, 10–12]. In some cases, however, specificity can be changed. For example, the activity of a multifunctional phosphodiesterase from potato with 2′,3′- and 3′,5′-cyclic nucleotides can be selectively inhibited by thermal alkali treatments [12]. With a *Portuluca* phosphodiesterase, the relative activity with corresponding 2′,3′- and 3′,5′-isomers is altered by allosteric regulators [7], and with a *Lactuca* phosphodiesterase there is more than one type of catalytic site, with the activity at one site affecting the activity at another [6, 11].

It is now accepted that mammalian cyclic nucleotide phosphodiesterases can be classified into five categories [13] which include the relatively non-specific enzymes that, like the plant enzymes referred to above, hydrolyse a broad range of cyclic nucleotide monophosphates irrespective of their base constituents and position of their cyclic monophosphate bonds [14–17]. A variety of mechanisms regulating the activity of mammalian phosphodiesterases have been described [18–20], different enzyme forms have been demonstrated to be present simultaneously within the same tissue [21], and in some cases the phosphodiesterase activity was associated with other enzymes, for example, nucleotidases [22, 23], in multienzyme aggregates. In view of the complexity of the mammalian phosphodiesterase system, and as a precaution against misinterpretation [18], great importance is now attached to the need for investigations of the properties of individual enzymes at their maximum practical state of purification. Recently it has been concluded [11] that the higher plant phosphodiesterase system possesses at least a similar level of complexity to that of mammals, and hence we consider that further investigations of the plant system should be subject to similar precautions.

A multifunctional phosphodiesterase from *Lactuca* cotyledons [6, 11] showing several similarities in properties to its mammalian counterpart [20], and which is capable of hydrolysing 2′,3′- and 3′,5′-purine and pyrimidine cyclic nucleotides and possessing more than one catalytic site type, has been partially purified [6, 11]. The adopted purification protocol included a final affinity chromatographic step which achieved a considerable increase in purity but the recovery was such that enzyme availability prevented further purification even though isoelectric focussing indicated that the product still contained several non-phosphodiesterase protein contaminants. To overcome these problems, a new purification procedure has been developed. This includes an immunoaffinity column stage which utilizes polyclonal antibodies raised against the partially purified phosphodiesterase. Described here is the preparation of this immunoaffinity column, the purification of the *Lactuca* enzyme using the new protocol, and analysis of the activity of the resultant purified phosphodiesterase.

RESULTS

Preincubation for one hr of the crude antibody preparation, at a dilution of 100-fold, with a 150 μg sample of

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the *Lactuca* phosphodiesterase produced a 20% inhibition in the activity of the latter and thus confirmed that the procedure for raising antisera had been successful. Fractionation of the antiserum into immunoglobulin subclasses and individual examination of each showed that IgG1, IgG2A and IgG2B were the most effective inhibitors of phosphodiesterase activity (>87%) and they were therefore selected for use in the immunoaffinity matrix.

When the *Lactuca* phosphodiesterase preparation, at the Affi-gel Blue stage of purification [6] was applied to the immunoaffinity column, a small proportion of the activity was eluted immediately (Fig. 1, peak A). The major enzymic component of the applied phosphodiesterase was retained by the immunoaffinity column but could be eluted with 2 M $MgCl_2$ (peak B) before the elution of the major band of retained proteins. Relative to the enzyme preparation at the ammonium sulphate stage of purification, the recovery of enzymic activity in peak B was 13% when assessed with cyclic AMP as substrate and 7% with cyclic GMP. A 77- or 40-fold increase in purity was observed, again depending upon choice of substrate (Table 1). The immunopurification brought about a significant change in the relative activity of the preparation towards the two substrates, the ratio of which had remained virtually constant throughout the earlier stages of purification. In a parallel purification, examination of the activities of both peaks A and B, with each of the two cyclic nucleotide substrates, indicated that peak A retained the approximately equal activities with cyclic AMP and cyclic GMP seen at the earlier stages of purification. In contrast, peak B had a greatly enhanced relative activity with cyclic AMP as substrate (Table 2). The immunoaffinity stage thus selects for a phosphodiesterase having a greater affinity for cyclic AMP. Kinetic analysis of the peak B preparation indicated K_m values comparable to those obtained by the previous method of purification [11]. The V_{max} (7 μmol/

