

Adenosine 5'-tetrphosphate phosphohydrolase from yellow lupin seeds: purification to homogeneity and some properties

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Adenosine 5'-tetrphosphate phosphohydrolase (EC 3.6.1.14) has been purified to homogeneity from the meal of yellow lupin (*Lupinus luteus*) seeds. The enzyme is a single polypeptide chain of 25 ± 1 kDa. It catalyses the hydrolysis of a nucleoside 5'-tetrphosphate to a nucleoside triphosphate and orthophosphate, and hydrolysis of tripolyphosphate but neither pyrophosphate nor tetraphosphate. A divalent cation, Mg^{2+} , Co^{2+} , Ni^{2+} or Mn^{2+} , is required for these reactions. The pH optimum for hydrolysis of adenosine 5'-tetrphosphate (p_4A) is 8.2, V_{max} is 21 ± 1.7 μ mol/min per mg of protein and the K_m for p_4A is 3 ± 0.6 μ M. At saturating p_4A concentrations, the rate constant

for the reaction is 8.5 ± 0.7 s^{-1} [at 30 °C, in 50 mM Hepes/KOH (pH 8.2)/5 mM $MgCl_2$ /0.1 mM dithiothreitol]. p_4A and guanosine 5'-tetrphosphate are hydrolysed at the same rate. Adenosine 5'-pentaphosphate (p_5A) is degraded 1/200 as fast and is converted into ATP and two molecules of orthophosphate, which are liberated sequentially. This contrasts with the cleavage of p_5A by the lupin diadenosine tetraphosphate hydrolase (EC 3.6.1.17), which gives ATP and pyrophosphate. Zn^{2+} , F^- and Ca^{2+} ions inhibit the hydrolysis of p_4A with I_{50} values of 0.1, 0.12 and 0.2 mM respectively.

INTRODUCTION

Cells contain several rare adenine nucleotides in addition to AMP, ADP and ATP. Foremost among them are the diadenosine polyphosphates: diadenosine 5',5'''- P^1 , P^3 -triphosphate (Ap_3A), diadenosine 5',5'''- P^1 , P^4 -tetraphosphate (Ap_4A) [1], Ap_5A and Ap_6A [2–4], as well as adenosine 5'-tetrphosphate (p_4A) and adenosine 5'-pentaphosphate (p_5A) [5–8], which have been studied less extensively. There is no information on the occurrence and biological role of p_4A and p_5A in plants. In animal tissues, the concentration of p_4A (2 μ M) is three to four orders of magnitude lower than that of ATP [5,7]. In sporulating yeast cultures, p_4A and p_5A constitute 2% and 1.5% respectively of the ATP content, whereas in vegetatively growing cells or early during sporulation the content of those nucleotides is less than 0.1% that of ATP [6]. As yet, no specific enzymes have been identified that are capable of the synthesis of any particular member of this class of uncommon nucleotides. Rather, these nucleotides seem to be products or by-products of certain ligases and transferases (reviewed in [8,9]). In contrast, in both prokaryotes and eukaryotes there exist many enzymes that bring about the selective degradation of individual dinucleoside polyphosphates, e.g. Ap_3A hydrolase (EC 3.6.1.29), (asymmetrical) Ap_4A hydrolase (EC 3.6.1.17), (symmetrical) Ap_4A hydrolase (EC 3.6.1.41) and Ap_4A phosphorylase (EC 2.7.7.53) [10–12]. In addition, there is one enzyme that is highly specific for the hydrolysis of nucleoside 5'-tetrphosphates, namely nucleoside 5'-tetrphosphate phosphohydrolase (EC 3.6.1.14) [13].

It has been known for some time that the Ap_4A and Ap_3A hydrolases from seeds of yellow lupin can degrade p_4A [14]. More recently we obtained homogeneous preparations of the lupin Ap_4A hydrolase by using a different preparation procedure [15] and found that they additionally catalyse the hydrolysis of

p_4A to ATP and P_i (A. Guranowski and E. Starzyńska, unpublished work). However, in recent assays of degradation of p_4A in different fractions of yellow lupin extracts, we noticed that most of the activity that transformed p_4A into ATP was in fractions distinct from those containing the Ap_4A hydrolase activity. The high abundance of this p_4A -degrading activity in this plant material, along with the fact that the only existing publication on a p_4A phosphohydrolase is a 30-year-old description of a partly purified protein of animal origin [13], encouraged us to purify and determine the properties of this essentially unknown enzyme. We also wished to investigate the relationship between Ap_4A and p_4A hydrolases.

