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Acid Pyrophosphatase from Red Kidney Beans

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ABSTRACT

Partial purification of acid pyrophosphatase activity from dried red kidney beans was achieved. The crude enzyme was found to adhere to plastic and was very unstable. These problems were solved by extraction with low pH and high-ionic-strength buffers. This extraction procedure separated acid pyrophosphatase activity into three parts. One of these activities appears to correspond to the purple phosphatase isolated by other workers (*1–3*). The other two fractions showed both general phosphomonoesterase and pyrophosphatase activity, but were most active with pyrophosphate and were used for further characterization. The pH optimum for the enzyme was approx 5.5–6.0 with pyrophosphatase, and it exhibited substrate inhibition with pyrophosphate and ATP at low pH. The partially purified acid pyrophosphatase was estimated to be a dimer of approx 98 kDa (mol wt estimated by gel filtration on Sephacryl S-200) with no detectable carbohydrate or iron content. Of the cations tested for their effect on pyrophosphatase activity, iron was the most inhibitory, followed by magnesium and zinc.

Index Entry: Enzyme purification.

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INTRODUCTION

Acid pyrophosphatase has recently become an enzyme of interest to the molecular biologist because of its ability to hydrolyze the phosphoric acid bonds present in the cap structures found at the 5′ ends of many eukaryotic RNAs leaving a 5′-phosphorylated terminus (*4*). The RNA can then be dephosphorylated with alkaline phosphatase and labeled to serve as a hybridization probe (*5,6*). Thus far, cultured tobacco callus cells have been the only commercial source for this enzyme.

A purple acid phosphatase has previously been isolated from red kidney beans (*2*). This phosphatase has general phosphomonoesterase activity accompanied by pyrophosphatase activity. Early reports (*7,8*) had claimed a high acid pyrophosphatase activity present in beans, but no further investigations had been performed. Hence, we decided to find out if the pyrophosphatase activity of the purple phosphatase was owing to a contaminating pyrophosphatase, and if so, whether a method could be found to isolate this enzyme in high purity. If so, acid pyrophosphatase from red kidney beans could be a viable alternative source of the enzyme.

MATERIALS AND METHODS

Partial Purification with Acetate Buffer

All steps were conducted at 4°C

Step 1: extraction of crude enzyme. Fifty grams of dried red kidney beans were finely ground in a blender and 500 mL 50-m*M* sodium acetate buffer (pH 4.2) added. The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C.

Step 2: filtration with polyvinylpolypyrrolidone. The yellowish supernatant was applied to a CM52 cation exchanger column (1.5×16 cm) that had previously been equilibrated with 50 m*M* sodium acetate buffer, pH 4.2. The effluent containing the pyrophosphatase activity was titrated to pH 8.0 with 1*M* NaOH before being passed through a polyvinylpolypyrrolidone column (2.8×14.5 cm).

Step 3: anion exchange. The filtrate was then loaded onto a DEAE-Sephacel column (1.8×16.4 cm) preequilibrated with a running buffer of 10 m*M* Tris-HCl, pH 7.0. High pyrophosphatase acivity was found in the clear effluent from the column. When a 0–0.2*M* NaCl salt gradient was run, a yellow fraction that also showed some pyrophosphatase activity eluted off the column. Finally, a purple fraction was eluted off the column with 50 m*M* Tris buffer, pH 7, 100 m*M* NaCl and 0.1% Tween-20, followed by 0.5*M* acetate buffer (pH 4.0).

Table 1
Partitioning of Pyrophosphatase Activity
among Fractions from DEAE-Sephacel Column (Low-pH Extract)

Fraction	Volume, mL	Total pyrophosphatase activity, mIU
Clear fraction	610	197 640
Yellow fraction	78.4	1 905
Purple fraction	5.6	334

Step 4a: dialysis of yellow fraction. The yellow fraction from the DEAE-Sephacel column was desalted using an ultrafiltration apparatus. The buffer was exchanged with 1 vol of 50 m*M* Tris-HCl, pH 7.0, removing, in the process, the yellow pigment. The resultant clear solution was then dialyzed (Spectrapor dialysis tubings, mol-wt cutoff 6000–8000 daltons) against distilled water for about 16 h.

