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INHIBITION OF ACID PHOSPHATASE ISOFORMS PURIFIED FROM MATURE SOYBEAN (*GLYCINE MAX*) SEEDS

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The four soybean seed acid phosphatase isoforms AP1, AP2, AP3A and AP3B were competitively inhibited by phosphate, vanadate, fluoride and molybdate, using *p*-nitrophenylphosphate as substrate. The four isoforms were not significantly affected by compounds that can interact with SH residues or by pyridoxal phosphate. These results indicated that cysteine and lysine residues are not present in the active site of the four soybean seed acid phosphatase isoforms. The inhibition constant values for phosphate, vanadate, fluoride and molybdate at pH 5.0 were respectively: AP1 (250, 12.8, 1.7, 0.05 μM), AP2 (800, 10, 500, 0.025 μM), AP3A (250, 24.2, 250, 0.032 μM), AP3B (2400, 36.9, 750, 0.05 μM).

Keywords: Acid phosphatase; Soybean seed; Inhibitors

Abbreviations: *p*CMB, *p*-choromercurybenzoate; *p*NPP, *p*-nitrophenylphosphate

INTRODUCTION

Acid phosphatases (E.C. 3.1.3.2) are a group of enzymes that catalyze the hydrolysis of phosphate monoesters. The enzymes are widely distributed in mammalian body fluids and tissues, plants and microorganisms.

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In particular, plant acid phosphatases have been characterized in roots,[1] tubers,[2] seed,[3] aleurone layer,[4] leaves[5] and maize scutellum.[6] These phosphatases, usually present in multiple forms, display different biochemical properties and exhibit a broad specificity at optimum pH below 6.0. Many roles have been ascribed to these enzymes in plants, including the release of inorganic phosphate to the environment. Phosphorus not only plays a vital role in energy transfer and in metabolic regulation, but is also an important macromolecular constituent, such as in phospholipids, proteins and nucleic acids. The growth and development of plants are particularly dependent upon the availability of phosphate, under conditions of phosphate limitation.[7]

In relation to inhibitors, some compounds display different effects on acid phosphatases isolated from several sources. Competitive inhibition by phosphate[8,9] and uncompetitive, noncompetitive or mixed competitive inhibition by fluoride[10,11] have been reported. Potent competitive inhibition of plant acid phosphatases by phosphate analogues such as arsenate, molybdate, tartrate and vanadate has also been reported.[12–14] Feedback inhibition of acid phosphatases by phosphate may represent a general form of cellular regulation of these enzymes.

However, in general, acid phosphatase inhibition studies have been restricted only to the most active isoform. In this work we describe the effect of phosphate, vanadate, fluoride and molybdate as inhibitors of four acid phosphatase isoforms purified from quiescent soybean seed.

MATERIALS AND METHODS

Enzymes and Other Reagents

Soybean seed acid phosphatases (AP1, AP2, AP3A and AP3B) were purified 910-, 180-, 15- and 73-fold, with specific activities of 50, 10, 0.84 and 2 μmol min^{-1} mg^{-1} of protein, respectively, as previously described.[15] *p*NPP was purchased from Sigma Chemical Company. All other chemicals were of the highest purity available.

Methods

Enzyme Assay

The reaction mixture (1 mL) contained 100 mM sodium acetate buffer (pH 5.0), 0.25 and 1 mM pNPP, and enzyme (0.06, 0.5, 3.2 and 2.2 μg for AP1, AP2, AP3A and AP3B, respectively). After 10 min of incubation at

37°C, the reaction was stopped by addition of 1 mL of 1 M NaOH and the absorbance was read at 405 nm. The molar extinction coefficient of *p*-nitrophenolate ion, at 405 nm, at this pH is $1.8 \times 10^4\,M^{-1}\,cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol of substrate to product per min.

Determination of Inhibition Constants

The inhibition constants were determined from Dixon plots.[16] The enzyme activities were determined in the presence of two constant concentrations of *p*NPP and varying concentrations of the inhibitors. The K_i values were obtained from the intersection of the curves read of from the abscissa axis.