The present report describes a purification procedure yielding homogeneous p_4A phosphohydrolase from yellow lupin seeds and some of its properties, including the mode of cleavage of the p_4A homologue, p_5A , by these two hydrolases from higher plants.

EXPERIMENTAL

Materials

Plant material

Yellow lupin (*Lupinus luteus* var. Topaz) seeds were purchased from the Plant Breeding Station (Wiatrowo near Poznań, Poland.)

Enzymes

Inorganic pyrophosphatase (yeast) was purchased from Sigma. Lysyl-tRNA synthetase (*Escherichia coli*) was kindly supplied by Professor Sylvain Blanquet and Dr. Pierre Plateau (École Poly-

Abbreviations used: Ap_3A , diadenosine 5',5'''- P^1 , P^3 -triphosphate; Ap_4A , diadenosine 5',5'''- P^1 , P^4 -tetraphosphate; p_4A , adenosine 5'-tetrphosphate; p_5A , adenosine 5'-pentaphosphate; p_4G , guanosine 5'-tetrphosphate; PPP_i , tripolyphosphate.

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technique, Palaiseau, France). The preparation of Ap_4A phosphorylase from yeast [12] and preparations of Ap_4A hydrolase [14], adenosine kinase [16] and 5'-methylthioadenosine hydrolase [17] were from yellow lupin seeds. These enzymes were used as analytical tools and/or as molecular mass standards during gel filtration.

Chemicals

Pyrophosphate, tripolyphosphates, tetrapolyphosphates, AMP, ADP, ATP, p_4A as its tricyclohexylammonium salt and guanosine 5'-tetraphosphate (p_4G) as its Tris salt were from Sigma. [2,8- ^3H]Adenosine 5'-triphosphate (specific radioactivity 40 mCi/mmol) was from Amersham Life Science. p_5A and labelled p_4A were synthesized enzymically by the use of lysyl-tRNA synthetase (see below).

Chromatographic media

DEAE-Sephacel, Sephadex G-50 superfine, Sephadex G-75 superfine, Sephadex G-200 and MonoQ columns were from Pharmacia. The dye-ligand adsorbent Black C-2 was purchased from Cambio (Cambridge, U.K.). The packing material for hydrophobic interaction chromatography, Toyopearl butyl-650S, was from Tosoh Corporation (Tokyo, Japan). TLC aluminium sheets precoated with silica gel containing fluorescent indicator were from E. Merck.

Buffers

Buffers A, B, C and D, containing 1 mM 2-mercaptoethanol and 5% (v/v) glycerol in 10, 20 and 50 mM potassium phosphate buffer, pH 6.8, and in 50 mM Tris/HCl buffer, pH 8.0, respectively, were used during enzyme purification.

Chromatographic systems

TLC

Silica gel thin-layer chromatograms were developed routinely for 30 min at room temperature in dioxane/ammonia/water (6:1:6, by vol.). This system gives satisfactory separation of ATP ($R_f = 0.30$) from p_4A ($R_f = 0.17$) and p_5A ($R_f = 0.10$) [8].

Ion-exchange chromatography

Preparative separation of p_5A and p_4A from ATP and Ap_4A was performed on a 1 ml MonoQ column with an ammonium bicarbonate gradient (5–500 mM, pH 8.5) as described previously [18]. The MonoQ column was also used for studies on substrate specificity with respect to nucleotides (see below).

Enzymic syntheses

The following syntheses based on previous observations that lysyl-tRNA synthetase, in the presence of Zn^{2+} , effectively adenylates various compounds containing a pyrophosphate moiety [5,9].

Synthesis of p_5A

The incubation mixture contained 50 mM Hepes/KOH, pH 8.0, 10 mM MgCl_2 , 5 mM ATP, 0.02 mM dithiothreitol, 10 mM KCl, 2 mM lysine, 0.14 mM ZnCl_2 , 1 mg/ml BSA, inorganic pyrophosphatase (0.1 unit/0.1 ml), 10 mM tetrapolyphosphate and lysyl-tRNA synthetase from *E. coli* (1 μg /0.1 ml). After 5 h incubation at 30 °C, the sample was kept at 100 °C for 4 min

then chilled, centrifuged and applied to a MonoQ column. The p_5A fractions were collected and freeze-dried.

Synthesis of labelled p_4A

Aliquots (0.1 ml) of the original stock solution of [2,8- ^3H]ATP were evaporated to dryness in a Speed-Vac system and then the labelled ATP was resolubilized in 0.1 ml of the mixture containing all the components described above, replacing tetrapolyphosphate with tripolyphosphate (10 mM). After 1 h of incubation at 30 °C, the sample was kept for 4 min at 100 °C, then chilled, centrifuged and subjected to chromatography on a MonoQ column. The p_4A fractions were collected and freeze-dried. Under these conditions, approx. 60% of the total radioactivity was recovered in p_4A .

