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Full Length Research Paper

Purification and characterization of three low-molecular-weight acid phosphatases from peanut (*Arachis hypogaea*) seedlings

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The maximum acid phosphatasic activity was detected in peanut seedlings the 5th day of germination. At least, three acid phosphatases were identified and purified by successive chromatography separations on DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-200 HR, and Phenyl-Sepharose HP to apparent homogeneity from developing five days old peanut seedlings. These enzymes designated acid phosphatase PI, PIIa and PIIb had native molecular weights of approximately 25.3, 22.4 and 24 kDa, respectively by gel permeation. SDS-PAGE of the purified acid phosphatase PI resolved two closely protein bands that migrated to approximately 14 and 12 kDa. Thus, this acid phosphatase likely functions as a heterodimer. Acid phosphatases PIIa and PIIb migrated as single band (each) with a similar molecular weight estimated to 21 kDa. The three enzymes had a similar optima pH (5.0) and temperature (55°C), and appeared to be stable in the presence of non-ionic detergents such as Triton X-100, Nonidet P 40 as well as Na^+ and K^+. Substrate specificity indicated that the three acid phosphatases hydrolyzed a broad range of phosphorylated substrates. However, natural substrates such as ADP and ATP were the compounds with highest rate of hydrolysis for acid phosphatase PI, while acid phosphatase PIIa exhibited phytasic activity. These results indicate that each purified acid phosphatase from peanut seedlings played a peculiar role during germination.

Key words: acid phosphatase; seedling; peanut; *arachis hypogaea*; germination; low-molecular-weight.

INTRODUCTION

Acid phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) are widely distributed in plants and animals. Many authors have purified and characterized acid phosphatases from tubers (Kamenan, 1984; Gellatly et al., 1994; Kusudo et al., 2003; Kouadio, 2004) , seeds (Ullah and Gibson, 1988; Olczak et al., 1997; Granjeiro et al., 1999), roots (Panara et al., 1990), leaves (Staswick et al., 1994), bulbs (Guo and Pesacreth, 1997) and seedlings (Yenigun and Guvenilir, 2003) . Acid phosphatases are enzymes that catalytically break down a wide variety of phosphate esters and exhibit pH optima below 6.0 (Vincent et al., 1992).

In plant roots, acid phosphatases seem to be involved in the solubilization of macromolecular organic phosphates in soils which can then be utilized by plants (Panara et al., 1990). From tubers, Kamenan (1984) and Kouadio (2004) have reported an important role of acid phosphatases concerning the transport of phosphate in the metabolic phenomena taking place during the preservation of yam (*Dioscorea cayenensis rotundata*)

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and cocoyam (*Xanthosoma* sp.) tubers. From seeds and seedlings, the physiological function of the acid phosphatases is to provide inorganic phosphate to the growing plant during germination and many different phosphate esters of sugars and substrates stored in the seed and seedling need to be hydrolyzed during germination and growth (Gahan and McLean, 1969; Schultz and Jensen, 1981; Akiyama and Suzuki, 1981; Hoehamer et al., 2005).

In this study, different chromatographic methods were employed to purify three acid phosphatases from peanut seedlings, and their properties were examined.

MATERIALS AND METHODS

Materials

Peanut seeds were obtained locally in Cote d'Ivoire. *para*nitrophenylphosphate (*p*NPP), inorganic pyrophosphate (PPi), adenosine-5'-monophosphate (AMP), adenosine-2'-3'-cyclomonophosphate, adenosine-5'-diphosphate (ADP), adenosine-5'- triphosphate (ATP), , uridyl-5'-phosphogalactose, nicotinamide adenine dinucleotide (NAD); nicotinamide adenine dinucleotide phosphate (NADP), galactose-1-phosphate, glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), phenylphosphate and sodium phytate were obtained from sigma-Aldrich. DEAE-Sepharose CL-6B, CM- Sepharose CL-6B, Sephacryl S-200 HR and Phenyl-Sepharose HP were purchased from Pharmacia Biotech. Standard proteins were obtained from BioRad. All the other reagents used were of analytical grade.

Peanut seeds germination

Peanut seeds were sterilized with 1 % (v/v) sodium hypochloride solution for 10 min, washed with water three times and soaked in water for 24 h. After hydratation, germination of peanut seeds was performed on moist cotton cloth during 13 days in a room at ambient temperature (28°C) with a relative humidity (85 %). Germinating peanut seeds were daily watered. Samples were taken every two days, and then seedlings were separated to cotyledons for enzyme activity assays.

Enzyme extraction

Peanut seedlings (15 g) were ground in a prechilled mortar in 30 ml of 20 mM sodium acetate buffer (pH 5.0) containing NaCl 0.9 % (w/v). The homogenate was subjected to sonication using a TRANSSONIC T420 for 10 min and then centrifuged at 6,000 rpm for 30 min. The supernatant filtered through cotton was used as the crude extract and conserved at 4°C.