Step 4b: treatment of clear effluent. The clear effluent from the DEAE-Sephacel column was dialyzed overnight against distilled water using Spectrapor dialysis tubings (mol-wt cutoff 6000–8000 daltons). One hundred milliliters of this solution were then treated with a mixture of 50 mL each of chilled acetone and ethyl-acetate. The solutions were mixed carefully and centrifuged at 15,000 rpm for 15 min at 4°C. The resultant white pellet was re-suspended in 100 mL of 50 m*M* Tris-HCl buffer, pH 7.0, and centrifuged again under the same conditions. The supernatant was then loaded onto a DEAE-Sephacel column (1.8×6.4 cm) equilibrated with 50 m*M* Tris-HCl buffer, pH 7.0, and the enzyme eluted with a 0–0.2*M* salt gradient.

The partitioning of pyrophosphatase activity between the three fractions as described in step 3 is shown in Table 1. The enzymes purified from the yellow fraction (step 4a) and the clear effluent (step 4b) were used separately for characterization of the acid pyrophosphatase.

Enzyme Assays

Enzyme assays were based on the formation of inorganic phosphate by action of the enzyme on various substrates. Phosphate released was routinely estimated by the Fiske-SubbaRow (*9*) phosphate assay, except when detergent was present in the enzyme solution. In such instances, the Heinonen-Lahti assay (*10*) was used instead.

The standard reaction mixture contained 30 m*M* citrate buffer, pH 5.0, 1 m*M* Tetra-sodium pyrophosphate, and enzyme in a 1-mL reaction mixture. Typically, the mixture was incubated for 10 min at 37°C and quenched with either Fiske-SubbaRow stopping reagent (Fiske-SubbaRow

assay) or an aliquot of citric acid (Heinonen-Lahti assay). Two controls were provided: one without enzyme and the other without substrate. One International Unit (IU) of enzyme was defined as the amount that liberated 1 μmol inorganic phosphate/min.

Protein Determination

All protein determinations were performed by the Slater method (*11*) with the exception that the protein content of fractions eluted off columns was estimated by reading their absorbance at 280 nm.

pH Profile

Thirty-millimolar solutions of buffers ranging from pH 2.5 to 9.5 were used to assay for pyrophosphatase activity. Buffers for pH 2.5–6.0 and pH 6.5–9.5 were sodium acetate and Tris-HCl, respectively. One hundred-microliter aliquots of enzyme and 1 m*M* PP_i were used throughout the assay.

Substrate Specificity

One-hundred-microliter aliquots of enzyme preparation were used for the assay at pH 6.0. The reaction mixtures contained either 1 m*M* PP_i, 2.5 m*M* ATP, 5 m*M* *p*-nitrophenyl phosphate, or 10 m*M* β-glycerophosphate. Activity values for each preparation were subjected to a one-way analysis of variance, and the ratio of the activity with each substrate calculated.

Molecular-Weight Measurement

A 2.5×37.8 cm Sephacryl S-200 column was equilibrated with 50 m*M* Tris-HCl and 100 m*M* NaCl, pH 7.5. The measurement was conducted at 4°C. A calibration curve was constructed using blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A, and RNase A.

Kinetics

The response of various enzyme fractions to varying concentrations of ATP and pyrophosphate was tested. The concentration of substrate was varied between 0–5 m*M*, while pH was kept constant at 5.5. One hundred-microliter aliquots of enzyme preparation were used. An activity vs substrate curve was drawn along with a double-reciprocal plot. K_m and V_{max} were determined using the Eisenthal and Cornish-Bowden plot.

Influence of Metal Ions

Reaction mixture were made up to 4, 10, 20 m*M* calcium chloride, iron (II) sulfate, and zinc acetate. One hundred-microliter aliquots of enzyme were incubated in pH 5.5 buffer containing the metal ions for 10

min before starting the reaction with 1 m*M* PP_i. Magnesium concentration, on the other hand, was varied from 0–10 m*M* in the reaction mixture (while PP_i was kept constant at 1 m*M* and pH 5.5).