Enzyme assays

Assay A

During enzyme purification, the activity of the lupin p_4A hydrolase was monitored by TLC analysis of the conversion of p_4A into ATP. The incubation mixture (50 μl) contained 50 mM Hepes/KOH, pH 8.2, 5 mM MgCl_2 , 0.1 mM dithiothreitol, 0.5 mM p_4A and 10 μl of analysis fraction. Incubation was performed at 30 °C and, after an appropriate period (10–30 min), 3 μl aliquots were transferred to the origin of TLC chromatograms and developed as described above. After being dried, the nucleotide spots were detected under UV illumination. For a typical chromatogram see Figure 1. This qualitative approach was sufficient for making quick decisions about which fractions from one step of enzyme purification could be collected and used in a following step.

Assay B

The effects of different factors were investigated in the appropriately modified mixture, which contained labelled p_4A . For estimation of the K_m for p_4A , the concentration of [^3H] p_4A varied between 2 and 60 μM (1 pmol = 200 c.p.m.). For initial velocity measurements, 5 μl aliquots were transferred at intervals (usually after 3, 6, 9 and 12 min of reaction) to the origin of TLC silica sheets. ATP standard was added and the chromatograms were developed for 90 min. The ATP areas were cut out, immersed in 7 ml of scintillation mixture, and counted for radioactivity. Assay B was also used for evaluation of the developed purification procedure summarized in Table 1. In the crude extract and the $(\text{NH}_4)_2\text{SO}_4$ fraction the disappearance of labelled p_4A was monitored because these fractions contained various ATP-converting activities.

Assay C

For comparison of the velocities of hydrolysis of p_5A and p_4A , the time course of ATP accumulation was monitored at 254 nm by chromatography of the incubation mixture on a MonoQ column connected to a Pharmacia FPLC system. The mixture contained either 0.5 mM p_5A or p_4A and the reaction was started with a rate-limiting amount of the p_4A hydrolase. At intervals (0, 10, 20 and 30 min for p_4A , and 0, 30, 60 and 120 min for p_5A) the reaction was terminated by transferring a 5 μl aliquot into 100 μl of water preheated to 95 °C. After 3 min at that temperature, samples were cooled and centrifuged, and 50 μl portions of the supernatant were injected into a 1 ml MonoQ column equilibrated with 50 mM ammonium bicarbonate (pH 8.5) and chromatographed as described above. Velocities of hydrolyses were calculated on the basis of the ATP produced.

Assay D

A phosphate release assay was also used for quantitative measurements of the p_4A hydrolase activity. The incubation mixture was scaled up to 0.5 ml, and 100 μ l aliquots were withdrawn at appropriate time intervals for determination of the released orthophosphate with the modified Fiske and SubbaRow colorimetric method [19].

Other methods

Electrophoresis under non-denaturing conditions

This was performed in 7.5% (w/v) polyacrylamide gel by the method of Davis [20].

Molecular mass determination

The molecular mass of the native p_4A hydrolase was estimated by gel filtration as described by Andrews [21] and that of the denatured enzyme by SDS/PAGE by the method of Weber et al. [22]. In gel filtration, the following standard proteins/enzymes were used: cytochrome *c* from pig heart (12.4 kDa), and the following enzymes from yellow lupin seeds: Ap_4A hydrolase (18.5 kDa [14]), adenosine kinase (38 kDa [16]), 5'-methylthioadenosine nucleosidase (62 kDa [17]) and yeast Ap_4A phosphorylase (40 kDa [12]). Chromatography was performed on a long column (1.14 cm \times 113 cm) equilibrated with 50 mM potassium phosphate, pH 6.8, containing 1 mM 2-mercaptoethanol and 5% (v/v) glycerol. Samples (1 ml) were applied and fractions (2 ml) were collected.

Protein determination

Protein concentration was determined by the turbidimetric tannin method [23].