Enzyme assay

Acid phosphatase activity was based on conversion of *p*NPP into *p*-nitrophenol (*p*NP). The standard acid phosphatase assay was performed in a total volume of 250 l, containing 100 mM sodium acetate buffer (pH 5.0), substrate *p*NPP (5 mM) and enzyme preparation (25 l). The reaction mixture was incubated at 37°C for 10 min, then 2 ml of Na_2CO_3 (2%, w/v) were added to stop the reaction and absorbances were measured at 410 nm using a spectrophotometer GENESIS. *p*NP was used as standard. Under the above experimental conditions, one unit of enzyme activity was defined as 1 mol of *p*NP released per min. Specific activity was defined as the units of enzyme activity per mg of protein.

When substrates other than *p*NPP were used in the assay, the liberated inorganic phosphate was determined by the method of Taussky and Shorr (1953) with KH_2PO_4 as standard.

Protein estimation

Protein elution profiles from chromatographic columns were monitored by measuring fractional absorbance at 280 nm. The concentration of purified enzyme was determined according to Lowry et al. (1951). BSA was used as the standard protein.

Enzyme purification

All the purification procedure was carried out in cold room. The crude extract from peanut seedlings (five days old) was loaded onto a DEAE-Sepharose CL-6B column (2.6 x 6.0) equilibrated with 20 mM sodium acetate buffer pH 5.0. Unbound proteins were removed by washing the gel with two bed volumes of equilibration buffer. Bound proteins were then eluted over stepwise gradient (0.3, 0.5 and 1 M) NaCl, in 20 mM sodium acetate buffer, pH 5.0 (flow rate 1 ml/min, 3.0 ml fractions).

On the one hand, pooled unbound acid phosphatase activity (Peak 1) was loaded onto a CM-Sepharose CL-6B column (2.6 x 4.0) equilibrated with 20 mM sodium acetate buffer pH 5.0. The column was washed with the same buffer at flow rate of 1 ml/min. Acid phosphatase activity was eluted with a stepwise salt gradient (0.2, 0.4, 0.6 and 1 M) NaCl in 20 mM sodium acetate buffer pH 5.0. Fractions of 2.0 ml were collected, and to the pooled active fractions, solid ammonium sulphate was slowly added to give a final concentration of 4.2 M (80% saturation). The pellet obtained after centrifugation (6,000 rpm) was resuspended in 1 ml of 20 mM sodium acetate buffer pH 5.0 and applied to Sephacryl S-200 HR column (1.6 x 65) equilibrated with the same buffer. Fractions of 1 ml were collected at a flow rate of 0.25 ml/min and those containing acid phosphatase activity were pooled. The activity of pooled fractions was satured to a final concentration of 1.7 M sodium thiosulfate and applied on a Phenyl-Sepharose HP column (1.4 x 4.6) previously equilibrated with 20 mM sodium acetate buffer pH 5.0 containing 1.7 M sodium thiosulfate. The column was washed with equilibration buffer and the proteins retained were then eluted with a reverse stepwise gradient of sodium thiosulfate concentration (from 0.9 to 0 M) in the same sodium acetate buffer at a flow rate of 0.33 ml/min. Fractions of 1 ml were collected. The active fractions pooled were dialyzed overnight against 20 mM sodium acetate buffer pH 5.0 and constituted the purified enzyme.

On the other hand, the bound acid phosphatase activity (Peak 2) eluted from DEAE- Sepharose CL-6B at the first step was also subjected to 80% saturation with ammonium sulphate. The precipitate obtained after centrifugation (6,000 rpm) was resuspended in 1 ml of 20 mM sodium acetate buffer and loaded onto the same Sephacryl S-200 HR column in the same experimental conditions described above. Each acid phosphatase activity peak obtained was satured to a final concentration of 1.7 M sodium thiosulfate and loaded onto a Phenyl-Sepharose HP column in the same procedure as above. The active fractions pooled and dialyzed overnight against 20 mM sodium acetate buffer constituted the purified enzyme.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out by the method of Laemmli (1970) on 12% (w/v) acrylamide gels under denaturing and non-denaturing

conditions. In denaturing conditions, samples were incubated for 5 min at 100°C with SDS-PAGE sample buffer containing 2-mercaptoethanol. Concerning non-denaturing conditions, samples were mixed just before running with sample buffer without 2-mercaptoethanol and SDS. Gels were stained with Coomassie brilliant blue R-250. The molecular-mass standard makers (Bio-Rad) comprising phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soya bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) were used.

Native molecular-size determination

The purified enzyme was applied to a gel TSK QC-PAK GFC 200 HPLC column equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing sodium azide 0.05% (w/v) to estimate molecular-size. Molecular-size standards used were -amylase (200,000 Da), BSA (66,000 Da), ovalbumin (45,000 Da) and cytochrome C (12,400 Da).