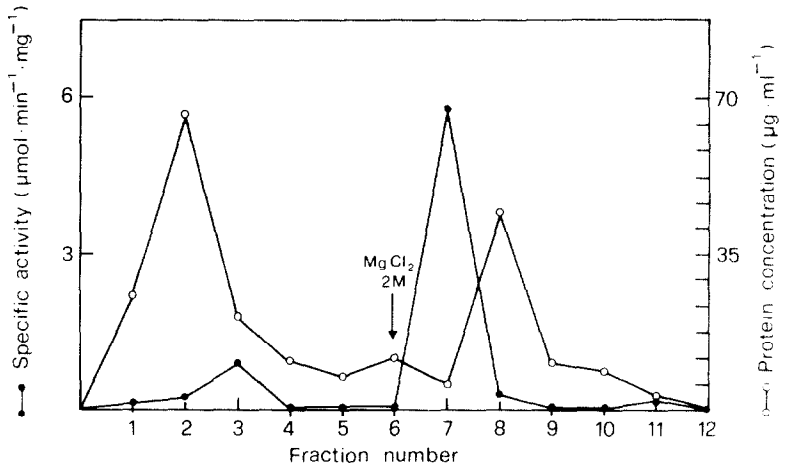

Fig. 1. Immunoaffinity chromatography of *Lactuca* cotyledon cyclic nucleotide phosphodiesterase. Partially purified extract was applied to a polyclonal 'antiphosphodiesterase' immunoaffinity column, washed with buffer and eluted with the addition of $MgCl_2$ as described in the Experimental. Fractions (0.8 ml) were collected and assayed for phosphodiesterase activity with 1.25 mM 3',5'-cyclic AMP as substrate (●). The protein concentration of each fraction was also determined (○). Fractions 2–4 constitute peak A and fractions 6–8 constitute peak B.

Table 1. Substrate specificity of *Lactuca* cyclic nucleotide phosphodiesterase at different stages of purification

	Substrates						Ratio of specific activity
	3',5'-Cyclic AMP			3'5'-Cyclic GMP			AMP/GMP
Purification step	Specific activity (nmol/min/mg)	Fold purification	% recovery	Specific activity (nmol/min/mg)	Fold purification	% recovery	
$(NH_4)_2SO_4$ ppt.	23	1	100	17	1	100	1.3
Sephadex G-200	63	3	45	64	4	51	1.0
Affi-Gel Blue	110	5	18	128	8	28	0.9
Immunoaffinity*	1768	77	13	687	40	7	2.6

*Peak B.

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

Table 2. Substrate specificity of the two peaks of cyclic nucleotide phosphodiesterase from the immunoaffinity column

Substrate	Peak A		Peak B	
	Specific activity (nmol/min/mg)	Ratio of specific activities (cAMP/cGMP)	Specific activity (nmol/min/mg)	Ratio of specific activities (cAMP/cGMP)
3′,5′-Cyclic AMP	368	1.3	1275	3.5
3′,5′-Cyclic GMP	270		362	

