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PURIFICATION AND CHARACTERIZATION OF A CYCLIC NUCLEOTIDE-REGULATED 5′-NUCLEOTIDASE FROM POTATO

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Summary

A procedure is presented for the rapid purification of a 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) from potato tubers, involving ammonium sulphate fractionation and chromatography on phosphocellulose, DEAE-cellulose and Sephadex G-75. Application of this procedure results in a 6000-fold purification of the 5′-nucleotidase and the final preparations are virtually homogeneous, yielding only one protein band on electrophoresis in polyacrylamide gels in non-dissociating or dissociating conditions. The 5′-nucleotidase has a molecular weight of 50 000 from gel filtration experiments. Sodium dodecylsulphate-polyacrylamide gel electrophoresis of the purified 5′-nucleotidase reveals one major band of molecular weight 25 000.

The 5′-nucleotidase is competitively inhibited by cyclic nucleotides, having micromolar K_i values for cyclic AMP and cyclic GMP at pH 5.0 and pH 8.0. The enzyme has a pH optimum of 5.0 with 5′-GMP as substrate. While 5′-AMP and 3′-AMP are hydrolyzed at comparable rates at pH 5.0, at pH 8.0 the rate of hydrolysis of 3′-AMP is only 4% of that with 5′-AMP. ADP, ATP and 2′-AMP are very poor substrates for the enzyme. The nucleotidase has micromolar K_m values for nucleoside 5′-monophosphates other than 5′-NMP. A wide variety of divalent cations activate the 5′-nucleotidase.

Introduction

There is mounting evidence for a growth-regulatory role of cyclic nucleotides in higher plants (for references see Polya [1], Bachhofen [2] and Lin [3]) and direct evidence for the occurrence of cyclic AMP in plants [3—12], although apparent demonstrations of the existence of adenyl cyclase in cell-free plant extracts [13,14] are not unambiguous [15]. Various phosphodiesterases in higher plants that can hydrolyze cyclic nucleotides have been resolved al-

though the physiological significance and specificities of these enzymes remain to be determined [3]. At present little is known about specific sites of action of cyclic nucleotides in higher plants. No evidence has been obtained as yet for the presence in higher plants of cyclic nucleotide-activated protein kinases of the kind found in animal cells [16], although protein kinases that are not activated by cyclic nucleotides have been demonstrated in higher plants [17,18].

Two highly specific sites of action of cyclic nucleotides in higher plants have been demonstrated. Cyclic GMP (but not cyclic AMP) stimulates polypeptide synthesis in a cell-free system from wheat embryo and acts at micromolar concentrations to promote binding of GTP to the translation elongation factor I [19]. A 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) has been highly purified from wheat seedlings in this laboratory and shown to be competitively inhibited by various cyclic nucleotides including cyclic AMP and cyclic GMP [1,20]. This 5′-nucleotidase has micromolar K_i values for cyclic nucleotides and has micromolar K_m values for 5′-AMP and 3′-AMP at pH 5.0.

In order to purify and characterize this type of 5′-nucleotidase from higher plants, potato tuber was chosen as a convenient bulk source for extraction of the enzyme. Partial purifications of the potato 5′-nucleotidase have been reported [21—24] but the best purifications were only 200—400-fold [22,23]. This paper describes the 6000-fold purification of the potato 5′-nucleotidase to homogeneity and physical and kinetic properties of the enzyme.

Methods

Enzyme assays

All assays were routinely made at 30° C. 5′-Nucleotidase was assayed routinely in a medium (1 ml) containing 1 mM substrate in 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. With *p*-nitrophenylphosphate as substrate, the reaction was terminated by addition of 2 ml of 0.1 M NaOH and the *p*-nitrophenol estimated from the absorbance at 400 nm [25]. With other phosphomonoesters as substrates reactions were terminated by addition of 12% perchloric acid and P_i was measured by the method of Allen [26]. ATPase and ADPase were measured by phosphate release in media containing 1 mM ATP, 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$ or 1 mM ADP, 0.1 M Tris (Cl^-, pH 8.0) and 4 mM $MgCl_2$. Phosphodiesterase activity was determined from release of *p*-nitrophenol employing bis-(*p*-nitrophenyl)-phosphate as substrate at 1 mM concentration in the standard reaction medium at pH 5.0 (0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$). Cyclic AMP phosphodiesterase was determined in a 100-μl medium containing 1 mM cyclic AMP, 0.1 M acetate (Na^+, pH 5.0), 4 mM $MgCl_2$ and 5 μCi/ml cyclic [8-^{3}H] AMP (spec. act. 20.7 Ci/mmol). Reaction products were resolved by chromatography on polyethyleneimine thin-layer plates developed in a solvent system containing 5% borate and 0.5 M ammonium acetate (pH 7.5). The 5′-AMP cyclic AMP and adenosine spots were detected under ultraviolet light, cut out and counted at 5% efficiency using a scintillation fluid containing 0.5% PPO and 0.03% POPOP in toluene. Poly-

ethyleneimine thin layers were prepared according to the procedure of Randerath and Randerath [27]. Rates of phosphomonoester hydrolysis were linear functions of protein concentration under the conditions used. Assay conditions were chosen so that less than 10% of substrate was hydrolyzed and phosphate ester hydrolysis was a linear function of time. All assays were corrected by use of appropriate zero time controls. Protein was determined using Folin reagent and employing crystalline bovine serum albumin as a standard [28].