Temperature and pH optima

The effect of pH on the enzyme activity was determined by measuring the hydrolysis of the substrate *p*NPP in a series of buffers at various pH values ranging from pH 3.6 to 6.0. The buffers used were sodium acetate buffer (100 mM) from pH 3.6 to 5.6 and sodium citrate buffer (100 mM) from pH 4.6 to 6.0. The pH values of each buffer were determined at 25°C.

The effect of temperature on acid phosphatase activity was performed in 100 mM acetate buffer pH 5.0 over a temperature range of 30 to 80°C using *p*NPP (5 mM) under the standard test conditions.

pH and temperature stabilities

The pH stability of each enzyme was studied in pH range 3.6 to 6.0 in 100 mM buffers. The buffers were the same as in the study of the pH and temperature optima (above). After 1 h preincubation at 37°C, aliquots were taken and immediately assayed for residual phosphatasic activity.

The thermal inactivation of the enzyme was determined at 37°C and 55°C after exposure to each temperature for a period from 10 to 120 min. The enzyme was incubated in 100 mM acetate buffer (pH 5.0). Aliquots were withdrawn at intervals and immediately cooled. Concerning thermal denaturation tests, the aliquots of enzyme were preincubated at different temperatures ranging from 30 to 80°C for 15 min. Residual activities, determined in the three cases at 37°C under the standard test conditions, are expressed as percentage activity of zero-time control of untreated enzyme.

Substrate specificity and kinetic parameters determination

The substrate specificity of the three acid phosphatases was determined by incubating each enzyme with various phosphorylated substrates (10 mM) at 37°C in 100 mM sodium acetate buffer (pH 5.0) for 30 min excepted sodium phytate which was incubated at 50°C for 2 h . The hydrolysis of these substrates was determined by titration of inorganic phosphate according to Taussky and Shorr (1953) method.

The Kinetic parameters (K_M, V_{max} and V_{max} / K_M) were determined in 100 mM sodium acetate buffer (pH 5.0) at 37°C. The hydrolysis of substrat *p*NPP was quantified on the basis of released *p*NP, as in the standard enzyme assay. The hydrolysis of other substrates was quantified by determination of released inorganic phosphate by the method of Taussky and Shorr (1953). K_M and V_{max} were determined from a Lineweaver-Burk plot using different concentrations of phosphorylated substrates.

Effect of some chemical agents

To determine the effect of various compounds (metal ions, detergents and dithiol-reducing agents) as possible activators or inhibitors of the purified acid phosphatases, the enzymatic solutions were preincubated at 37°C for 30 min with the compounds and then the activity was assayed. The substrate *p*NPP (5mM) was added to the medium and incubated at 37°C for 10 min. The residual activity was assayed as the standard conditions.

RESULTS

Evolution of acid phosphatase activities during peanut seeds germination

Peanut seeds were germinated in illuminated and aired room at ambient temperature (below 28°C). When *p*NPP was used as substrate, maximum acid phosphatasic activity (0.6 units/mg) was obtained the 5th day of germination (Figure 1). Above this day, the specific activity of acid phosphatase decreased progressively during germination time. This activity was around 0.2 units/mg the 13th day.

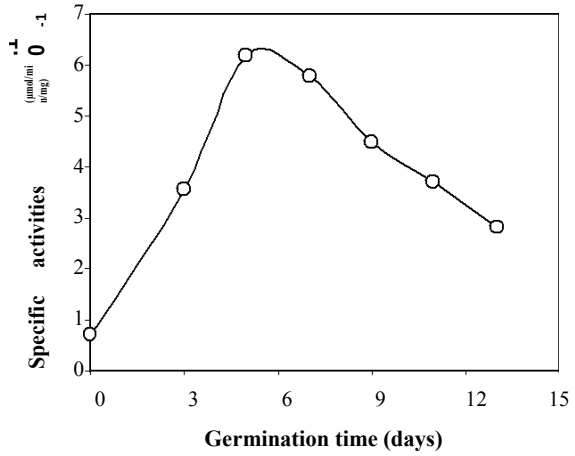

Figure 1. Expression of acid phosphatase activities during peanut seeds germination. Enzyme activities were determined every two (2) days, as described in materials and methods section, using *p*NPP as substrate. The experiments were performed in triplicate and the values given are the averages.

Purification

The results of the purification of acid phosphatase activities from peanut seedlings are summarized in Table 1. Three iso-enzymes were purified from the crude extract. The purification protocol involved four steps of chromatography for one iso-enzyme (PI) and three steps for the others (PIIa and PIIb).

Table 1. Purification of acid phosphatases PI, PIIa and PIIb from peanut seedlings.