RESULTS

Purification of adenosine 5'-tetrphosphate phosphohydrolase

All operations were performed at 4 °C. Meal (1 kg) obtained from yellow lupin seeds was extracted with 3 litres of buffer A for 30–40 min. The slurry was then centrifuged for 20 min at 20000 *g*. From the supernatant, referred to as crude extract, the p_4A hydrolase was precipitated with $(NH_4)_2SO_4$ (50–70% satd.). The precipitate was dissolved in buffer B and the resulting solution was dialysed extensively against this buffer overnight. After centrifugation (15 min at 30000 *g*), the dialysate was applied to a DEAE-Sephacel column (5 cm \times 35 cm) equilibrated with buffer B. The column was washed with 3 litres of this buffer followed by a linear gradient of 0–0.5 M KCl in the same buffer (total volume 7 litres). The p_4A activity appeared at 0.20–0.25 M KCl. Active fractions were pooled and brought to 70% saturation with solid $(NH_4)_2SO_4$. The precipitate obtained was collected by centrifugation, dissolved in buffer C and applied to a Sephadex G-200 column (5 cm \times 90 cm) equilibrated with the same buffer. Active fractions, which appeared at $V_e/V_0 = 2.25$ were pooled and applied to a dye–ligand column Black C-2 (1.5 cm \times 10 cm) equilibrated with buffer C. The column was then washed with 30 ml of buffer C. The adsorbed p_4A hydrolase was eluted with buffer D containing 1 M KCl (see Figure 1). The active fractions (fractions 24–28) were pooled, brought to 1.5 M with $(NH_4)_2SO_4$ and applied to a Toyopearl butyl-650S column (0.5 cm \times 2 cm) equilibrated with buffer D containing 1.5 M $(NH_4)_2SO_4$. The p_4A hydrolase that adsorbed on that hydrophobic gel was eluted stepwise with a reverse gradient of

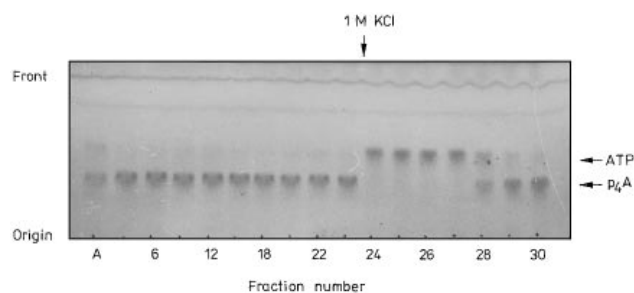


Figure 1 Analysis of adenosine 5'-tetrphosphate phosphohydrolase activity in eluates from Black C-2 agarose column by TLC

The complete assay mixture (final volume 50 μ l) contained 50 mM Hepes/KOH, pH 8.2, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.5 mM p_4A and the indicated analysis fraction. After incubation at 30 °C for 15 min, 3 μ l aliquots were spotted on silica gel sheets containing fluorescent indicator. The chromatogram was developed for 40 min in dioxane/ammonia/water (6:1:6, by vol.) and photographed under short-wave UV illumination. Lane A shows the pooled Sephadex G-200 fraction as applied to the dye–ligand column (1.5 cm \times 10 cm). Twenty 12 ml breakthrough fractions, three 10 ml wash fractions and seven 3 ml fractions eluted with 50 mM Tris/HCl buffer, pH 8.0, containing 2-mercaptoethanol, 5% (v/v) glycerol and 1 M KCl were collected and the fractions indicated were analysed for p_4A hydrolase activity.

Table 1 Purification of adenosine 5'-tetrphosphate phosphohydrolase from yellow lupin seeds

Purification was performed from 1 kg of lupin meal. Procedures were as described in the Results section. One unit of enzyme activity is the amount that hydrolyses 1 μ mol of p_4A /min under standard assay conditions. The yields are based not on p_4A hydrolase activity but on total hydrolysis of p_4A ; therefore the true yields of the p_4A hydrolase are greatly diminished by non-specific pyrophosphatases present in the crude extract and $(NH_4)_2SO_4$ fractions.

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	1860	37 200	1041*	0.028	1	100
$(NH_4)_2SO_4$ (50–70% satd.)	105	7 350	492*	0.067	2.4	47
DEAE-Sephacel	410	410	50†	0.122	4.3	4.8
Sephadex G-200	235	7	27†	3.857	137	2.6
Black C-2	6	1.4	20†	14.285	510	1.9
Toyopearl butyl-650S	0.3	0.012	0.25†	20.833	744	0.024

* Determined by the disappearance of p_4A .

† Calculated from the formation of ATP.

$(NH_4)_2SO_4$ in buffer D. Elution used two 1 ml portions for each step and the $(NH_4)_2SO_4$ concentration was decreased by 0.25 M per step. The peak of enzyme activity appeared at 0.75 M $(NH_4)_2SO_4$. These active fractions were dialysed first against buffer D and then concentrated by dialysis against buffer D containing 50% (v/v) glycerol. The final preparation was stored at –20 °C. No loss of enzyme activity was observed over at least six months. This purification procedure is summarized in Table 1.