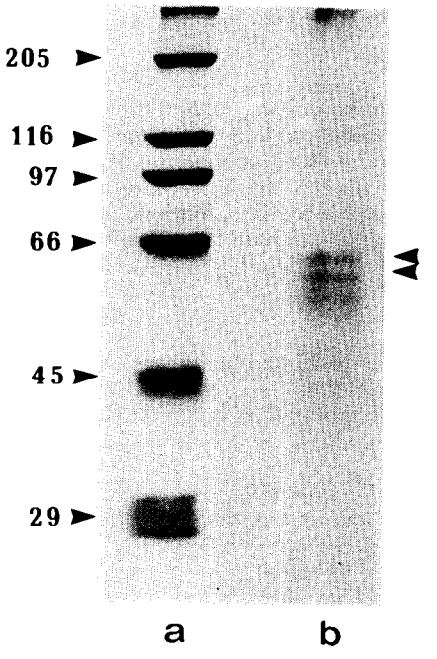

Fig. 2. Electrophoresis on SDS polyacrylamide gel (10%) of immunopurified cyclic nucleotide phosphodiesterase (lane b). Lane a is a concomitant separation of M_r makers: myosin 205 000, β-galactosidase 116 000, phosphorylase-*b* 97 000, bovine albumin 66 000, ovalbumin 45 000, carbonic anhydrase 29 000. The arrows indicate bands corresponding to M_r of 62 300 and 65 000 respectively.

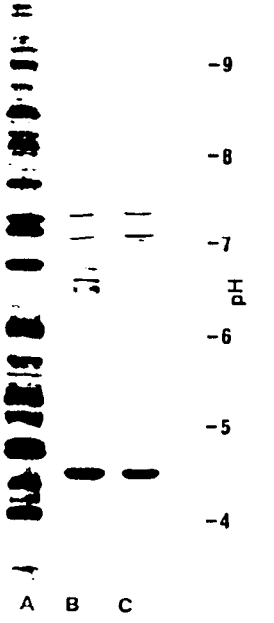

Fig. 3. Isoelectrofocussing of cyclic nucleotide phosphodiesterase preparations after immunoaffinity chromatography. Lane A is that of the protein not binding to the affinity matrix (i.e. peak A, Fig. 1). Lane B is that of the protein which binds to the matrix (i.e. peak B, Fig. 1). Lane C is that of the same protein as in Lane B but one-third of the amount was applied.

min/mg protein) was, however, an order of magnitude greater than that obtained earlier [6, 11].

SDS electrophoretic analysis of peak B showed the presence of two protein bands with apparent M_r of 61 300 and 65 000 respectively (Fig. 2). The M_r so indicated coincides with that previously reported by us [6, 11] and since several bands are evident in the preparation obtained by the previously employed affinity chromatographic procedure, the results also indicate that fewer contaminating proteins remain in the immunoaffinity preparation. Examination of peaks A and B by isoelectrofocussing demonstrated the effectiveness of the immunoaffinity column in selectively purifying the phosphodiesterase (Fig. 3). Lane A, derived from peak A, revealed the presence of at least 28 proteins in the fractions which did not bind to the immunoaffinity column. Five of these, with pI of 4.2, 4.4, 4.7, 5.3 and 8.0, respectively, contained phosphodiesterase activity. Lanes B and C, representing 15 and 5 μl samples of peak B, respectively, show a maximum of six protein bands of which only one (pI 4.5) contained any phosphodiesterase activity and which, from the staining pattern, could be seen to comprise the bulk of the total protein.

One of the proteins removed by the immunoaffinity step possesses the 5′-and 3′-nucleotidase activities; Fig. 4 shows that without exogenous 3′- and 5′-nucleotidase, only the immunopurified preparation did not release nucleoside products. Since the intermediate products of hydrolysis are stable, this preparation provides a much superior means of examining the ability of the phosphodiesterase to cleave the 2′,3′- and 5′-bonds. In general, 2′,3′-cyclic nucleotides were hydrolysed at a greater rate than 3′,5′-cyclic nucleotides, irrespective of the base present. Hydrolysis of 2′,3′-cyclic UMP and 2′,3′-cyclic