Purification steps	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Yield (%)	Purification factor
Crude extract	227	141	0.62	100	1
DEAE-Sepharose CL-6B					
Acid phosphatase PI	75	49.1	0.65	34.9	1.1
Acid phosphatase PII	23.2	32.8	1.4	22.6	2.2
CM-Sepharose CL-6B					
Acid phosphatase PI	2.2	26.9	12	19.1	19.3
Sephacryl-S 200 HR					
Acid phosphatase PI	0.5	13.4	25.7	9.5	41.6
Acid phosphatase PIIa	0.6	5.7	10	4.1	16.0
Acid phosphatase PIIb	0.6	8.1	13.5	5.8	21.8
Phenyl-Sepharose HP					
Acid phosphatase PI	0.13	7.1	54.7	5	92.0
Acid phosphatase PIIa	0.16	4.9	30.5	3.5	49.5
Acid phosphatase PIIb	0.2	6.8	36	4.9	58.2

[a]One unit equals 1 mol of *p*NP release per min.
Values given are the averages of at least three experiments.

Two peaks of acid phosphatase activity were resolved on the DEAE-Sepharose CL-6B column, when washing the column with 20 mM sodium acetate buffer (pH 5.0) and at 0.2 M NaCl concentration and, they were designated acid phosphatase PI and acid phosphatase PII, respectively.

The acid phosphatase PI fractions were subjected to cation-exchange chromatography on CM-Sepharose CL-6B column. A single peak of acid phosphatase PI activity was eluted at 0.3 M NaCl. The pooled fractions, after this step, were loaded onto a gel filtration chromatography on Sephacryl S- 200 HR column. One peak showing acid phosphatase PI activity was resolved. The enzyme present in this peak was further purified 92 fold in a final step using hydrophobic chromatography on phenyl-Sepharose HP at 0.7 M sodium thiosulfate with 5.0% yield.

The peak of acid phosphatase PII resolved on the DEAE-Sepharose CL-6B column step was applied to a Sephacryl S-200 HR gel. Two peaks showing acid phosphatase activity were eluted and were designated acid phosphatase PIIa and acid phosphatase PIIb. These two acid phosphatase activities were ultimately purified using hydrophobic interaction on phenyl-Sepharose HP gel. Acid phosphatase PIIa was eluted at 0.4 M sodium thiosulfate and acid phosphatase PIIb at 0.2 M sodium thiosulfate with 49.5 fold and 58.2 fold, respectively.

Each acid phosphatase iso-enzyme (PI, PIIa, PIIb) showed a single protein band on native-polyacrylamide gel electrophoresis staining with Coomassie brillant blue R-250 (Figure 2).

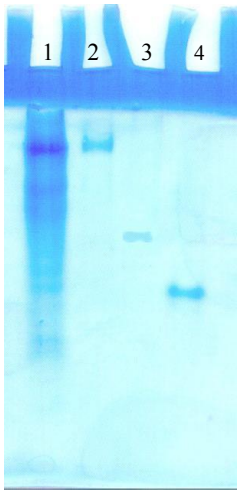

Figure 2. Native-PAGE analysis of the purified acid phosphatases from peanut seedlings. The samples were loaded onto a 12% gel. Lane 1, crude extract of peanut seedlings; lane 2, acid phosphatase PI; lane 3, acid phosphatase PIIa; lane 4, acid phosphatase PIIb.

Molecular weight estimation

After SDS-PAGE analysis under reducing conditions, two closely migrating bands were observed for acid phosphatase PI (Figure 3) and their apparent molecular weight were estimated to 14 and 12 kDa. The molecular weight of native acid phosphatase PI was estimated to be

Table 2. Some physicochemical characteristics of acid phosphatases PI, PIIa and PIIb from peanut seedlings.

Physicochemical properties	PI	PIIa	PIIb
Optimum temperature (°C)	55	55	55
Optimum pH	5.0	5.0	5.0
pH stability	4.6-5.6	5.0-5.6	4.6-5.6
Molecular weight (kDa)			
SDS-PAGE	26	21	21
Gel filtration	25.3	.22.4	24
Q_{10}	1.53	1.54	1.94
Activation energy (kJ/mol)	32.7	30.2	50.3

Values given are the averages of at least three experiments.

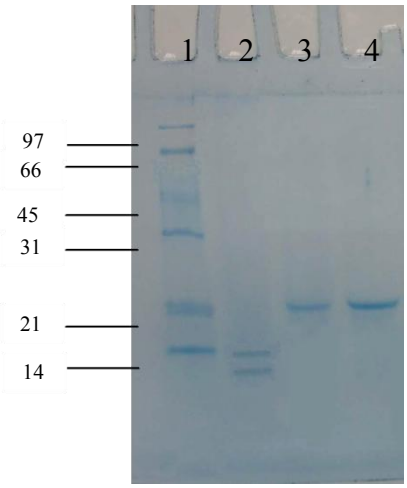

Figure 3. SDS-PAGE analysis of the purified acid phosphatases from peanut seedlings. The samples were loaded onto a 12% gel. Lane 1, molecular weight markers; lane 2, acid phosphatase PI; lane 3, acid phosphatase PIIa; lane 4, acid phosphatase PIIb. Numbers on the left indicate the molecular mass (kDa) of the makers.

around 25.3 kDa. These results indicated that the purified enzyme was a dimeric protein.