Purity, molecular mass and subunit structure

The peak fraction of the lupin p_4A hydrolase obtained from the Toyopearl butyl-650S column was subjected both to electrophoresis under non-denaturing conditions and to SDS/PAGE under denaturing and reducing conditions. In the former, the enzyme protein showed a single band, indicating that it does not

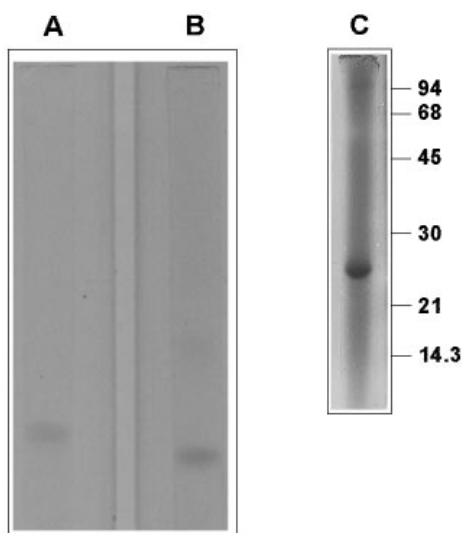


Figure 2 Electrophoresis of purified lupin seed adenosine 5'-tetrphosphate phosphohydrolase

Electrophoresis of the purified enzyme (A) and BSA (B) under non-denaturing conditions was performed in a 7.5% (w/v) gel by the method of Davis [20]. SDS/PAGE of the hydrolase (C) was performed in a 12% (w/v) gel by the method of Weber et al. [22]. Approx. 5 μ g of the purified enzyme was run and the gels were stained with Coomassie Brilliant Blue. The positions of the following standard proteins run under denaturing conditions and their molecular masses (in kDa) are shown at the right: phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

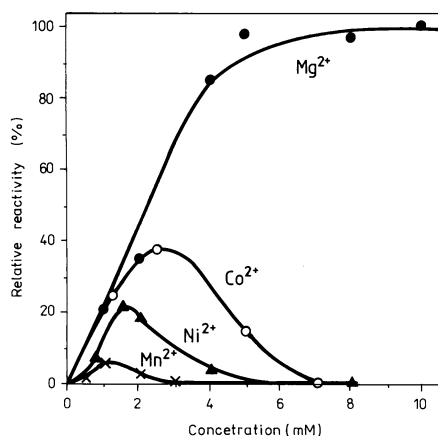


Figure 3 Effect of certain divalent metal ions acting as cofactors of lupin adenosine 5'-tetrphosphate phosphohydrolase

Initial rates of p_4A hydrolysis were measured in mixtures containing 50 mM Hepes/KOH, pH 8.2, 0.5 mM [3H] p_4A , 1% (v/v) glycerol, 0.4 mg/ml BSA, various concentrations of Mg^{2+} (●), Co^{2+} (○), Ni^{2+} (▲) or Mn^{2+} (×) as chlorides, and rate-limiting amounts of the enzyme.

form multimers (Figure 2A). [Under the same conditions, BSA showed multiple bands (Figure 2B).] Under SDS/PAGE the enzyme migrated as a single polypeptide of 25 ± 1 kDa (Figure 2C). The native enzyme exhibited the same molecular mass when chromatographed on a Sephadex G-75 superfine column. These results indicate that lupin p_4A hydrolase is a single polypeptide chain.

Requirement for divalent cation

The lupin p_4A hydrolase exhibited an absolute requirement for a divalent cation for the hydrolysis of p_4A and triphosphatase (see below, in the Substrate specificity section).

In the former reaction, we have checked the following cations, used as their chlorides: Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cd^{2+} and Zn^{2+} . Of these, Mg^{2+} seemed to be the preferred cation. The optimum concentration was reached at 5 mM and did not change practically up to 20 mM. Ni^{2+} and Mn^{2+} were weaker activators than Mg^{2+} , whereas Co^{2+} was active over a rather narrower concentration range (Figure 3). Regarding the triphosphatase activity of the lupin hydrolase, we have studied the effects of Mg^{2+} and Ni^{2+} only (see below).

pH optimum

This parameter was estimated in 50 mM Mes, Hepes, Bicine and 2-(*N*-cyclohexylamino)ethanesulphonic acid buffers (all adjusted with KOH) covering the pH range from 6.5 to 10. The p_4A hydrolase activity plotted against pH dependence exhibited a typical bell-shaped curve (results not shown), with a pH optimum at approx. 8.2. Half-maximal activity was observed at pH 7.6 and 9.4. In separate experiments we ascertained that the hydrolase was stable at the extremes of pH used for at least 30 min and that the observed effects of pH on the enzyme activity were reversible over the pH range investigated.