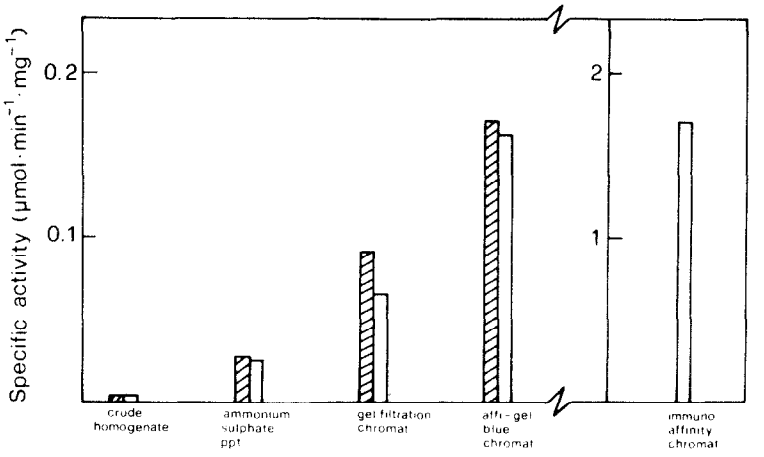

Fig. 4. Contamination by endogenous nucleotidase activities of cyclic nucleotide phosphodiesterase preparations at various stages of purification. Samples (2–750 μg protein) from the various purification stages were tested for phosphodiesterase activity toward 1.25 mM 3′,5′-cyclic AMP in presence (■) or absence (□) of exogenous nucleotidase. The assay for phosphodiesterase is a coupled assay requiring nucleotidase as the second enzyme. Unless this is present as a contaminant, it has to be supplied exogenously. Incubation conditions are described in Experimental. With the immunoaffinity-purified fraction, no activity could be detected unless nucleotidase was added.

Table 3. Hydrolysis of cyclic 2′,3′ and 3′,5′ nucleotides catalysed by the immunopurified *Lactuca* cyclic nucleotide phosphodiesterase

	Nucleoside monophosphate formed (nmol/min/mg protein)				
	2′	3′	5′	Ratio 3′/2′	Ratio 3′/5′
2′,3′-Cyclic CMP	92	171	nd	1.9	
2′,3′-Cyclic UMP	121	118	nd	1.0	
2′,3′-Cyclic GMP	35	42	nd	1.2	
2′,3′-Cyclic AMP	64	nd	nd		
3′,5′-Cyclic UMP	nd	67	66		1.0
3′,5′-Cyclic AMP	nd	22	47		0.5
3′,5′-Cyclic GMP	nd	23	30		0.8
3′,5′-Cyclic CMP	nd	19	31		0.6

nd = not detected

GMP yielded 2′- and 3′-nucleoside monophosphates in approximately equal amounts. With 2′,3′-cyclic CMP, there was an approximate 2:1 ratio in favour of the 3′-isomer, and with 2′,3′-cyclic AMP only 3′-AMP was released (Table 3). Those 2′,3′-cyclic nucleotides with a pyrimidine base were hydrolysed with greater velocity than those with a purine base. In general, hydrolysis of 3′,5′-cyclic nucleotides yielded both 3′- and 5′-products with the corresponding 5′-nucleotide as the major product in each case. Cyclic UMP was an exception in that the 3′-nucleotide was the main product. Again, greatest activity was observed when the base component of the substrate was a pyrimidine, namely uracil, although of the 3′,5′-cyclic nucleotides examined the least activity was observed with cyclic CMP.