Acid phosphatases PIIa and PIIb showed a single protein band each. Their molecular weights were similar and estimated to 21 kDa. On the other hand, gel permeation chromatography with gel TSK QC- PAK GFC 200 showed that purified acid phosphatases PIIa and PIIb had molecular weight of 22.4 and 24 kDa, respectively.

Effects of pH and temperature

The effect of pH and temperature on the three acid phosphatase activities is shown in Table 2, and Figures 4 and 5. The three enzymes were most active at pH 5.0 and 55°C. At 37°C, the three enzymes were stable over a pH range 4.6-5.6 (Table 2).

The thermal inactivation studies indicated that at pH 5.0, the three acid phosphatases remained fully stable for 120 min at 37°C (data not shown). But at 55°C (optimum temperature) acid phosphatases PI, PIIa and PIIb were less stable and lost 60 , 80 and 95% of their hydrolytic activity after 120 min of preincubation (Figure 4).

The thermal denaturation was investigated by incubation of the three enzymes at various temperatures for 15 min. The midpoint of the temperature-stability curve was at around 60 - 65°C for the three enzymes (Figure 5). The enzymes were fairly stable at temperature up to 55°C. Above 55°C, their activities declined rapidly as the temperature increased, but the enzymes were not completely inactivated even at 80°C.

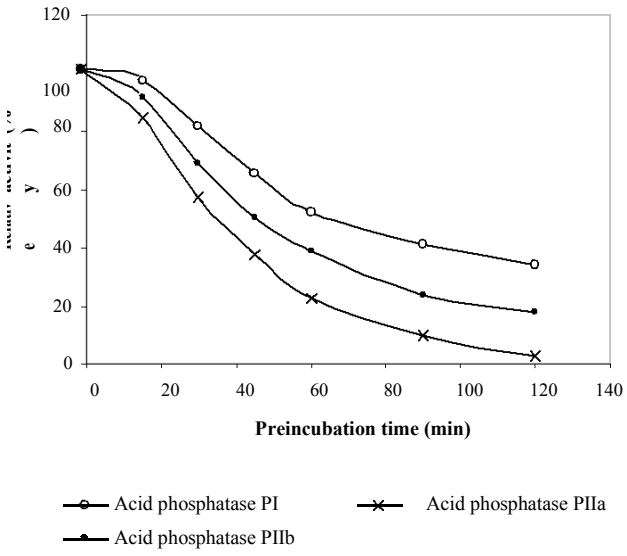

Figure 4. Thermal inactivation of acid phosphatases PI, PIIa and PIIb from peanut seedlings. The enzymes were preincubated at 55°C in 100 mM sodium acetate buffer (pH 5.0). At the indicated times, aliquots were withdrawn and the residual activity was measured at 37°C under the standard assay conditions.

Table 3. Substrate specificity of purified acid phosphatases PI, PIIa and PIIb from peanut seedlings.

Substrates	Hydrolysis activity %		
	PI	PIIa	PIIb
p-Nitrophenylphosphate	100	100	100
Phenylphosphate	93.10	85.2	89
Sodium pyrophosphate	87	56.1	66.35
Glucose-1-phosphate	21.4	0	0
Glucose-6-phosphate	30.4	0	0
Adenosine-2':3' cyclomonophosphate	9	0	63
Adenosine-5'-monophosphate	51.5	25.5	25.2
Adenosine-5'-diphosphate	103.4	100.2	81.5
Adenosine-5'-triphosphate	116.7	80.7	76.9
Uridyl-5'-phosphogalactose	0	25.5	21
Galactose-1-phosphate	0	44.7	8.8
-NAD[a]	16.5	26.6	15.4
NADP[b]	36	61.7	28
Sodium phytate[c]	0	20.1	0

[a]Nicotinamide adenine dinucleotide.
[b]Nicotinamide adenine dinucleotide phosphate.
[c]Substrate incubated with the enzyme solution at 50°C for 2 h.
Values given are the averages of at least three experiments.

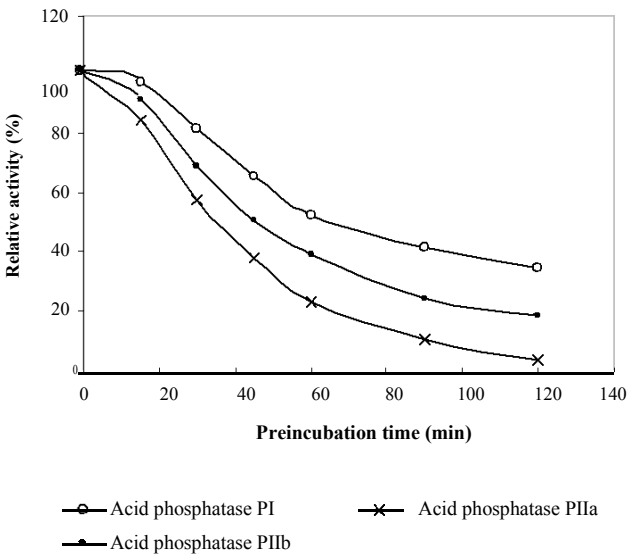

Figure 4. Thermal inactivation of acid phosphatases PI, PIIa and PIIb from peanut seedlings. The enzymes were preincubated at 55°C in 100 mM sodium acetate buffer (pH 5.0). At the indicated times, aliquots were withdrawn and the residual activity was measured at 37°C under the standard assay conditions.