Native Polyacrylamide Gel Electrophoresis

Disk-PAGE slab gels were prepared according to Davis (*12*). Electrophoresis was carried out at constant current (16 mA) with the maximum voltage set at 100 V for 2 h 45 min. Proteins in the gels were stained with 0.025% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 7% (v/v) acetic acid. Pyrophosphatase activity in the gel was detected by the malachite green method of Zlotnick and Gottlieb (*13*). A modified periodic acid-Schiff stain for glycoproteins separated by nondenaturing polyacrylamide gel electrophoresis was also performed (*14*). Finally, nonheme iron proteins were visualized by soaking the gel slice in a solution of 100 m*M* potassium ferricyanide in 50 m*M* Tris-HCl and 50 m*M* NaCl, pH 7.5, for 20 min. The gel was rinsed well with distilled water, and immersed in a solution of 10% (v/v) methanol and 10% (w/v) trichloroacetic acid. The gel slice was left in this solution in the dark, for about 1 h. Pale blue bands marked the position of iron-containing proteins. Protein bands were scanned using the Ultroscan XL Laser Densitometer with data collection and processing performed by Gelscan XL software on an IBM AT.

RESULTS

Purification

Crude extracts of the enzyme were very unstable, formed flocculent precipitates with Fiske-SubbaRow stopping reagent and adhered to any plastic encountered. These included polyallomer, polycarbonate, and polypropylene. The low-pH extraction solved these problems, but the resultant crude extract browned very quickly, making it necessary to pass it through polyvinylpolypyrrolidone in an effort to remove phenolics. Three variously colored fractions were obtained when the low-pH acetate extract was passed through an DEAE-Sephacel column. A clear effluent was obtained when the crude extract was first loaded onto the column, while a yellow band was eluted from the column using a 0–0.2*M* NaCl gradient. However, a brownish purple band required the addition of running buffer containing 0.1% Tween-20 followed by washing with 0.5*M* acetate buffer to elute it from the column. Table 1 shows a major portion of the pyrophosphatase activity associated with the clear effluent. This portion of the enzyme activity required organic extraction (step 4a) or treatment with Tween 20 (subsequent observation) to adhere to DEAE-Sephacel. This finding suggested that the enzyme was originally bound to lipid in such a

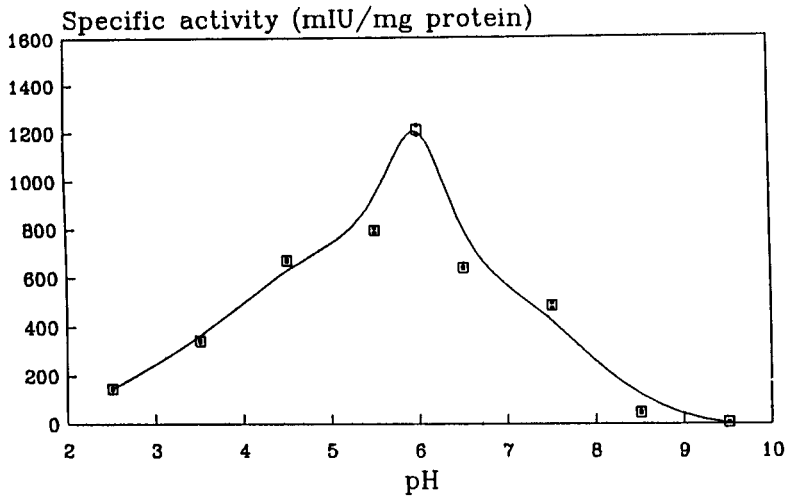


Fig. 1. Effect of pH on the activity of red kidney bean acid-IPPase obtained from the yellow fraction.

way that its surface became effectively uncharged. Both treatments might have removed lipid from the enzyme, exposing its negatively charged groups. The yellow fraction contained a substantial amount of pyrophosphatase activity. Its color was the result of a pigment that eluted from the Sephacel at the same time as the enzyme. The pigment could be removed by ultrafiltration and was later shown to have no pyrophosphatase activity whatsoever. The purple fraction possessed a high-phosphate monoesterase activity, but very little pyrophosphatase activity and was thought to correspond to the purple phosphatase of Beck et al. (2,3). This fraction was not studied further.