Michaelis and rate constants

The effect of the concentration of p_4A on the initial velocity was studied with [3H] p_4A in the concentration range 2–60 μ M, at 5 mM $MgCl_2$. The p_4A hydrolase showed Michaelis–Menten-type kinetics in the reaction leading from p_4A to ATP and P_i . V_{max} was 21 ± 1.7 μ mol/min per mg of protein and the apparent K_m for p_4A computed from an Eadie–Hofstee plot was 3 ± 0.6 μ M (results not shown). Given the molecular mass of the hydrolase (25 kDa), the calculated rate constant for p_4A was 8.5 ± 0.7 s^{-1} .

Substrate specificity

Specificity for nucleotides

A range of nucleotides and dinucleotides was tested as potential substrates of the lupin p_4A hydrolase: ADP, ATP, p_4A , p_4G , p_5A , Ap_3A and Ap_4A . After prolonged (5 h) incubation, reaction mixtures containing the above nucleotides were monitored by TLC as described in the Experimental section. Only p_4A , p_4G and p_5A were substrates.

The nucleoside tetraphosphates, at 0.5 mM, were hydrolysed at the same rate. By means of appropriate adjustments of the enzyme concentration, we have determined the relative velocities of hydrolysis of p_5A and p_4A , each substrate being used at 0.5 mM concentration. We found that p_5A was hydrolysed at 1/200 the rate of p_4A . Because of this difference, it is not possible from the experiments reported here to establish whether the p_4A produced by hydrolysis of p_5A was released from the enzyme before further, rapid hydrolysis to ATP or whether it was cleaved a second time within the same enzyme–substrate complex.

Analysis of the mechanism of lupin p_4A hydrolase degradation of p_5A to ATP

One can consider two possible modes for the degradation of p_5A : (1) slow conversion of p_5A into p_4A and subsequent fast conversion into ATP, or (2) slow conversion of p_5A into ATP with concomitant release of PP_i . We employed inorganic pyrophosphatase and a standard colorimetric method for P_i es-

timination [19] to distinguish between these two processes. A reaction mixture (0.25 ml total volume), in which at least 60% of a 0.3 mM solution of p_5A had been converted into ATP during a 30 min incubation by approx. 0.01 unit of the p_4A hydrolase, was divided into two 120 μ l portions.

An excess of inorganic pyrophosphatase (0.1 unit in 5 μ l) was added to one of them and an equal volume of water to the other. After incubation for 5 min at 30 °C, 100 μ l aliquots of each sample were transferred into 100 μ l of 10% (w/v) trichloroacetic acid to stop the reactions, chilled in ice and centrifuged. Their P_i content was then estimated colorimetrically in 100 μ l of the supernatants. The two samples showed practically the same absorbances and the equivalents of P_i liberated amounted to 1.18 and 1.12 respectively, indicating that no PP_i was liberated by the p_4A hydrolase from p_5A . Thus the enzyme removes two P_i residues consecutively from the p_5A nucleotide, which establishes (1) as the mode of cleavage.

These experiments were complemented by several controls. First, the mixture containing 0.3 mM p_5A was incubated for 30 min at 30 °C in the absence of the p_4A hydrolase, then divided into two as before, incubated for 5 min with or without the inorganic pyrophosphatase and finally subjected to P_i analysis as described above. Secondly, the mixture containing 0.3 mM p_5A was treated with trichloroacetic acid at zero time, i.e. without incubation, and analysed for P_i . All the control samples showed the same P_i concentration. This showed three things: first, that the stock sample of p_5A , which is a rather unstable compound, was contaminated with P_i (up to 15%), secondly, that p_5A is not a substrate for inorganic pyrophosphatase, and thirdly that the nucleotide is not degraded during incubation in the absence of the p_4A hydrolase. In a separate experiment (see below) we have shown that the hydrolase does not degrade pyrophosphate. To establish the validity of these experiments further, we have used the same protocol to examine the action of the lupin Ap_4A hydrolase on p_5A . Pilot experiments showed that p_5A is indeed a substrate for Ap_4A hydrolase, with a rate of degradation similar to that of p_4A and Ap_4A [12].