Gel electrophoresis

Non-dissociating polyacrylamide gel electrophoresis was carried out in disk gels (9 × 0.8 cm) containing 5% acrylamide, 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. The upper and lower buffer reservoirs contained 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. Polyacrylamide gels were pre-electrophoresed to remove persulphate and samples were electrophoresed at 4° C for 15 h at a current of 2—3 mA per disk gel. Protein bands were stained for 5 h with 1% Coomassie Brilliant Blue in a solution, subsequently used for destaining, containing methanol/acetic acid/glycerol/water (5 : 1 : 1 : 17, v/v). Phosphatase was detected using a staining solution containing 0.1% α-naphthylphosphate, 0.1% Fast Red TR, 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$.

Dissociating polyacrylamide gel electrophoresis was carried out according to the procedure of Shapiro et al. [35], employing disk gels (9 × 0.8 cm) containing 5% acrylamide, 0.1% sodium dodecylsulphate and 0.1 M sodium phosphate (pH 7.1). Gels were pre-electrophoresed to remove persulphate. Samples (10—100 μg of protein) were denatured by heating for 5 min at 90—100° C in a medium containing 1% sodium dodecylsulphate, 0.1 M sodium phosphate (pH 7.5) and 1% 2-mercaptoethanol. Electrophoresis was carried out at room temperature at 5 mA per disk gel over 6 h. Gels were washed for 10 h in 12.5% trichloroacetic acid and then in destaining solution (see above) to remove sodium dodecylsulphate before staining and destaining as described above.

Starch gel electrophoresis was carried out employing slab gels (20 × 12 cm) containing 10% electrostarch in the appropriate buffer (ionic strength approx. 0.1). The same buffer used for preparation of the starch gel was also routinely used in the cathode and anode buffer reservoirs. Samples (30 μl) were introduced into slots formed by the gel mould and electrophoresis was carried out at 4° C for 12 h with currents of approx. 20 mA per gel. Gels were sliced into equal halves and phosphatase was detected on the starch gels as described above in the presence or absence of 0.1 mM cyclic AMP. 5′-AMPase activity was detected by the Gomori staining procedure. Starch gels were washed at 4° C in 0.1 M acetate (Na^+, pH 5.0) containing 4 mM $MgCl_2$ and then incubated with 1 mM 5′-AMP in the same buffer containing 0.1 mM $Pb(NO_3)_2$. The zone of lead phosphate precipitated in the region of the 5′-nucleotidase was detected as a black precipitate of lead sulphide on addition of 0.1% $(NH_4)_2S$.

Molecular weight determinations

The molecular weight of the native 5′-nucleotidase was determined by gel filtration on a Sephadex G-75 column (2 × 64 cm) employing γ-globulin, cytochrome *c*, myoglobin, ovalbumin, bovine serum albumin and Blue Dextran

2000 as standards. The column was eluted at the rate of 0.25 ml/min with a buffer containing 0.1 M KCl, 10 mM Tris (Cl^-, pH 8.0), 0.1 mM EDTA and 0.03% 2-mercaptoethanol.

The subunit molecular weight was determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis employing horse cytochrome *c*, bovine serum albumin and γ-globulin as standards as described by Shapiro et al. [29].

Materials

Potato tubers, grown in the Kinglake district of Victoria, were purchased locally and stored at 4°C before processing. Phosphate esters, Coomassie Brilliant Blue and Fast Red TR were obtained from the Sigma Chemical Company; acrylamide, *N,N,N',N'*-tetramethylethylenediamine and *N,N'*-methylenebisacrylamide from Eastman Kodak Company; and phosphocellulose (Cellex-P, 0.93 mequiv/g) and DEAE-cellulose (Cellex-D, 0.67 mequiv/g) were obtained from Calbiochem. Phosphocellulose and DEAE-cellulose were prepared for use by washing in 0.5 M NaOH or 0.5 M HCl respectively and then in 0.5 M HCl or 0.5 M NaOH respectively. After washing in water the ion-exchanger suspensions were adjusted to pH 8.0. Cyclic [8-^{3}H] AMP was obtained from the Radiochemical Centre, Amersham, Great Britain. All other reagents were of analytical reagent grade.

Purification of the 5'-nucleotidase

All steps of the purification were carried out at 4°C unless otherwise specified. Cyclic AMP-sensitive 5'-nucleotidase was conveniently determined by measuring the *p*-nitrophenylphosphatase activity inhibited by addition of 0.1 mM cyclic AMP at pH 5.0 as described above. The procedure described below is a rapid protocol refined from numerous purification attempts and has been repeatedly and successfully reproduced.