Properties of the Acid Pyrophosphatase

pH Profile: Figure 1 shows the pH profile for pyrophosphate obtained with the enzyme purified from the yellow fraction. It shows a sudden increase in activity at about pH 6.0, which forms the (narrow) pH optimum of the enzyme. The enzyme purified from the clear effluent shows essentially the same results (not shown).

Substrate specificity: The activity of the enzyme purified from the yellow fraction with various substrates is displayed in Table 2. It can be seen that the acid pyrophosphatase has a broad substrate specificity at pH 6, hydrolyzing many types of phosphate esters. However, maximal activity was obtained with pyrophosphate. The enzyme purified from the clear effluent showed very similar results (not shown). At the same substrate concentrations used, both enzymes from the yellow fraction and the clear effluent displayed the following ratio for activity with PP_i, ATP,

Table 2
Substrate Specificity of the Enzyme from the Yellow Fraction

Substrate	Enzyme activity, mIU/mg protein
ATP	958.54
Sodium pyrophosphatase	1238.526
p-nitrophenyl phosphate	460.934
β-glycerophosphate	21.96

p-nitrophenyl, and β-glycerophosphate: 56(56):43(41):21(17.5):1(1) (numbers in brackets apply to the clear effluent). There is no significant difference between the ratios for the two enzymes, leading us to suspect that the yellow fraction and the clear effluent may contain the same enzyme. It was more convenient to work with the yellow fraction than the clear effluent, since the latter required further organic extraction or Tween 20 treatment. Thus, all subsequent studies were done using the enzyme obtained from the yellow fraction after dialysis (step 4a).

Molecular weight: The mol wt of the enzyme from the yellow fraction as estimated by gel filtration on Sephacryl S-200 in the absence of detergent was 98 kDa. When the solution was treated with Tween-20, and then passed through the same column, the estimated mol wt was 55 kDa and the enzyme retained its catalytic activity. The 55-kDa value was believed to represent the mol wt of the monomeric form of the enzyme.

Kinetic studies: The influence of PP_i and ATP concentration on the enzyme from the yellow fraction at pH 5.5 is shown by activity vs substrate curves in Fig. 2. Upward curving reciprocal plots characteristic of substrate inhibition were obtained with both substrates (Fig. 3). Apparent K_m and V_{max} values were estimated by constructing Eisenthal and Cornish-Bowden plots (not shown). Results show that the enzyme has a greater affinity for ATP; however, the V_{max} pyrophosphatase activity is about twice as large as that for ATPase activity, meaning that a faster turnover of PP_i molecules as compared to ATP molecules is possible.

Influence of metal ions: The results are summarized in Table 3. Iron (II) ions were found to inhibit pyrophosphatase activity drastically even when present in 4-m*M* concentration. As the concentration of iron (II) was increased, pyrophosphatase activity fell to nearly zero. ATPase activity, however, was less affected. Zinc, again, inhibits pyrophosphatase activity at 10-m*M* concentration, but has no effect on ATPase activity. Calcium had no effect at all on either pyrophosphatase or ATPase activity. Magnesium has a slight inhibitory effect on pyrophosphatase activity (< 10%) above 6 m*M*.