Comparison of the intermediate products of the enzymic hydrolysis at different stages of purification of the enzyme (Fig. 5) showed that the ratio of their concentrations did change (Table 4) during the purification process. Although it has been reported [10] that pH changes can affect the relative yield of the intermediate hydrolysis products, in these experiments the pH remained constant and was not therefore instrumental in this change. It can be concluded that the changes in ratios were due either to the absence of nucleotidase activity in the immunoaffinity preparation or to the fact that a mixture of phosphodiesterases are under consideration, and that one or more of these enzymes has been selectively removed by immunoaffinity chromatography. Another possibility is that an inhibitor of cyclic AMP phosphodiesterase activity has been removed. Table 5 compares the rate of hydrolysis of cyclic nucleotide substrates by multifunctional phosphodiesterases from various animal and plant sources. The phosphodiestrase from *Pisum sativum* [5] and potato

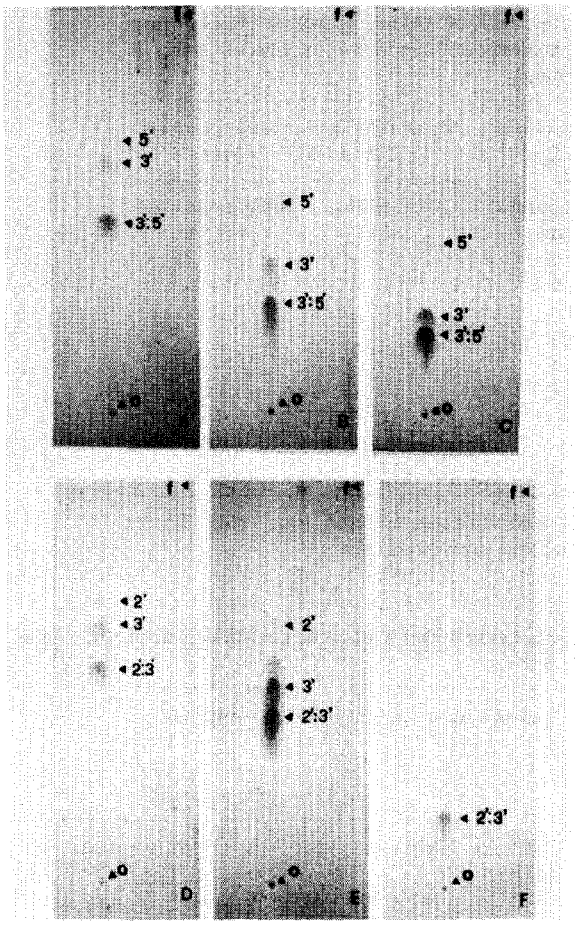

Fig. 5. TLC separations of the intermediate products of hydrolysis of different cyclic nucleotides. The plates were photographed in UV light. Cyclic nucleotide phosphodiesterase at the Affi-gel Blue chromatographic stage of purification was used to hydrolyse: A, 3′,5′-cyclic CMP; B, 3′,5′-cyclic GMP; C, 3′,5′-cyclic AMP; D, 2′,3′-cyclic CMP; E, 2′,3′-cyclic GMP; and F, 2′,3′-cyclic AMP. F and O indicate, respectively, solvent fronts and sample origins. The intermediate products from 2′,3′-cyclic AMP were present but in insufficient concentration to be recorded photographically. Incubations and TLC conditions are reported in the Experimental.

tuber [8] show similar relative orders of hydrolysis for both 2′,3′- and 3′,5′-cyclic nucleotide substrates, with no apparent preference for purine or pyrimidine derivatives. The enzyme from pig liver [17] also showed a similar order of preference for 2′,3′- and 3′,5′-substrates, but with a preference for pyrimidines. The *Lactuca* enzyme, purification of which is described above, differs from the other multifunctional phosphodiesterases in that it exhibits a significantly different rate of hydrolysis with 2′,3′-substrates than it does with 3′,5′-substrates; 2′,3′-cyclic CMP is the most rapidly hydrolysed and 3′,5′-cyclic CMP the least rapidly hydrolysed (Table 3). With the *Lactuca* phosphodiesterase, no preference towards either purine or pyrimidine derivatives is evident with 3′,5′-cyclic nucleotides but with 2′,3′-substrates those with a pyrimidine base are favoured.