Substrate specificity and kinetic properties

The results are summarized in Table 3. Acid phosphatases PI, PIIa and PIIb hydrolyzed a broad range of substrates such as phosphorylated substrates and phosphorylated sugars. The highest activity of acid phosphatase PI was observed with ATP and ADP followed by the synthetic substrate *p*NPP. Acid phosphatases PIIa and PIIb hydrolyzed energetic substrates, phosphorylated sugars and their highest activity was obtained with *p*NPP. PIIb also hydrolyzed sodium phytate. Concerning phosphorylated sugars, the nature of the sugar residue on the substrate seems critical for the three enzymes activity (Table 3).

The kinectic parameters for the three acid phosphatases were studied using *p*NPP, phenylphosphate, ADP and ATP as substrates (Table 4). With the four substrates, acid phosphatases PI, PIIa and PIIb obeyed the Michaelis-Menten equation. The K_M and V_{max} values are reported in Table 4. The catalytic efficiency of the enzyme given by the V_{max}/K_M ratio is much higer for the synthetic substrate (*p*NPP) than for the natural subtrates (Table 4).

Effect of metal ions, reducing and detergent agents

The influence of various metal ions and chelating agents on the purified acid phosphatases PI, PIIa and PIIb is presented in Table 5. The three enzymes showed different comportments in the presence of these compounds. Peanut seedlings acid phosphatase PI was activated by Sr^{2+}, Ca^{2+}, Ba^{2+} and Cu^{2+}, while Mg^{2+}, Zn^{2+} and EDTA had an inhibitory effect (Table 5). The other ions had little effect or none. Concerning acid phosphatase PIIa, its activity was enhanced by Mg^{2+}, Sr^{2+} (at 5 mM). Cu^{2+} and Zn^{2+} were inhibitory, while the other cations and EDTA tested had only a slight effect or none.

Table 4. Kinetic parameters of purified acid phosphatases PI, PIIa and PIIb from peanut seedlings towards *p*NPP, phenylphosphate, ADP and ATP.

Substrates	Acid phosphatase PI			Acid phosphatase PIIa			Acid phosphatase PIIb		
	K_M	Vmax	Vmax/K_M	K_M	Vmax	Vmax/K_M	K_M	Vmax	Vmax/K_M
*p*NPP	0.49	58.84	120.08	0.79	35.34	44.73	0.76	38.02	50.02
phenylphosphate	0.38	1.27	3.34	1.52	0.93	0.61	1.31	1.30	0.99
ADP	1.48	13.8	9.32	4.63	18.7	4.03	5.71	32.4	5.72
ATP	1.16	23.2	27.75	2.88	10.8	3.75	1.77	10.03	5.66

The Michaelis constants (K_M) and the maximum velocities (V_{max}) are expressed as mM and units/mg protein, respectively.
Values given are the averages of at least three experiments.

Table 5. Effect of some metal ions and chelating agent on the activity of acid phosphatases PI, PIIa and PIIb from peanut seedlings.

Reagent	Concentration (mM)	Relative activity (%)		
		PI	PIIa	PIIb
Control	0	100	100	100
Na^+	1	100	100	100
	5	110	118	104
K^+	1	125	100	100
	5	127.3	120	106
Mg^{2+}	1	93.4	124.9	92.6
	5	66.5	143.6	90.8
Sr^{2+}	1	197	102	91.5
	5	193	131	45.7
Fe^{3+}	1	113.2	100	83.8
	5	129	85	76
Ca^{2+}	1	186	89.2	95.6
	5	244	80.2	86.2
Ba^{2+}	1	156.2	100	87.5
	5	223.3	115	78.8
Cu^{2+}	1	120.23	30.3	50.9
	5	190	30	25.7
Zn^{2+}	1	64.8	64.7	48.5
	5	19	15	3.1
EDTA	1	74.5	98.3	103.1
	5	40	92.2	85

Values given are the averages of at least three experiments.

Acid phosphatase PIIb activity was inhibited by Sr^{2+}, Cu^{2+} and Zn^{2+}. However, the common inhibitor ion of the three acid phosphatases was Zn^{2+}, while K^+ and Na^+ were the common activator ions.