Potato tubers were stored at 4°C prior to processing. Tubers (2—5 kg) were peeled, washed and cut into segments approx. 5 × 3 × 2 cm. These segments were homogenized in a National fruit juice extractor and the juice immediately brought to 50 mM Tris (Cl^-, pH 8.0), 1 mM EDTA and 0.1% 2-mercaptoethanol. The juice was allowed to stand for 5 min to permit a starchy precipitate to settle and then filtered through Miracloth to remove much of the remaining starchy material. A phosphocellulose suspension at pH 8.0 (equivalent to 1 g dry weight of Cellex-P per 1 kg of potato extracted) was added to the filtered homogenate and the suspension stirred and then filtered on a Buchner funnel. The filtrate was discarded and the phosphocellulose was resuspended in 500 ml of Buffer A (10 mM Tris (Cl^-, pH 8.0), 1 mM EDTA and 0.03% 2-mercaptoethanol) and collected again on a Buchner funnel. The phosphocellulose was then washed twice in this fashion with 500 ml of 0.1 M ammonium sulphate in Buffer A and then washed twice with 250 ml of 0.5 M ammonium sulphate in Buffer A to remove the cyclic AMP-sensitive 5'-nucleotidase. The filtrate containing 5'-nucleotidase was centrifuged at 30 000 × *g* for 10 min to remove a slight haze and the supernatant then brought to 100% ammonium sulphate saturation. The resulting precipitate was collected by centrifuging at 30 000 × *g* for 10 min and dissolved in about 10 ml of Buffer B

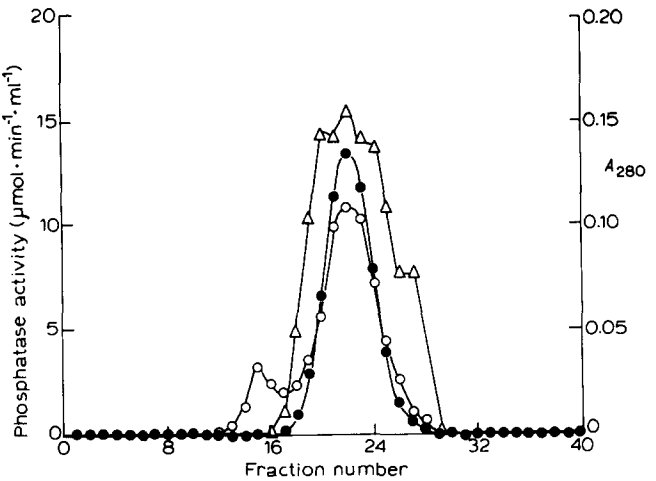

Fig. 1. Final gel filtration of the highly purified 5′-nucleotidase on Sephadex G-75. The 5′-nucleotidase was eluted in Buffer B as described in Methods. ●———●, *p*-nitrophenylphosphatase (μmol/min per ml); ○———○, A_{280nm}; △———△, ratio of *p*-nitrophenylphosphatase/A_{280nm}.

(0.5 M ammonium sulphate in Buffer A). This solution was applied to a Sephadex G-75 column (2 × 64 cm) equilibrated with Buffer B, and eluted with Buffer B at the rate of 0.2 ml/min. The active fractions were pooled (20 ml) and dialyzed against 1 l of Buffer A with two changes for 4 h. The dialyzed solution was applied to a DEAE-cellulose (Cellex-D, 0.67 mequiv/g) column (1.5 × 6 cm) equilibrated with Buffer A. The 5′-nucleotidase is not retained by DEAE-cellulose in these conditions and was washed off with Buffer A. The active fractions were pooled, brought to 0.5 M ammonium sulphate concentration and then concentrated to about 3 ml using an Amicon pressure-pack pressure filtration device with a UM-10 membrane. The concentrated solution containing 5′-nucleotidase was applied to a Sephadex G-75 column equilibrated with Buffer B and eluted with Buffer B at flow rate of 0.2 ml/min. This final elution is shown in Fig. 1. The constant specific activity fractions were pooled and checked for homogeneity electrophoretically. A representative purification schedule is presented in Table I.

TABLE I

PURIFICATION OF THE CYCLIC AMP-SENSITIVE 5′-NUCLEOTIDASE

Purification of the cyclic AMP-sensitive 5′-nucleotidase was followed by assaying *p*-nitrophenylphosphatase at 30°C in the presence or absence of 0.1 mM cyclic AMP in an assay medium containing 1 mM *p*-nitrophenylphosphate, 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. 5′-Nucleotidase activity is expressed in terms of *p*-nitrophenylphosphatase activity inhibited by 0.1 mM cyclic AMP.

Fractionation	Total 5′-nucleotidase (μmol/min)	Total protein (mg)	Specific activity (μmol/min per mg of protein)
Filtered homogenate	875	34506	0.025
Phosphocellulose	565	90	6.3
Sephadex G-75 (1)	229	8.6	26.6
DEAE-cellulose	106	2.2	48
Sephadex G-75 (2)	73	0.5	145

Highly purified (but not homogeneous) preparations of the 5′-nucleotidase were used for some kinetic studies (see Tables IV and V) while attempts at final purification were in progress. The purification procedure involved steps similar to those described above but with the inclusion of chromatography on an agarose (A 0.5, mesh 50—100) column (2 × 70 cm) in Buffer A. The 5′-nucleotidase was retarded on this column and was eluted with the salt peak. The 5′-nucleotidase preparations from this procedure were purified approx. 2000-fold and were kinetically indistinguishable from homogeneous preparations of the enzyme.