DISCUSSION

The activity obtained with the *Lactuca* phosphodiesterase preparation described above indicates the success of including a polyclonal antibody column in the purification protocol. This additional step produced an enzyme preparation with a higher specific activity than previously obtained [6, 11] and one which contains fewer protein contaminants. Comparison of the isoelectrophoretogram of peak B with that previously obtained after affinity chromatography on Affi-Gel Blue [11] showed that both of the protein bands exhibiting phosphodiesterase activity had a pI of 4.5 but in contrast to the six bands apparent in peak B after immunoaffinity chromatography, a much larger number of proteins were in evidence after affinity chromatography on the less specific Affi-Gel Blue matrix [11]. A major benefit of the enzyme preparation after immunoaffinity chromatography is the absence of nucleotidase and non-specific phosphatase activity. This facilitates a more effective examination of the isomeric products of phosphodiesterase activity with both 2′,3′- and 3′,5′-cyclic nucleotide substrates. In some respects the *Lactuca* phosphodiesterase is similar to other plant and mammalian multifunctional phosphodiesterases, for example each hydrolyses 2′,3′-cyclic nucleotides more rapidly than their corresponding 3′,5′-isomers. There are however significant differences. For example, with the mammalian enzyme [17] affinity for the 2′,3′- and 3′,5′-cyclic nucleotides was found to depend upon the nature of the base as well as upon the positions of the cyclic phosphate bonds, and the similarity of the relative hydrolysis rates with the 2′,3′- and 3′,5′-isomers of several purine and pyrimidine cyclic nucleotides has been interpreted as an indication of a single active site. In contrast, our previous kinetic data [11] for *Lactuca* phosphodiesterase suggested that more than one catalytic site existed and that each catalytic site could be specific for the hydrolysis of a single substrate. The data presented here, in which the enzyme displays a different order of relative hydrolysis rates with purines and pyrimidines dependent upon the 2′,3′- or 3′,5′-linkage of the phosphate residue, further support this hypothesis, suggesting that the 2′,3′- and 3′,5′-cyclic nucleotide catalytic sites exist as two different types of site, which would account for the different K_i values obtained with 2′,3′- and 3′,5′-cyclic nucleotides and the mixed type inhibition observed [11]. If this is the case, then the *Lactuca* phosphodiesterase would be unique amongst the multifunctional phosphodiestdrases thus far reported. Further investigation of the mechanism of recognition of the cyclic nucleotide isomer and of the properties of this enzyme should yield valuable information concerning its function, which in turn should give further indication of the physiological significance of cyclic nucleotides in higher plants.

EXPERIMENTAL

Materials. Radioactively labelled cyclic nucleotides and other chemicals were obtained from the sources previously listed [6, 11]. Seeds of *Lactuca sativa* cv Arctic King (C. Sharpe and Co. Ltd, Sleaford, Lincs) were surface sterilized, germinated and their cotyledons detached and homogenized at 4° as described earlier [6, 11]. CNBr-activated Sepharose 4B was obtained from Pharmacia; Sepharose CL4B-Protein A and Freund's complete adjuvant were purchased from Sigma.

Table 4. Analysis of the molar ratio of intermediate products of enzymic hydrolysis obtained from various cyclic nucleotide substrates with the phosphodiesterase preparation at different stages of purification

Substrates	Molar ratio of hydrolysis products 3′/2′ monophosphate		Molar ratio of hydrolysis products 3′/5′ monophosphate	
	Affi-Gel Blue stage	Immunopurified enzyme	Affi-Gel Blue stage	Immunopurified enzyme
2′,3′ Cyclic CMP	2.0	1.9	—	—
2′,3′ Cyclic UMP	2.6	1.0	—	—
2′,3′ Cyclic GMP	2.3	1.2	—	—
2′,3′ Cyclic AMP	1.4	nd	—	—
3′,5′ Cyclic UMP	—	—	2.5	1.0
3′,5′ Cyclic AMP	—	—	3.0	0.5
3′,5′ Cyclic GMP	—	—	2.2	0.8
3′,5′ Cyclic CMP	—	—	2.8	0.6

nd = Not detected. The absolute amounts to which these ratios refer are taken from Table 3 for the immunopurified enzyme and from [11] for the Affi-Gel Blue-purified enzyme