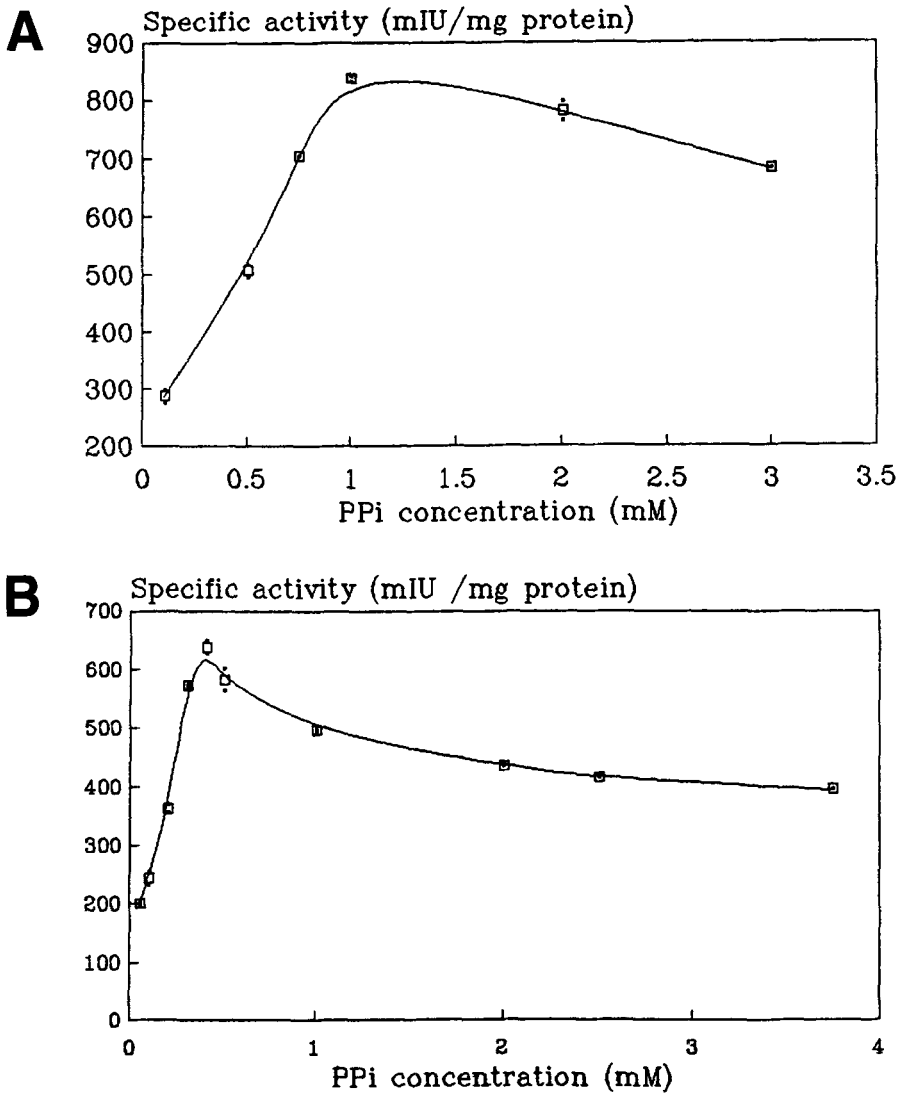
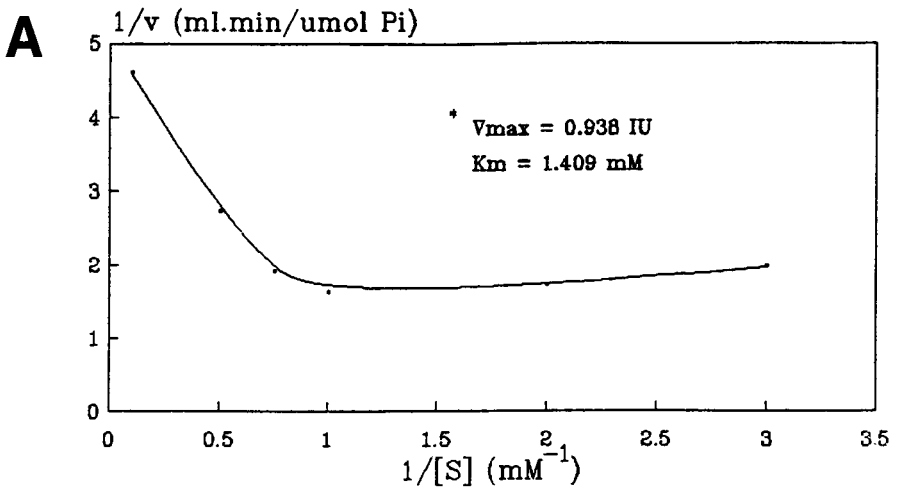