RESULTS AND DISCUSSION

Inhibition studies showed that tartrate did not affect the enzyme activities, but fluoride, phosphate, vanadate and molybdate exhibited typical phosphatase inhibition. As expected, soybean seed acid phosphatases were not inhibited by tartrate, since plant acid phosphatases are not usually inhibited by this compound.[17,18] The lack of effect of a chelator (EDTA) evidently substantiates the finding that the enzyme activities were independent of cations.[15] Absence of inhibition by *p*CMB and pyridoxal 5-P, at micromolar concentrations (Table I), suggested that neither SH groups, nor lysine

TABLE I Effect of potential inhibitors on the soybean seed acid phosphatase activities

Inhibitor	*Relative activity* (%)			
	AP1	*AP2*	*AP3A*	*AP3B*
o-Vanadate 100 μM	25 (±2)	47 (±2)	59 (±3)	63 (±1)
m-Vanadate 100 μM	28 (±1)	43 (±2)	57 (±2)	62 (+2)
*p*CMB 100 μM	100 (±4)	104 (±3)	106 (±2)	102 (±4)
*p*CMB 1 mM	31 (±2)	77 (±2)	84 (±4)	66 (±3)
H_2O_2 1 M	99 (±2)	101 (±3)	98 (±1)	96 (±4)
Molybdate 100 μM	2 (±1)	5 (±2)	6 (±1)	13 (±3)
Phosphate 1 mM	73 (±4)	84 (±3)	86 (+2)	88 (±4)
Phosphate 10 mM	18 (±2)	26 (±2)	32 (±2)	41 (±3)
Tartrate 5 mM	98 (±3)	101 (±4)	102 (±5)	101 (±4)
Pyridoxal-5P 100 μM	102 (±4)	97 (±5)	94 (±3)	96 (+2)
EDTA 5 mM	100 (±3)	93 (±5)	98 (±3)	102 (±5)
Fluoride 1 mM	72 (±3)	37 (±1)	38 (±5)	52 (±4)
Fluoride 5 mM	32 (±2)	15 (±2)	14 (±4)	22 (±3)

The inhibitors were added to the assay mixture and the enzyme activities were determined as described in Materials and Methods, using *p*NPP as substrate. In the absence of inhibitors the activities were considered as 100%. All the data were obtained in triplicate and are shown with their standard deviations (in parentheses).

residues were involved in the catalysis mechanism or in supporting the active conformation. The absence of enzyme inactivation in the presence of H_2O_2 correlates well with the noninvolvement of SH groups in the active site.

The inhibition constants (K_i) were determined for the strongest inhibitors (phosphate, vanadate, fluoride and molybdate) and in order to define the type of inhibition, we used different concentrations of inhibitor and two concentrations of *p*NPP, which were kept constant.

Phosphate is a typical competitive inhibitor for acid phosphatases (Figure 1). The values obtained for AP1, AP2 and AP3A (Table II) were similar to those observed for acid phosphatases from cotyledons of