Table 6 shows the effect of reducing agents on the purified acid phosphatases PI, PIIa and PIIb. With exception of DL-dithiothreitol which displayed significant stimulatory effect on acid phosphatase PIIa, all other reducing agents tested were found to be inhibitory for the three acid phosphatases.

The influence of various detergents on the three acid phosphatase activities was studied (Table 7). The presence of anionic or non-ionic detergents on the three enzyme activities involved different results. At 0.1% concentration, cationic detergents showed at around 17 to 49% inhibition of acid phosphatase PI activity. SDS is a strong inhibitor of the three enzymes. All the non-ionic detergents tested were activators of the three acid phosphatases.

DISCUSSION

Germination brings out the synthesis or activation of enzymes responsible for the degradation of seeds

Table 6. Effect of some reducing agents on the activity of acid phosphatases PI, PIIa and PIIb from peanut seedlings.

Reducing agents	Concentration (%)	Relative activity (%)		
		PI	PIIa	PIIb
DL-dithiothreitol	0.1 (w/v)	64.34	136.55	98.70
*p*CMB[a]	0.1 (w/v)	69.49	62.99	72.81
DTNB[b]	0.1 (w/v)	39.83	9.1	13.26
L-cyteine	0.1 (w/v)	72.72	100	85.27
-mercaptoethanol	0.1 (v/v)	69.78	75.98	92.8

[a]Sodium parachloromercuribenzoate.
[b]5,5'-dithio-2,2' dinitro -dibenzoïc acid.
Values given are the averages of at least three experiments.

Table 7. Effect of some detergents on the activity of acid phosphatases PI, PIIa and PIIb from peanut seedlings.

Detergents	Concentration	Relative activity (% of control)		
		PI	PIIa	PIIb
Anionic				
SDS	0.1 % (w/v)	10	22	3
Taurocholic acid sodium salt	1 % (w/v)	114	136.3	168.3
Polyoxyethylene-9-lauryl ether	1 % (w/v)	125	120.4	147.4
Non ionic				
Triton X-100	1 % (v/v)	132.5	124.5	169.6
Nonidet P 40	1 % (v/v)	107.8	166.7	146.5
Cationic				
Tretradecyl Trimethyl Ammonium Bromide	0.1 % (w/v)	50.6	100	150.4
Hexadecyl Trimethyl Ammonium Bromide	0.1 % (w/v)	87.8	103	126.1

Values given are the averages of at least three experiments.

reserves. Among these enzymes, acid phosphatases are involved in the metabolic processes of germination and maturation of plants. They are constituvely expressed in seeds during germination, and have their activities increase with germination to release the reserve materials for the growing embryo (Biwas and Cundiff, 1991; Thomas, 1993) . In the present study, acid phosphatase activities increased and reached the maximum on the 5th day of peanut seeds germination at temperature below 28°C. Prazeres et al. (2004) reported that, when *p*NPP was used as substrate, maximum acid phosphatase activity of soybean seedlings were detected on the 6th and 9th days, for germination temperatures of 28°C and 20°C, respectively. These results suggest that temperature and seed species have an influence on germination phenomena. However, 3 to 9 days of germination seems to be the meantime corresponding to rapid cell growth and division regarding peanut seedlings acid phosphatase activity (Hegeman and Grabau, 2001).

Several studies have been devoted to purification of acid phosphatases from cotelydons of germinating seeds (Ullah and Gibson, 1988; Basha, 1984; Granjeiro et al., 1999). However, few works concerning purification of seedlings acid phosphatase have been so far reported (Bhargava and Sachar, 1987; Yenigun and Guvenilir, 2003). To better understand the role played by acid phosphatase during peanut seeds germination, three acid phosphatases from the crude extract of peanut seedlings were purified to homogeneity by four chromatographic process and their properties were examined.

SDS- PAGE exhibited two closely subunits for the purified acid phosphatase PI with molecular weights estimated to 14 and 12 kDa, and by gel filtration, the native enzyme had 25.3 kDa. These results indicated that the native functional enzyme was a heterodimer. The molecular weights of acid phosphatases PIIa and PIIb by gel filtration (22.4 and 24.0 kDa, respectively), somewhat larger than that obtained by SDS-PAGE analysis (21 kDa), suggested that, these enzymes were monomeric. In comparison to other molecular weights of purified plant acid phosphatases, peanut seedlings acid phosphatases were lower than those from potato tuber (100 kDa) (Gellatly et al., 1994), peanut seed cotyledons (240 kDa) (Basha, 1984), tomato cell culture (92 kDa) (Paul et al.,

1987), barley roots (79 kDa) (Panara et al., 1990), wheat seedlings (35 kDa) (Chen and Tao, 1989) and soybean seed (51, 58, 52 and 30 kDa) (Ferreira et al., 1998). Accordingly, peanut seedlings acid phosphatases were low molecular weight acid phosphatases. However, these acid phosphatases had higher molecular weight than the purified acid phosphatase from bovine heart (18 kDa) (Zhang and Van-Etten, 1990).