Results

Purification of 5′-nucleotidase from potato tubers

The purification schedule described above yields preparations with a specific activity at pH 5.0 of 145 μmol/min per mg of protein (in terms cyclic AMP-inhibited *p*-nitrophenylphosphatase at pH 5.0) and 193 μmol/min per mg of protein (with 5′-AMP as substrate). Starch gel electrophoresis of the crude filtered homogenate at pH 5.0 and staining for phosphatase activity as described in Methods reveals only one phosphatase band that is sensitive to addition of cyclic AMP to the enzyme-staining reagent. This band has the same mobility as the purified enzyme. Accordingly the overall purification of the enzyme can be calculated from the data in Table I. The final purification was 5800-fold with respect to the filtered homogenate. The absolute amount of 5′-nucleotidase present in potato tubers can be calculated from the data in Table I to be approx. 1 mg/kg wet weight.

Immediate treatment of the filtered homogenate with phosphocellulose and elution of proteins from the ion exchanger by Buchner filtration results in 4—5-fold better yields of the 5′-nucleotidase than procedures originally used involving more conventional application to and elution from phosphocellulose columns. This filtration procedure also results in a very rapid 250-fold purification of the 5′-nucleotidase since 5 kg potato tubers can be processed past the phosphocellulose stage in this way in several hours.

The extensive washing of the phosphocellulose with 0.1 M ammonium sulphate in Buffer A removes several phosphodiesterases than can utilize cyclic nucleotides as substrates at low pH. After phosphocellulose treatment, the phosphatase activity is completely inhibited by cyclic AMP. Gel filtration of the phosphocellulose-purified 5′-nucleotidase was carried out at high ionic strength (0.5 M $(NH_4)_2SO_4$ in Buffer A) in order to prevent association of the enzyme with other proteins. When this step is carried out at lower ionic strength (e.g. 0.1 M KCl in Buffer A), 5′-nucleotidase is distributed throughout the elution profile as compared with elution of the enzyme as one sharp peak of activity at high ionic strength (0.5 M ammonium sulphate in Buffer A). This gel filtration step eliminates major protein contaminants of higher and lower molecular weight than the 5′-nucleotidase. The subsequent passage through DEAE-cellulose is required to remove a highly active ATPase from the preparation. This contaminant has the same molecular weight as the 5′-nucleotidase and thus cannot be removed by gel filtration. However the ATPase binds to DEAE-cellulose at pH 8.0 whereas the 5′-nucleotidase is not bound. This ATP-

ase hydrolyzes ADP and ATP at comparable rates at pH 8.0 and corresponds to the previously reported "low-ratio" potato apyrase [30]. A final passage through Sephadex G-75 removes the residual major high-molecular-weight contaminant and provides the bulk of the 5′-nucleotidase in fractions of approximately constant specific activity (Fig. 1).

Purity of the 5′-nucleotidase

The final Sephadex G-75 gel filtration step yields a one-to-one correspondence between peaks of cyclic AMP-sensitive phosphatase activity and absorbance at 280 nm. The peak 5′-nucleotidase fractions have specific activities in the range of 174–193 μmol 5′-AMP hydrolyzed/min per mg of protein from which one can estimate a purity of about 95%. Electrophoresis of the purified enzyme in 5% polyacrylamide gels in 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$ yields one band of cyclic AMP-sensitive phosphatase activity coincident with one protein band; no other protein bands were observed. After electrophoresis of the purified 5′-nucleotidase in starch gels in 0.1 M acetate (Na^+, pH 5.0) containing 4 mM $MgCl_2$, only one band of cyclic AMP-sensitive α-naphthylphosphatase is detected. This band is coincident with 5′-nucleotidase activity detected using 5′-AMP as substrate and the Gomori stain for P_i as described under Methods. No other 5′-nucleotidase bands are present. A further criterion for the purity of the 5′-nucleotidase was obtained from dissociating polyacryl-

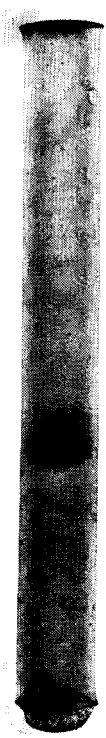

Fig. 2. Dissociating polyacrylamide gel electrophoresis of the purified 5′-nucleotidase. 50 μg of purified 5′-nucleotidase was denatured and electrophoresed in a 0.1% sodium dodecylsulphate/5% polyacrylamide gel as described in Methods.

amide gel electrophoresis in the presence of 0.1% sodium dodecylsulfate. Only one major protein band is observed (Fig. 2). As discussed below, the molecular weight of this polypeptide is 25 000 i.e. half the native molecular weight. In some preparations a minor protein contaminant with a molecular weight of 38 000 can be detected on dissociating gel electrophoresis but a further high-resolution gel filtration on Sephadex G-75 removes this trace contaminant.

The presence of enzyme contaminants in the purified 5′-nucleotidase preparations was checked. 3′-Nucleotidase, a persistent contaminant of previous potato 5′-nucleotidase preparations (e.g. see Klein [23]) and ATPase [30] are removed in the purification. Phosphodiesterase activity of the purified 5′-nucleotidase, measured at pH 5.0 with 1 mM bis-(*p*-nitrophenyl)-phosphate or 1 mM cyclic AMP as described in Methods, is less than 0.0005 of the 5′-nucleotidase activity measured with 1 mM 5′-AMP in the same conditions.