We therefore constituted the same reaction mixture described above but containing Ap_4A hydrolase (1 m-unit) in place of p_4A hydrolase. After 30 min approx. 60–70% conversion of 0.3 mM p_5A into ATP had taken place. The sample was divided as before and one aliquot subjected to incubation with inorganic pyrophosphatase. The latter sample showed a much higher P_i content than the untreated sample; the equivalents of P_i liberated were 1.16 and 0.06 respectively. This result thus provides an additional positive control for the experiments with p_4A hydrolase.

Specificity for polyphosphates

The nucleoside tetraphosphate phosphohydrolase from rabbit muscle degrades not only nucleoside 5'-tetraphosphates but also inorganic tripolyphosphate [13]. The preferred cation in that reaction is Ni^{2+} . We therefore examined whether the plant hydrolase exhibits a similar tripolyphosphatase activity. We applied the phosphate release assay for monitoring the time-course degradation of 0.5 mM pyrophosphate, tripolyphosphate and tetrapolyphosphate. Of these, only tripolyphosphate behaved as a substrate of the lupin p_4A hydrolase. In the standard incubation mixture containing 5 mM $MgCl_2$, the tripolyphosphate was degraded at approx. one-third the rate of p_4A .

In contrast with the rabbit enzyme, tripolyphosphatase activity of the lupin p_4A hydrolase was stimulated much more strongly by Mg^{2+} than by Ni^{2+} . (Studies on the tripolyphosphatase activity are in progress and detailed results will be published elsewhere).

Inhibition studies

Sensitivity to thiol reagents

The Black C-2 fraction of the lupin p_4A hydrolase (0.2 ml aliquot) was subjected first to gel filtration on a small Sephadex G-50 superfine column (2 ml total volume) to remove 2-mercaptoethanol. Next, 50 μ l aliquots of the enzyme sample were incubated for 30 min at 25 °C with 0.1 mM *p*-hydroxymercuribenzoate (a control sample had no additives). Then a 10 μ l aliquot of each sample was assayed in the standard incubation mixture for retained activity. Whereas the removal of 2-mercaptoethanol did not affect the enzyme, the anti-thiol reagent fully inhibited p_4A hydrolysis. Treatment of the inactivated enzyme sample with 10 mM dithiothreitol for 20 min restored enzymic activity almost completely. These results suggest that some thiol group function is essential for lupin p_4A hydrolase.

Effect of Zn^{2+} , F^- and Ca^{2+}

In studies on two other lupin enzymes specific for uncommon nucleotides, it has been shown that Ca^{2+} [14] and F^- [24] ions inhibit Ap_4A hydrolase, and Zn^{2+} cation [14] inhibits Ap_3A hydrolase. In the present study, all three ions seemed to be inhibitors of the lupin p_4A hydrolase. Assaying them in the standard incubation mixture (at 5 mM $MgCl_2$), we determined the following I_{50} values: Zn^{2+} , 0.1 mM; F^- , 0.12 mM; Ca^{2+} , 0.2 mM.

DISCUSSION

It has previously been established that p_4A is one of several possible substrates for such plant enzymes as phosphodiesterase type I (EC 3.1.4.1), Ap_4A hydrolase (EC 3.6.1.17), Ap_3A hydrolase (EC 3.6.1.29) [14] as well as apyrase (EC 3.6.1.5) [25]. (In this study we found that the preparation of apyrase obtained from yellow lupin seedlings [25] degraded p_4A at approx. 1/20 of the rate at which it degrades ATP). However, in monitoring the fractions of yellow lupin seed that were eluted from the DEAE-Sephacel column used by us routinely for enzyme purifications [17,26,27], we observed that the fractions that were eluted with the KCl gradient between Ap_3A hydrolase (appearing at approx. 0.15 M KCl) [27] and Ap_4A hydrolase (appearing at 0.35 M KCl) exhibited an elevated and very specific p_4A hydrolase activity. In the course of purification of that hydrolase to homogeneity, we tested various chromatographic media that had proved useful for the purification of other nucleotide-metabolizing enzymes [12,14,17,26,27]. The best results were obtained with a dye-ligand agarose, Black C-2 manufactured by Cambio. (Black C-2 is classified by Cambio as a member of group 5, having the highest binding avidity for protein). We found that the p_4A hydrolase could not be displaced from that resin by affinity elution with different ligands such as p_4A/Mg^{2+} or Ap_4A/Mg^{2+} but was eluted at high salt concentration. Because elution of the enzyme from Black C-2 by a 0–1 M KCl gradient was accompanied by some tailing, we used, after washing the bulk of unadsorbed protein, stepwise elution with 1 M KCl (Figure 1). Despite substantial purification of the enzyme protein at the Black C-2 step, the preparation was not homogeneous under electrophoresis.