The effect of pH on the three acid phosphatases was investigated in the range of pH 3.6- 6.0. The three enzymes were stable in a range of 4.6- 5.6 with a similar pH optimum of 5.0. This stability is a good compromise for hydrolysis of natural substrates and biosynthesis reaction which need to be performed for a long time by the three enzymes. This pH optimum determined for the peanut seedlings acid phosphatases was largely consistent with other pH optima (5.0 – 6.0) (Ferreira et al., 1998; Duff et al., 1989; Haas et al., 1991).

The three acid phosphatases from peanut seedlings were optimally active at 55 C. However, they were sensitive to temperature above 50°C and lost 60 to 95% of their catalytic activity after 120 min preincubation. This attitude seems to be a general character of plant acid phosphatases. Although, acid phosphatase from soybean seeds exhibited maximum catalytic activity at 60°C, this enzyme lost drastically and fully its activity at 68°C after 10 min (Ullah and Gibson, 1988).

The three purified enzymes hydrolyzed broad phosphorylated substrates to various degrees. Similar observations have been reported for acid phosphatase from sweet potato (Kusudo et al., 2003). This indicated that the activity of each enzyme was not restricted towards a single substrate. However, natural substrates such as ADP and ATP had the highest relative rate of hydrolysis, while phosphorylated sugars showed a low rate of hydrolysis for acid phosphatase PI. A higher rate of ADP and ATP hydrolysis by acid phosphatases was also observed in tobacco cells (Pan and Chen, 1988) and rice seedlings (Tso and Chen, 1997). Sodium phytate hydrolysis is a significant character of acid phosphatase PIIa. These observations showed that each purified acid phosphatase from peanut seedlings played a peculiar role during germination. Thus, we suggest that acid phosphatase PI seems to be involved in energy transfer, metabolic regulation and biosynthesis of lipid reserves in peanut seeds. The importance of the nature of the sugar concerning the phosphorylated sugars should also be noted, because it seems critical for the enzyme activities. Indeed, the type of sugar residue could influence the rate of hydrolysis of phosphorylated sugars when they were used as substrates for the three enzymes. Acid phosphatase PIIa exhibited phytasic activity. Thus, this enzyme could cleave phosphate moieties from phytic acid (myo-inositol-hexakisphosphate) present in leguminous plant (peanut, soybean), thereby generating myo-inositol, inorganic phosphate and some multivalent cations. It is well known that phytic acid (phytate) chelates multivalent cations and some proteins, thereby rendering these biologically unavailable to the animal (Harland and Morris, 1995). Moreover, myo-inositol via this oxidation pathway is directed to cell wall polysaccharide biosynthesis (Loewus and Murthy, 2000), being important for cell wall elongation and growth.

For the three enzymes, kinetic study correlates well with substrate specificity and showed a Lineweaver -Burk plot. Among the substrates used, the purified acid phosphatases PI, PIIa and PIIb showed the highest catalytic efficiency (Vmax/Km) toward *p*NPP. The Km value (0.49 mM) of acid phosphatase PI is similar to the Km value of acid phosphatase AP1 from soybean seeds toward *p*NPP (Ferreira et al., 1998). Judging from the Vmax/Km, ATP was considered as a better natural substrate for acid phosphatase PI than phenylphosphate which showed the lowest Km value (0.38 mM). The relative high activity toward ATP and ADP indicates the possibility of application of acid phosphatase PI to synchronous enzyme- reaction system which need energy resulting of hydrolysis of these substrates.

The purified acid phosphatases PI, PIIa and PIIb were sensitive to ions at various degrees depending on ions nature and isoenzymes. The requirement of metallic ions for acid phosphatase activity has also varied according to plant species, developmental stage, and isoenzymes heterogeneity (Scandalois, 1974; Panara et al., 1990; Tso and Chen, 1997).

However, for the three enzymes, Zn^{2+} was inhibitor and, K^{+} and Na^{+} were activators. The inhibition of acid phosphatase activity by Zn^{2+} has been previously reported in other plant species such as pea (Mizuta and Suda, 1980) and tobacco (Pan et al., 1987). The stimulatory effect showed by anionic and non-ionic detergents make the latter useful when extracting peanut seedlings acid phosphatases by giving best stability. Triton X-100 is an activator for the three enzymes. Shekar et al. (2002) have also reported the same effect of Triton X-100 on the lysophosphatidic acid phosphatase activity from developing peanut cotyledons.

The three acid phosphatases were sensitive to reducing agents except the activating effect of dithiothreitol on acid phosphatase PIIa. These chemicals must be eliminated after treatment of substrate when this needs to be reduced before its hydrolysis by the enzyme.

Although, the three acid phosphatases purified from peanut seedlings had some identical physicochemical properties (pH and optimum temperature), their molecular weight, kinetic properties, substrates specificities and effect of various chemicals confirmed that the three enzymes were different.

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