Molecular weight determinations and subunit composition

The molecular weight of the 5′-nucleotidase was determined by gel filtration on a calibrated Sephadex G-75 column. In the conditions used (the column was eluted with 0.1 M KCl in Buffer A at 4° C) the molecular weight is 50 000. An additional confirmation of this result derives from the coincidence of peaks of "low-ratio" apyrase ATPase and cyclic AMP-sensitive phosphatase on gel filtration through Sephadex G-75 at high ionic strength in Buffer B. The "low ratio" apyrase is composed of one polypeptide with a molecular weight of 50 000 (Polya, unpublished data). Dissociating sodium dodecylsulphate-polyacrylamide gel electrophoresis experiments with the purified 5′-nucleotidase revealed only one major protein band (Fig. 2). The molecular weight of this polypeptide was determined by calibrating sodium dodecylsulphate-polyacrylamide gels with standard proteins of known subunit composition and known subunit molecular weight (Fig. 3). The molecular weight of the polypeptide is 25 000, indicating that the 5′-nucleotidase is composed of two polypeptides of the same molecular weight.

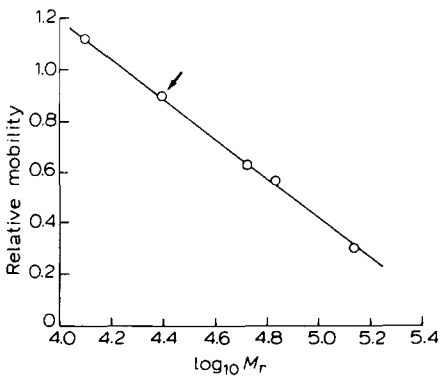

Fig. 3. Semilog plot of molecular weight against relative migration rate. The position of the 5′-nucleotidase polypeptide is arrowed. The polypeptide standards included bovine serum albumin, heavy and light chains of γ-globulin and the dimer and monomer of horse heart cytochrome *c*. Samples (20—100 μg) of purified 5′-nucleotidase and standards were denatured and electrophoresed in 0.1% sodium dodecylsulphate/5% polyacrylamide gels as described in Methods.

Electrophoretic behaviour of the 5′-nucleotidase

The 5′-nucleotidase is a basic protein binding tightly to phosphocellulose at pH 8.0 but not to DEAE-cellulose under the same conditions. The 5′-nucleotidase has approximately half the mobility of cytochrome *c* (towards the cathode) in polyacrylamide gel electrophoresis in 0.1 M acetate (Na^+, pH 5.0) in the presence of 4 mM $MgCl_2$. If electrophoresis is carried out in these conditions but in the absence of $MgCl_2$, the 5′-nucleotidase aggregates and most of the enzyme remains at the origin. The 5′-nucleotidase behaves similarly on electrophoresis in starch gel. Attempts to determine the isoelectric point of the 5′-nucleotidase by isoelectric focussing were unsuccessful but the isoelectric point can be crudely estimated to be in the pH range 8–9 from the direction of movement of the enzyme on starch gel electrophoresis at various pH values.

Stability of the 5′-nucleotidase

The 5′-nucleotidase was relatively stable even in very dilute solution (down to 10 μg/ml) when stored in Buffer A or Buffer B at 4° C. For example, storage of 10 μg/ml 5′-nucleotidase in Buffer A at 4° C resulted in loss of 37% of initial activity over 3 weeks. The 5′-nucleotidase is heat-labile; over 98% of activity is lost by incubation of the enzyme for 5 min at 50° C in Buffer A.

Competitive inhibition of the 5′-nucleotidase by cyclic nucleotides

The 5′-nucleotidase is competitively inhibited by cyclic AMP and cyclic GMP. Plots of reciprocal velocity ($1/v$) against reciprocal of substrate concentration (1/S) in the presence or absence of these cyclic nucleotides have common intercepts on the $1/v$ axis. In addition, there is a linear relation between reciprocal velocity and cyclic nucleotide inhibitor concentration at a given substrate concentration. The 5′-nucleotidase has micromolar K_i values for both cyclic nucleotides at pH 5.0 and pH 8.0 (Table II).

Substrate specificity

The 5′-nucleotidase has pH optima of 4.2 and 5.0 at 30° C when assayed in the standard medium with *p*-nitrophenylphosphate or 5′-GMP, respectively, as substrates (Fig. 4). While maximal rates of hydrolysis of both substrates are similar at pH 5.0, no hydrolysis of *p*-nitrophenylphosphate occurs at pH 7.0 while the rate of hydrolysis of 5′-GMP at pH 7.0 is 50% of that at pH 5.0.

TABLE II

COMPETITIVE INHIBITION OF THE POTATO 5′-NUCLEOTIDASE BY CYCLIC NUCLEOTIDES

K_i values were determined using *p*-nitrophenylphosphate or ribose 5′-phosphate as substrates at pH 5.0 or pH 8.0 as described in Methods. Standard errors for K_i values are indicated. These were computed by the least-squares method assuming competitive kinetics.