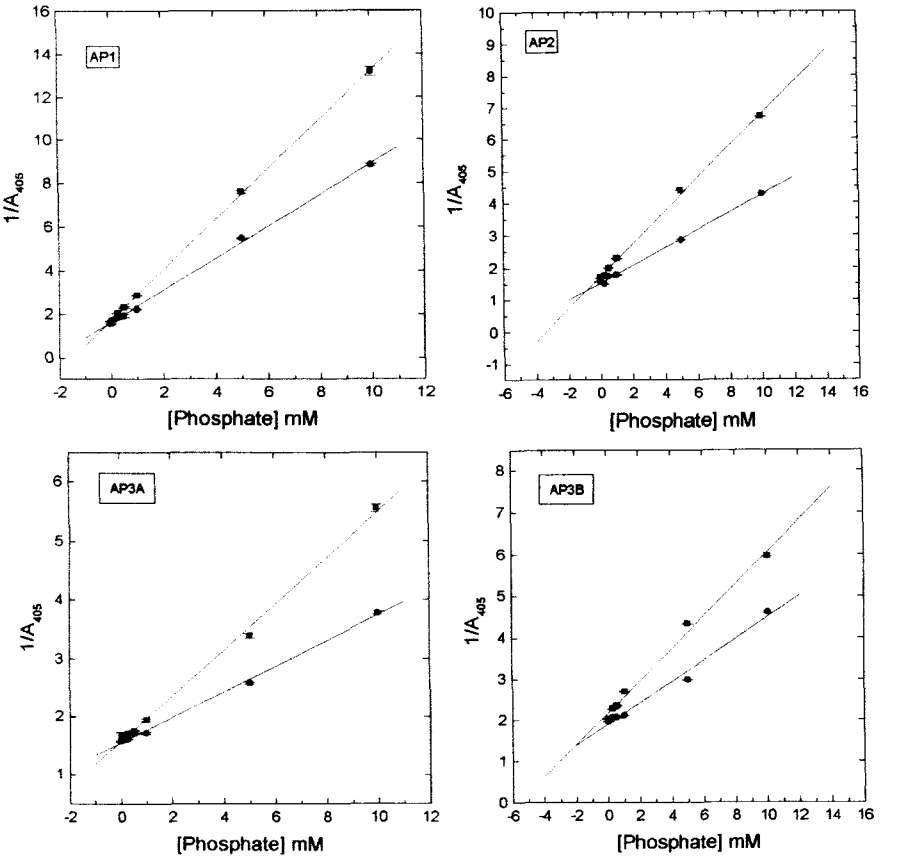

FIGURE 1 Determination of the K_i value for inorganic phosphate for the four acid phosphatase isoforms. The enzyme activity and the K_i value (method of Dixon) were determined as described in Materials and Methods, in the presence of 0.25 (■) and 1 mM (●) *p*NPP, and varying concentrations of phosphate. The experiments were performed in triplicate and bars represent the standard deviations.

TABLE II K_i values for different inhibitors of soybean seed acid phosphatases

Inhibitor	K_i (μM)				*Inhibition*
	AP1	*AP2*	*AP3A*	*AP3B*	
Phosphate	250	800	250	2,400	Competitive
o-Vanadate	12.8	10	24.3	36.9	Competitive
Fluoride	1,7	500	250	750	Competitive
Molybdate	0.05	0.025	0.032	0.05	Competitive

The K_i values were obtained from Figures 1–4, as described in Materials and Methods.