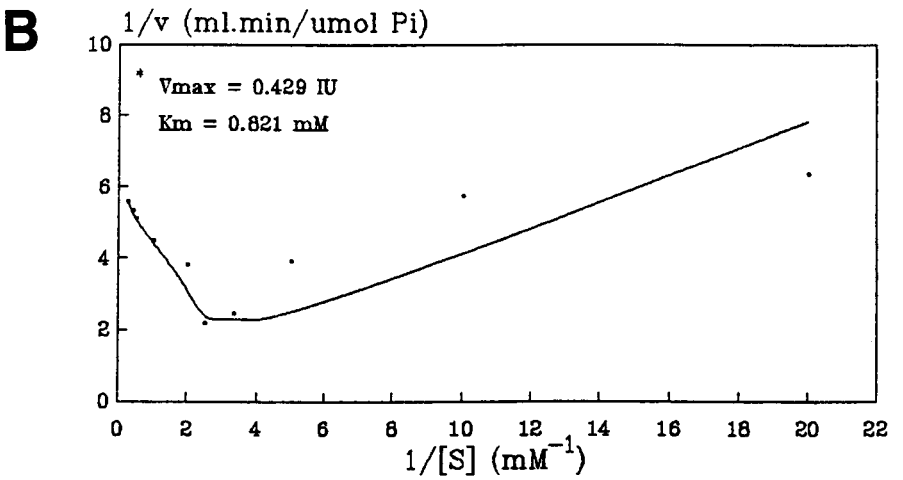
Fig. 2(**A**). Effect of PP_i concentration on the activity of red kidney bean acid-IPPase in pH 5.5 citrate buffer. (**B**) Effect of ATP concentration on the activity of red kidney bean acid-IPPase in pH 5.5 citrate buffer.

Electrophoresis of the Partially Purified Enzyme from the Yellow Fraction

The partially purified enzyme migrated as one major band of enzyme activity, accompanied by one smaller, faster-moving activity band, both of which corresponded to two bands at the same position on the Coomassie-blue-stained gel (Fig. 4). Two other faint bands could be seen on the Coomassie-blue-stained gel probably indicating the presence of trace



* Kinetic constants estimated from Eisenthal and Cornish-Bowden plot (not shown)



* Kinetic constants estimated from Eisenthal and Cornish-Bowden plot (not shown)

Fig. 3(**A**). Lineweaver-Burk plot of data from Fig. 3a showing substrate inhibition of PP_i of red kidney bean acid-IPPase. (**B**) Lineweaver-Burk plot of data from Fig. 3b showing substrate inhibition by ATP of red kidney bean acid-IPPase.

amounts of contaminants. Gels stained with the periodic acid-schiff stain showed no bands at all, indicating that the enzyme protein is probably not a glycoprotein. Similarly, staining for iron-containing proteins yielded no visible reaction. Since red kidney bean purple phosphatase is known to be a zinc-iron glycoprotein, this evidence appears to support the idea that the acid pyrophosphatase is not the same enzyme as the purple phosphatase.

Table 3
Effect of Metal Ions on Red Kidney Bean Acid-IPPase and ATPase[a]

Metal ion	Metal ion concentration, m*M*	Percent inhibition	
		IPPase	ATPase
Ferrous sulfate	0	0	0
	4	77	32
	10	89	58
	20	95	70
Zinc acetate	0	0	0
	4	0	0
	10	6	0
	20	35	0
Calcium chloride	0–20	0	0
Magnesium chloride	0	0	[b]
	4	0	[b]
	6	0	[b]
	8	4	[b]
	9	10	[b]

[a]Activity was measured with either 1 m*M* PP_i or 25 m*M* ATP at pH 5.5 (50 m*M* citrate buffer).
[b]Not tested.