Table 5. Comparison of the substrate specificity of the *Lactuca* phosphodiesterase with cyclic nucleotide phosphodiesterases from other sources

Source	Reference	Relative rate of hydrolysis of various cyclic nucleotide substrates %							
		2′,3′-Cyclic nucleotide				3′,5′-Cyclic nucleotide			
Pisum sativum	[5, 14]	UMP (100)	AMP (83)	GMP (51)	CMP (26)	UMP (100)	AMP (60)	GMP (38)	CMP (34)
Potato tuber	[8, 12]		AMP (100)	CMP (44)	GMP (20)		AMP (100)	CMP (100)	GMP (80)
Pig liver	[17, 20]	CMP (100)	UMP (46)	GMP (28)	AMP (24)	CMP (100)	UMP (29)	AMP (25)	GMP (19)
*Lactuca sativa**		CMP (100)	UMP (91)	GMP (29)	AMP (24)	UMP (100)	AMP (52)	GMP (40)	CMP (38)

* Present studies.

For such enzyme, relative rates of hydrolysis of the 2′,3′-isomers are expressed as a percentage of the rate with the preferred 2′,3′-substrate. Similarly, for each enzyme with a 3′,5′-substrate, relative rates are based on the rate with the preferred 3′,5′-substrate.

Extraction and partial purification electrophoresis of cyclic nucleotide phosphodiesterase. The phosphodiesterase activity was extracted from excised *Lactuca* cotyledons and partially purified by fractional precipitation with ammonium sulphate, followed by gel filtration, Affi-gel Blue affinity chromatography and non-denaturing gel electrophoresis. Details of the extraction and purification procedure have been previously described [6].

Preparation and purification of anti-phosphodiesterase sera. Purified cyclic nucleotide phosphodiesterase (0.7 mg protein) was dissolved in 2 ml of sterile saline solution and emulsified with 9 volumes of Freund's complete adjuvant. Aliquots (200 μl) of the emulsion were used to immunize 10 female 10-week-old Swiss white mice according to the protocol suggested in [24]. The mice were injected intraperitoneally and booster injections were given 14, 21, 28 and 35 days after the intial dose. After 50 days, the animals were killed by decapitation and a pooled sample (5 ml) of blood collected. The serum was immediately separated according to the procedure of [25].

Before purification, a sample of antiserum was tested for its ability to inhibit *Lactuca* phosphodiesterase. This was done by incubating the antiserum sample, at a dilution of 100-fold, for 1 hr with the enzyme and then applying the standard enzyme assay protocol. The antiserum (5 ml) was then fractionated into subclasses of IgG by affinity chromatography at 4° [25]. The affinity column consisted of a matrix of Protein A coupled to Sepharose CL-4B and was of 1 ml total volume. Immunoglobulin subclasses were dialysed overnight against 40 mM Tris–HCl buffer at pH 6.5 then tested for their ability to inhibit the *Lactuca* cyclic nucleotide phosphodiesterase. The enzyme preparation taken for this purpose was at the Affi-gel Blue stage of purification and the standard assay was used following 2 hr preincubation at 4° with the individual IgG fractions. The immunoglobulin subclasses IgG1, IgG2A and IgG2B, which were the most effective inhibitors, were pooled to yield an immunoglobulin fraction of 4.3 mg. This was used for production of an immunoaffinity matrix.

Preparation of immunoaffinity column. Using the procedure recommended by the supplier, CNBr-activated Sepharose 4B (1.5 ml) was coupled with the pooled immunoglobulin described above. The resultant coupled gel (4.5 ml) was transferred to a glass chromatography column (5 × 0.5 cm diameter) and equilibrated, by washing, with 5 ml of 20 mM Tris–HCl buffer at pH 7.5.