Nucleotide	pH	K_i (μM)
Cyclic AMP	5.0	3.4 ± 0.5
	8.0	4.5 ± 0.7
Cyclic GMP	5.0	0.8 ± 0.3
	8.0	2.0 ± 0.3

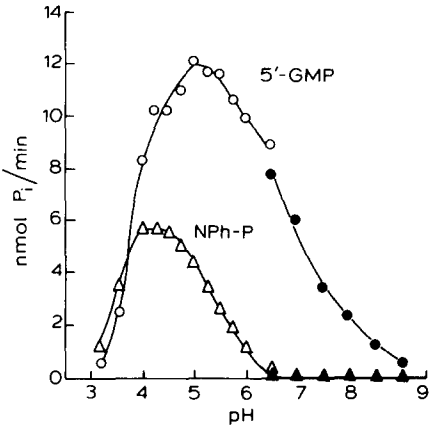

Fig. 4. Dependence on pH of potato 5'-nucleotidase activity. The 5'-nucleotidase was assayed with either 1 mM 5'-GMP (○———○, ●———●) or 1 mM *p*-nitrophenylphosphate (△———△, ▲———▲) as substrate in presence of 4 mM $MgCl_2$ and 0.1 M acetate buffer (○———○, △———△) or 0.1 M Tris buffer (●———●, ▲———▲) at various final pH values as indicated.

While 5'-AMP and 3'-AMP are hydrolyzed at comparable rates at pH 5.0 (Table III), the rate of 3'-AMP hydrolysis at pH 8.0 is less than 0.05 of the rate of 5'-AMP hydrolysis. The 5'-nucleotidase can catalyze the hydrolysis of 2'-AMP and ADP at pH 5.0, albeit at low rates (Table II), but at pH 8.0 rates of hydrolysis of these substrates are reduced to 0.0007 and 0.003, respectively, of the rate of hydrolysis of 5'-AMP. Rates of hydrolysis of ATP at pH 5.0 and pH 8.0 are 0.002 and 0.004 respectively, of the rates of hydrolysis of 5'-AMP in the same conditions. Previous studies have also demonstrated the specificity of this enzyme for nucleoside 5'-monophosphates at high pH [21,22].

Various phosphomonoesters, such as *p*-nitrophenylphosphate, ribose 5'-phosphate and β-glycerophosphate are hydrolyzed by the 5'-nucleotidase at rates comparable to those obtained with nucleosidemonophosphate substrates, but the K_m values for these substrates are in the millimolar range (Table IV).

TABLE III

RELATIVE RATES OF HYDROLYSIS OF NUCLEOTIDES BY THE 5'-NUCLEOTIDASE

Initial rates of hydrolysis of 1 mM nucleotides were obtained from time courses of hydrolysis determined at 30° C in 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. All rates are expressed relative to the rate of hydrolysis of 5'-AMP.

Nucleotide	Relative rate of hydrolysis at pH 5.0
5'-AMP	1.00
3'-AMP	0.56
3'-TMP	0.55
3'-UMP	0.091
3'-CMP	0.040
3'-GMP	0.034
2'-AMP	0.025
ADP	0.090
ATP	0.002

TABLE IV

SUBSTRATE SPECIFICITY OF THE 5′-NUCLEOTIDASE

The K_m values for nucleotides were calculated from the relationship $K_m = 1/I\ (1 + i/K_i)$ where K_i is the inhibitor constant for a competitive inhibitor and I the intercept on the 1/S axis in a $1/v$ versus 1/S plot determined in the presence of a concentration i of the competitive inhibitor cyclic AMP. The K_i of the 5′-nucleotidase for cyclic AMP used in the calculations ($3.4 \cdot 10^{-6}$ M) was the mean of determinations made using *p*-nitrophenylphosphate, ribose 5′-phosphate and β-glycerophosphate as substrates. *V* values are expressed relative to that for 5′-AMP at pH 5.0. All assays were conducted at 30°C in a medium containing substrate in 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$. Standard errors, computed as for the data in Table II, were in the range of 20—30% of calculated K_m values. These standard errors include the constant error associated with the K_i value used in the calculation of nucleotide K_m values.

Substrate	K_m (M)	Relative *V*
2′-Deoxy-5′-AMP	$0.9 \cdot 10^{-6}$	0.63
5′-AMP	$1.5 \cdot 10^{-6}$	1.00
2′-Deoxy-5′-IMP	$2.6 \cdot 10^{-6}$	0.63
3′-AMP	$3.4 \cdot 10^{-6}$	0.56
2′-Deoxy-5′-GMP	$4.0 \cdot 10^{-6}$	0.98
5′-IMP	$4.5 \cdot 10^{-6}$	1.11
5′-GMP	$6.1 \cdot 10^{-6}$	1.44
2′-Deoxy-5′-UMP	$6.8 \cdot 10^{-6}$	0.93
2′-Deoxy-5′-CMP	$9.1 \cdot 10^{-6}$	0.68
2′-Deoxy-5′-TMP	$9.6 \cdot 10^{-6}$	1.33
5′-CMP	$1.7 \cdot 10^{-5}$	0.40
5′-UMP	$1.8 \cdot 10^{-5}$	1.67
5′-NMP	$2.7 \cdot 10^{-4}$	1.46
p-Nitrophenylphosphate	$5.4 \cdot 10^{-4}$	1.14
Ribose 5′-phosphate	$6.6 \cdot 10^{-3}$	0.74
β-Glycerophosphate	$1.3 \cdot 10^{-2}$	0.10