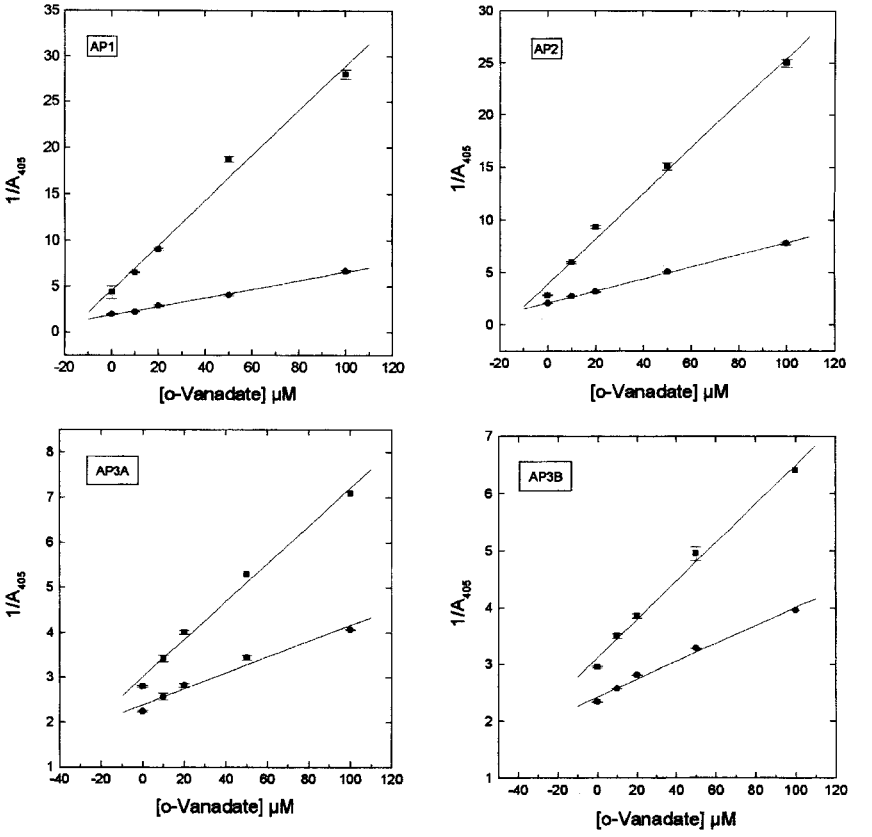

FIGURE 2 Determination of the K_i value for vanadate for the four acid phosphatase isoforms. The assay conditions were the same as described in Figure 1, at varying concentrations of vanadate. The experiments were performed in triplicate and bars represent the standard deviations.

germinating soybean seeds (280 μM)[8] and for the isoforms AP1, AP2 and AP3 of bean seeds (500, 250 and 120 μM).[9] The high K_i value 2400 μM obtained for AP3B was similar to that found for rice (2300 μM),[19] and lower than that described for potato tuber (7770 μM).[20]

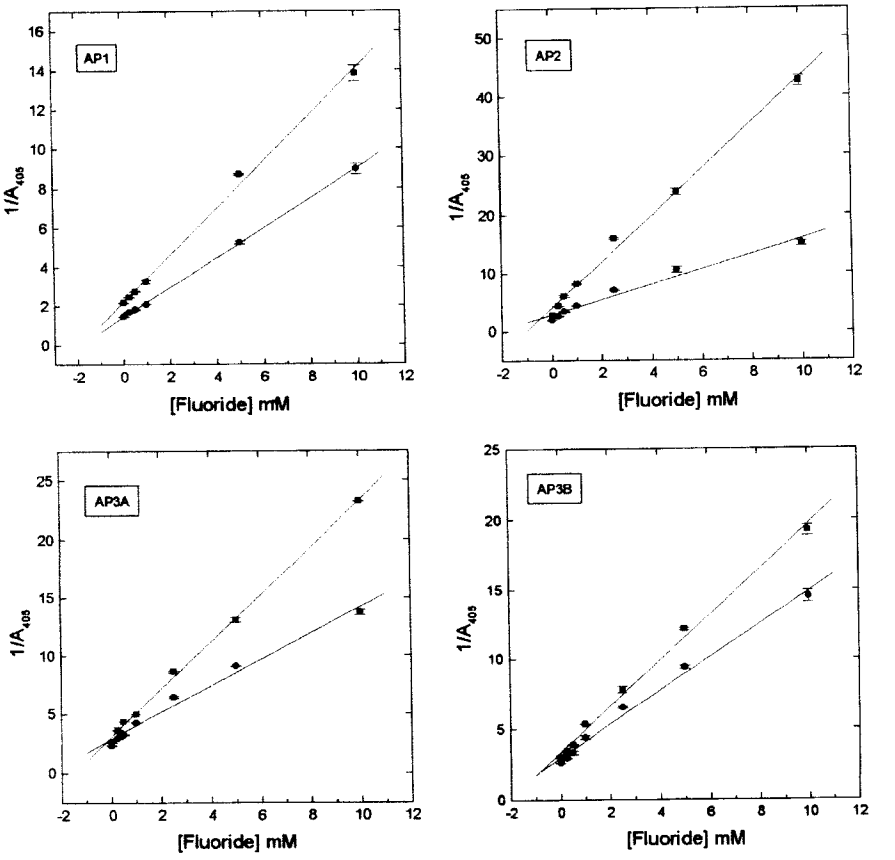

FIGURE 3 Determination of the K_i value for fluoride for the four acid phosphatase isoforms. The assay conditions were the same as described in Figure 1, at varying concentrations of fluoride. The experiments were performed in triplicate and bars represent the standard deviations.