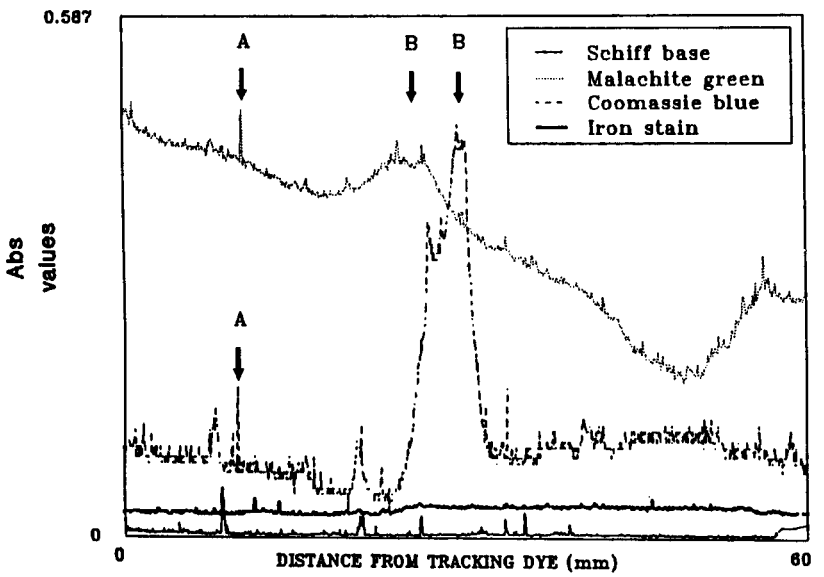


Fig. 4. Densitometer scan of nondenaturing polyacrylamide gel of partially purified yellow fraction as treated with stains for (1) glycoprotein (Schiff Base), (2) enzyme activity (Malachite green), (3) protein (Coomassie blue), and (4) iron.

Table 4
A Summary of the Properties of Red Kidney Bean Acid-IPPase
(as Reported in This Work)
and Purple Phosphatase (Beck et al., 1986, Nochumson et al., 1973)

Property	Acid-IPPase	Purple phosphate
Mol wt by gel filtration		
Dimers	97,724	141,000
Monomers	54,954	55,000[a]
Color	Colorless	Purple
Iron content	None	2 iron atoms/monomer
Attached carbohydrate	None	Glycoprotein
Amount of enzyme/g of bean meal	4.13 mg/g	2 mg/g
Substrate specificity	Wide	Wide
Substrate with highest K_m	ATP	ATP
Substrate with highest V_{max}	PP_i	Not reported
Substrate inhibition (ATP and PP_i)	Yes	Not reported
Effect of metal ions		
Magnesium	Inhibitory	Inhibitory
Calcium	No effect	Stimulatory[b]
Iron	Inhibitory	Not reported
Zinc	Inhibitory (slight)	Not reported

[a]Purple phosphatase monomer weight obtained from SDS-PAGE.
[b]Calcium is stimulatory only when no NaCl is present in solution; at 0.5 *M* NaCl, it becomes inhibitory (Nochumson et al., 1973).

DISCUSSION

Aside from tobacco pyrophosphatase, no other pyrophosphatases that are active at acid pH and do not require metal ions for activity are known. The existence of a red kidney bean purple phosphatase with broad substrate specificities made it important to compare the two (Table 4).

As can be seen, the two enzymes differ in a number of characteristics, yet they both share an affinity for ATP as a substrate. An optical spectrum scan of purified acid pyrophosphatase showed no sign of the characteristic peak at 560 nm, which had been reported for the purple phosphatase (2), and the acid pyrophosphatase was clear, not purple in color. Anion exchange after low-pH extraction separated clear and yellow extracts from a purple fraction, which showed activity with ATP, but very little with PP_i, and was suspected to be the purple phosphatase. Thus, available evidence leads us to conclude that the acid pyrophosphatase is a different enzyme from the purple phosphatase.

Our research has shown that red kidney bean acid pyrophosphatase is a dimeric protein of mol wt 98 kDa (as determined by gel filtration). Gel filtration with added Tween-20 in the running buffer has revealed a monomeric mol wt of 55 kDa. This is very similar to a number of higher plant acid phosphatases, including sunflower seed acid phosphatase with a dimeric mol wt of 103,000 daltons (*15*) and soybean seed acid phosphatase (monomeric mol wt 60 kDa) (*16*).

Unlike red kidney bean acid phosphatase, acid pyrophosphatase is neither a ferroproten nor a glycoprotein. Our work on the stability of the acid pyrophosphatase showed that crude extracts prepared in step 1 (*see* Materials and Methods) lost their activity very rapidly when distilled water or neutral Tris buffer was used as an extraction medium. If such enzyme solutions were left at room temperature or 4°C for longer than 12 h, they could lose as much as a quarter of their activity. The enzyme extracted with 50 m*M* sodium acetate buffer at pH 4.2 could be stored for a week at 4°C without any change in activity.