This enabled direct determination of substrate K_m values and K_i values for cyclic AMP from the kinetics of P_i release. With nucleosidemonophosphates as substrates this method of analysis was completely inadequate due to the insensitivity of the P_i analysis and the micromolar K_m values for these substrates. However using the K_i value for cyclic AMP, the K_m values for nucleoside monophosphates could be calculated from competition data as shown in Table IV. The validity of this procedure was checked by determining the K_m for 5′-AMP radiochemically, employing micromolar concentrations of the substrate. The K_m for 5′-AMP determined in the same conditions, but with the inclusion of 1% ethanol in the assay, is 2.0 ± 0.5 μM as compared to a value of 1.5 ± 0.3 μM calculated from competition kinetic data from experiments involving millimolar concentrations of 5′-AMP. The K_m for 5′-AMP of the wheat seedling 5′-nucleotidase determined radiochemically in the same fashion is $1.8 \cdot 10^{-6}$ M as compared to the value of $1.4 \cdot 10^{-6}$ M calculated from competition kinetic data [20].

The 5′-nucleotidase has a very marked specificity for nucleoside 5′-monophosphates as shown in Table IV. While all nucleoside 5′-monophosphates examined are hydrolyzed at comparable maximal rates, consistent differences in K_m values for certain groups of nucleotides are observed. Purine nucleotides have much lower K_m values than pyrimidine nucleotides and purine or pyrimidine 2′-deoxyribonucleotides have much lower K_m values than the correspond-

ing ribonucleotides. 5′-NMP is exceptional in that the K_m for this substrate is two orders of magnitude greater than for other purine ribonucleoside 5′-monophosphates. For both the ribonucleoside 5′-monophosphate series and the 2′-deoxyribonucleoside 5′-monophosphate series, the base specificity observed is A > I > G > U,C.

While 3′-AMP is hydrolyzed at less than 5% of the rate with 5′-AMP as substrate at pH 8.0, the 5′-nucleotidase has a micromolar K_m for 3′-AMP at pH 5.0 and the V for 3′-AMP hydrolysis at pH 5.0 is comparable to that for 5′-AMP (Table IV). While 3′-AMP and 2′-deoxy-3′-TMP are excellent substrates at low pH, 3′-UMP, 3′-CMP and 3′-GMP are hydrolyzed at much lower rates in these conditions (Table III).

The temperature dependence of rates of hydrolysis of various substrates catalysed by the 5′-nucleotidase was examined. Arrhenius plots of the data were linear over the temperature range of 19—55°C with 5′-UMP, 5′-GMP and *p*-nitrophenylphosphate as substrates and activation energies were 35.6, 37.1 and 47.4 kJ · mol^{-1} respectively.

Cation activation

The 5′-nucleotidase does not have an absolute dependence on an added divalent cation for activity but is markedly activated by a wide range of divalent cations (Table V). Mg^{2+} is the best activating cation in terms of stimulation of phosphatase activity. With *p*-nitrophenylphosphate as substrate, Mg^{2+} > Cu^{2+} > Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} in terms of stimulation of substrate hydrolysis. With 5′-GMP as substrate, Mg^{2+} > Cu^{2+}, Co^{2+} > Zn^{2+} > Fe^{2+} > Mn^{2+} > Ni^{2+} in terms of stimulation of the 5′-nucleotidase.

Hg^{2+}, Pb^{2+}, Cd^{2+}, Ba^{2+} and Ca^{2+} do not activate the 5′-nucleotidase. With either *p*-nitrophenylphosphate or 5′-GMP as substrate, both Pb^{2+} and Ca^{2+} inhibit the basal 5′-nucleotidase activity whereas Hg^{2+} and Ba^{2+} have little or no inhibitory effect on the basal activity at concentrations up to 0.8 mM.

TABLE V

CATION ACTIVATION OF HYDROLYSIS OF *p*-NITROPHENYLPHOSPHATE AND 5′-GMP BY THE 5′-NUCLEOTIDASE

Rates of hydrolysis of 1 mM *p*-nitrophenylphosphate (A) or 1 mM 5′-GMP (B) were determined in the presence or absence of cations in a medium containing 0.1 M acetate (Na^+, pH 5.0). Relative maximal rates are expressed relative to the rate with no added cation (1.0).

Cation	Cation concentration for half-maximal stimulation (μM)		Relative maximal rates	
	A	B	A	B
None	—	—	1.0	1.0
Mg^{2+}	80	110	9.4	9.4
Cu^{2+}	28	87	5.6	8.0
Co^{2+}	18	55	4.0	7.9
Ni^{2+}	12	63	3.8	6.4
Mn^{2+}	20	58	3.8	5.0
Zn^{2+}	10	46	3.7	3.8
Fe^{2+}	72	44	3.5	3.5

TABLE VI

INHIBITION OF 5'-NUCLEOTIDASE

5'-Nucleotidase was assayed at 30°C using either 1 mM *p*-nitrophenylphosphate or 1 mM 5'-GMP as substrate in a medium containing 0.1 M acetate (Na^+, pH 5.0) and 4 mM $MgCl_2$, as described in Materials and Methods.