Immunopurification of Lactuca sativa cyclic nucleotide phosphodiesterase. Cyclic nucleotide phosphodiesterase (250 μg protein in 0.5 ml 40 mM Tris–HCl, pH 6.5) purified to the Affi-gel

Blue chromatographic stage [6] was loaded onto the equilibrated immunoaffinity column. The latter was washed with 5 ml of 20 mM Tris–HCl (pH 7.5) and fractions of 0.8 ml collected. Attempts to elute the bound enzyme were made with 2 M NaCl, 2 mM EGTA and 2 M $MgCl_2$ in the same buffer. Elution and recovery was achieved using 5 ml of 2 M $MgCl_2$ in 20 mM Tris–HCl pH 7.5 and collecting 0.8 ml fractions. This procedure was adopted for routine use.

Assay of cyclic nucleotide phosphodiesterase activity. The assay procedure used routinely in this work was essentially that of ref. [6] with minor modifications. Enzyme samples containing 0.8–100 mg of protein were assayed in a reaction mixture containing 0.025 μCi of the appropriate [8-^{3}H]-labelled nucleotide. The composition of the incubation medium and incubation conditions were as previously described [6, 11]. The radioactivity of the incubation products (0.5 ml samples) was measured by liquid scintillation counting in 5 ml portions of Optiphase RIA scintillation fluid (LKB, South Croydon, U.K.); counting efficiency was 28%.

Determination of protein concentrations. Protein concentrations were measured by the Lowry method [26].

Analysis of the products of hydrolysis. The standard reaction mixture consisted of 5 mM Tris–HCl buffer (pH 6.5), containing 0.1 mM $CaCl_2$, 0.1 mM $FeCl_2$, 0.1 mM $MgCl_2$, 4–75 μg of enzyme preparation and 0.4 mM cyclic nucleotide substrate. After incubation at 30° for 25 min, the reaction was stopped by rapid freezing in liquid N_2 followed by freeze-drying. The freeze-dried material was resuspended by addition of 20 μl of distilled water and then chromatographed by TLC on cellulose layers according to the method of [17]. Spots were visualized under UV light and those cochromatographing with standards scraped off and eluted in 1.5 ml of 0.01 M HCl. After removing cellulose by filtration the reaction products were quantified by UV spectrophotometry.

Ultrafiltration. Protein preparations were concentrated by ultrafiltration under N_2 (50 MPa) through an Amicon YM 10 membrane used in either a model 12 or 52 stirred cell (10 or 65 ml capacity, respectively).

SDS-polyacrylamide gel electrophoresis. The procedure used routinely for M_r determination by SDS polyacrylamide gel electrophoresis was based on the methods of [26, 27]. Samples (16.9 μg protein) of enzyme preparation and M_r markers were applied to a 10% polyacrylamide gel slab (20 cm × 15 cm × 0.1 cm). Electrophoresis was carried out at 4° with a constant current of 50 mA until the tracking dye (Bromophenol Blue) had reached the bottom of the gel slab (*ca* 17 hr). The gels were stained for 4 hr in a 0.025% aqueous solution of Coomassie Blue containing 25% isopropanol and 10% HOAc. They were destained in 10% HOAc and the relative mobility of bands calculated.

Isoelectrofocussing. Protein samples (5–20 μl) from both peak A and peak B were applied as bands on commercially prepared layers (LKB Ltd, Croydon, UK) of polyacrylamide gel containing ampholyte with a pH range of 3.5–9.5. After isoelectrofocussing the gel was cut in half lengthwise and the separated proteins on one half were fixed and stained as previously described [11]. The replicate half of the gel was cut into pieces and the phosphodiesterase activity in each band determined as before [11].

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