Substrate specificity studies showed that the acid-IPPase had a wide specificity, being able to hydrolyze ATP, PP_i, *p*-nitrophenyl phosphate, and β-glycerophosphate. It showed very high affinity for ATP and slightly lower affinity for PP_i. Because of its higher K_m, the enzyme was thought to have less affinity for PP_i than ATP, although PP_i had the highest calculated maximal velocity of the two substrates tested.

Investigation of the kinetics of the red kidney bean acid pyrophosphatase showed that the enzyme displayed substrate inhibition with both PP_i and ATP. Explanations for the apparent substrate inhibition observed in red kidney bean acid pyrophosphatase fall into three categories.

1. "Substrate inhibition" arises because of artifacts in the assay methods. Naganna et al. (*8*) noted that pyrophosphatase in high concentrations greatly interferes with the phosphomolybdate color development in the Fiske-SubbaRow assay. However, "high concentrations" were defined as 30 m*M* and above, and the concentrations used in the assay were always lower than 10 m*M*. Hence, this was thought to be unlikely.
2. A single enzyme with two active sites with different catalytic properties is present. Possibly, each active site comes into play at different PP_i concentrations.
3. A single enzyme species is present that has more than one pathway for the breakdown of enzyme intermediate to product.

The acid pyrophosphatase has an optimum pH of 6.0. This pH optimum is extremely narrow, meaning that the enzyme functions with peak efficiency only very close to pH 6.0.

The effect of added metal ions and the metalloenzyme character of acid phosphatases have been of continual interest in the literature. With this in mind, the effect of metal ions on the activity of the acid pyrophosphatase

was tested. Iron at a concentration of 20 m*M* almost completely inhibited acid pyrophosphatase activity. It had less effect on ATPase activity.

Calcium has been found to have no effect on both pyrophosphatase and ATPase activities. Calcium (2–8 m*M*) was known to be stimulatory to red kidney bean purple phosphatase activity with ATP provided no salt was present in the assay mixture (*2*). At 0.5*M* NaCl, calcium became inhibitory. However, neither effect was seen with the red kidney bean acid pyrophosphatase.

Zinc ions were slightly inhibitory to the pyrophosphatase activity, but had no effect on ATPase activity. The inhibitory effect of zinc also tends to rule out the idea that the acid pyrophosphatase might be a Zn^{2+} acid pyrophosphatase, i.e., one that is activated by zinc ions like those previously found in rice (*17,18*) and maize (*19*).

Magnesium ions were found to be inhibitory to the pyrophosphatase activity as well. This was expected since magnesium inhibition is known to occur in potato acid pyrophosphatase as well as the various acid phosphatases, including red kidney bean purple phosphatase. It is interesting to note that magnesium concentrations that are not (or very slightly) inhibitory to the crude acid-IPPase extract are much more effective at inhibiting the purified extract. The actual mechanism of magnesium inhibition, or for that matter, iron (II) and zinc inhibition, can only be speculated. Two possibilities may be suggested. PP_i may bind to the cations, thus becoming too bulky or of the wrong conformation to enter the active site of the enzyme. As support for this idea, note that yeast alkaline inorganic pyrophosphatase requires the magnesium–pyrophosphate complex $MgPP_i^{2-}$ for activity, and this complex is readily formed in solution (*20,21*). Alternatively, the PP_i may compete with the ions for binding sites on the enzyme.

In conclusion, an enzyme having similar properties to tobacco pyrophosphatase has been isolated from red kidney beans. The red kidney bean enzyme has a higher relative maximal activity with pyrophosphatase rather than ATP compared to the tobacco pyrophosphatase, which has been shown to have higher relative activity with ATP than pyrophosphate (*4*). This implies that the red kidney bean pyrophosphatase may be more appropriate for hydrolyzing the pyrophosphate bonds in the 5′ caps of eukaryotic RNA, and this possibility is currently under investigation.

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