Substrate	Inhibitor	Concentration for 50% inhibition (mM)
5'-GMP	Cd^{2+}	0.06
	Pb^{2+}	0.16
	F^-	5.1
p-Nitrophenylphosphate	Cd^{2+}	0.26
	Pb^{2+}	1.0
	F^-	0.17

Inhibitors of the 5'-nucleotidase

Cd^{2+} and Pb^{2+} are potent inhibitors of the 5'-nucleotidase but have a much greater effect on hydrolysis of 5'-GMP than on hydrolysis of *p*-nitrophenylphosphate for a given non-saturating cation concentration (Table VI). Conversely F^- inhibited hydrolysis of 5'-GMP less severely than hydrolysis of *p*-nitrophenylphosphate (Table VI).

While Hg^{2+} (0.1—0.8 mM) inhibits hydrolysis of *p*-nitrophenylphosphate by about 30% at pH 5, in the same conditions up to 10 mM Hg^{2+} has no effect on hydrolysis of 5'-GMP. *p*-Chloromercuribenzoate (up to 0.1 mM) and dithioerythritol (1 mM) have no effect on the enzyme activity, further suggesting the absence of involvement of thiols in the catalytic mechanism [31]. Pyrophosphate inhibits *p*-nitrophenylphosphatase by 50% at 5 mM concentration. While F^- is a potent inhibitor of the 5'-nucleotidase (Table VI), Cl^-, SO_4^{2-} and NO_3^- have little significant effect on the enzyme activity at concentrations up to 10 mM.

The products of the nucleotidase reaction are inhibitory. Phosphate inhibits the hydrolysis of *p*-nitrophenylphosphate by 50% at 3.5 mM concentration. As described previously, nucleosides are non-competitive inhibitors of the 5'-nucleotidase from potato tubers [24] and wheat seedlings [1], the K_i for adenosine of the very similar wheat seedling enzyme being $2.8 \cdot 10^{-4}$ M.

Discussion

The potato 5'-nucleotidase closely resembles the 5'-nucleotidase from wheat seedlings in terms of its behaviour during purification, substrate specificities and competitive inhibition by cyclic nucleotides [1,20]. The K_i values for cyclic nucleotides of the two enzymes determined in the same conditions are very similar. In addition both the wheat [1] and potato [24] enzymes are non-competitively inhibited by nucleosides. These higher-plant 5'-nucleotidases differ from 5'-nucleotidases from other sources [32—35] in being competitively inhibited by cyclic nucleotides. The potato 5'-nucleotidase also differs from many other 5'-nucleotidases in its relative specificity for purine nucleoside 5'-monophosphates and in having micromolar K_m values for many nucleo-

side 5′-monophosphate substrates (Table IV). The potato 5′-nucleotidase is unusual in comparison with 5′-nucleotidases from other sources in that it is activated by a very wide range of divalent cations, including Cu^{2+}. 5′-Nucleotidases from other sources are activated by some but not all of the divalent cations that activate the potato 5′-nucleotidase [34]. The activation of the potato 5′-nucleotidase by a variety of transition metal ions and especially by Cu^{2+} suggests the involvement of a histidine imidazole in the catalytic mechanism [36]. No other 5′-nucleotidases appear to be activated by Cu^{2+} although a Cu^{2+}-activated phosphatase is present in tobacco leaves [37]. From the concentrations of divalent cations required for half-maximal activation of the 5′-nucleotidase (Table V), it seems likely that Mg^{2+} (or possibly Mn^{2+}) is the physiological activating cation.

While having no apparent relation to other eucaryotic cyclic AMP-binding proteins, the potato 5′-nucleotidase is very similar in many of its properties to the cyclic AMP-receptor protein [38,39] that is required, together with cyclic AMP, for transcription of catabolite-repressible operons in *Escherichia coli* [40]. Cyclic AMP-receptor protein has a similar molecular weight, a dimeric subunit composition of two equal-molecular-weight polypeptides and is purified in essentially the same way. Both proteins are very basic and bind very tightly to phosphocellulose at high pH. The dissociation constants for cyclic AMP and cyclic GMP, which are in the micromolar range for both proteins at pH 8.0, are also very similar. *E. coli* lactose operon DNA has been introduced into higher-plant cells and subsequently transcribed and translated as evidenced by the synthesis of *E. coli*-specific β-galactosidase in the transformed plant cells [41]. These "transgenosis" experiments have raised the possibility of the presence of cyclic AMP-receptor protein-like proteins in higher plants. Nevertheless it is possible that no such requirements hold for transcription of the lactose operon by higher-plant RNA polymerase and the similarities between the plant cyclic AMP-binding 5′-nucleotidase and cyclic AMP-receptor protein may be fortuitous. Attempts to demonstrate the existence of "cyclic AMP-receptor protein-like" proteins in higher plants through immunological cross-reaction with antibodies to the purified bacterial cyclic AMP-receptor protein have been unsuccessful, although cyclic AMP-binding activity has been detected in extracts from higher plants [42].

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