Hydrophobic interaction chromatography on Toyopearl butyl-650S resin proved to be the most efficient of the processes explored, finally giving homogeneous protein (Figure 2). In practice, the p_4A hydrolase tailed on that resin also and only the peak fraction that emerged at the highest enzyme-eluting $(NH_4)_2SO_4$ concentration (0.75 M) was homogeneous. Most of the p_4A hydrolase was eluted in further fractions and was

contaminated with other proteins. Fortunately, repeated chromatography of these fractions gave an improved yield of pure protein. The purification results depicted in Table 1 show a very low apparent yield (0.024%) for the purification of the p₄A hydrolase activity. However, because ATP is quickly cleaved by various enzymes present in the crude extract and the analysis is based on disappearance of p₄A, the activity measured is the sum of the activities of p₄A hydrolase plus, among others, Ap₄A hydrolase and non-specific phosphodiesterase. We have shown previously that the first of these impurities is precipitated in the 30–50%-saturated (NH₄)₂SO₄ fraction, whereas the second is removed by DEAE-Sephacel chromatography [14]. Therefore it is actually impossible to estimate the real purification yield for p₄A hydrolase. The fact that our preparation of homogeneous p₄A hydrolase does not degrade the nucleotides AMP, ADP, ATP, Ap₄A and Ap₃A nor PP_i shows that it is free of such enzymes as 5'-nucleotidase, phosphodiesterase, apyrase, adenylate kinase, Ap₄A hydrolase, Ap₃A hydrolase and inorganic pyrophosphatase.

To the best of our knowledge, this work describes the first homogeneous preparation of a nucleoside 5'-tetraphosphatase and gives some molecular characterization of the protein.

It should be mentioned here that rabbit polyclonal antibodies that we had previously raised against pure lupin Ap₄A hydrolase showed no cross-reactivity with the lupin p₄A hydrolase in ELISA (results not shown).

We determined the molecular mass of the p₄A hydrolase protein by both standard gel electrophoresis and gel-filtration methods. SDS/PAGE gave a molecular mass of approx. 24.5 kDa (Figure 2). Analysis of the elution pattern of the enzyme from Sephadex G-75 superfine gel filtration gave a molecular mass of 26.3 kDa. Although the molecular mass of the native rabbit muscle p₄A phosphohydrolase was not reported [13], we have used gel-filtration data to calculate the V_e/V_0 ratio as 2.12 (on Sephadex G-100) and conclude that the animal enzyme is similar in size to the lupin counterpart, i.e. approx. 25 kDa. Both lupin and rabbit enzymes show similarities with respect to optimum pH (approx. 8) and sensitivity to anti-thiol reagents. These two p₄A hydrolases show some difference in their absolute requirements for divalent metal cations. The rabbit enzyme was found to be twice as active with 1–5 mM CoCl₂ as with MgCl₂ [13]. However, the lupin enzyme is activated equally by MgCl₂ and CoCl₂ up to 2 mM concentration. At higher concentrations of divalent metal ion, activity reaches a maximum plateau value at 5 mM for Mg²⁺ but falls rapidly with any further increase in Co²⁺ concentration (Figure 3). The lupin enzyme has a lower K_m for p₄A (3 μM) than does the rabbit enzyme (27 μM).

Inhibition of the lupin p₄A hydrolase by Ca²⁺ and by F⁻ is similar to the effects exerted by these ions on the lupin Ap₄A hydrolase [14,24], whereas the inhibition observed with Zn²⁺ resembles its interaction with lupin Ap₃A hydrolase [14].

During this study we have demonstrated for the first time that there exist in higher plants at least two enzymes that can hydrolyse p₅A; it was opportune to make a comparison between the modes of cleavage of p₅A by the lupin p₄A hydrolase and Ap₄A hydrolase. As we have shown previously [18], the latter enzyme 'counts' the phosphate residues of its substrate, incorporates water at the fourth phosphorus from the bound adenosine moiety and acts as an *endo*-phosphatase. It is clear that there is good scope for the further deployment of phosphonate analogues of p₄A and p₅A to illuminate the details of the mechanism of action of this interesting enzyme.

Finally, the presence of such a specific nucleoside polyphosphate hydrolase with apparently well-conserved properties between two very distant eukaryotic species, lupin and rabbit, suggests that as with Ap₄A hydrolase [10,14,26] and Ap₃A hydrolase [10,14,27,28] (two distinct and specific hydrolases found in both animals and plants), the enzymes that control the cellular concentration of p_nN species have an important, albeit as yet undefined, role.

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