Vanadate is generally considered a phosphate analogue, since it can adopt a similar structure resembling the transition state of many phosphoryl transfer reactions.[21] The four isoforms were similarly inhibited by o- and m-vanadate (Table I) and presented competitive inhibition patterns (Figure 2). The K_i values showed that o-vanadate was a stronger inhibitor when compared with inorganic phosphate (Table II). Vanadate is a typical phosphotyrosine protein phosphatases inhibitor. It is worthwhile mentioning that the four soybean seed acid phosphatase isoforms efficiently hydrolyze tyrosine phosphate (results not shown).

The soybean seed acid phosphatases were competitively inhibited by fluoride (Figure 3), in contrast to other plant acid phosphatases

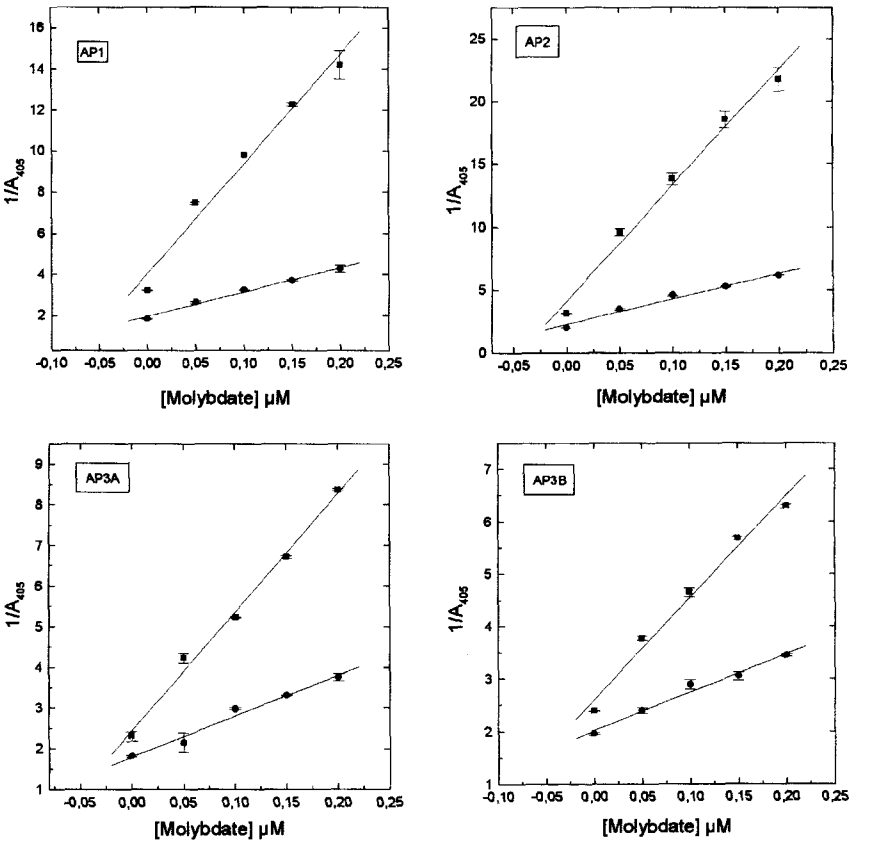

FIGURE 4 Determination of the K_i value for molybdate for the four acid phosphatase isoforms. The assay conditions were the same as described in Figure 1, at varying concentrations of molybdate. The experiments were performed in triplicate and bars represent the standard deviations.

where this compound usually demonstrated noncompetitive inhibition patterns.[10,11]

Among the inhibitors tested, molybdate demonstrated the strongest effect (Table II) and, similarly to the other compounds, gave a competitive inhibition pattern (Figure 4). Based on the inhibition constant values, our results suggest that molybdate presents higher affinity in relation to the acid phosphatase isoforms, when compared with fluoride (a weak inhibitor except for AP1), phosphate (a very poor inhibitor) and vanadate.

This work showed that the inhibition of the four soybean seed acid phosphatase was due to the interaction of molybdate, fluoride, vanadate and

phosphate with the active site of these isoforms. One very important conclusion was that inhibition by fluoride could be used to differentiate AP1 from the other three acid phosphatase isoforms.

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