Purification and characterization of two acid phosphatases from germinating peanut (*Arachis hypogaea*) seed

Jean Tia Gonnety[1], Sebastien Niamke[2]*, Betty Meuwiah Faulet[1], Eugène Jean-Parfait N'guessan Kouadio[1], Lucien Patrice Kouame[1]

[1]Laboratoire de Biochimie et Technologie des Aliments de l'Unité de Formation et de Recherche en Sciences et Technologie des Aliments de l'Universite d'Abobo-Adjamé, 02, BP 801 Abidjan 02, Côte d'Ivoire [2]Laboratoire de Biotechnologies, Filière Biochimie-Microbiologie de l'Unité de Formation et de Recherche en Biosciences de l'Université de Cocody-Abidjan, 22 BP 582 Abidjan 22, Côte d'Ivoire

Abstract
The maximum acid phosphatasic activity was detected in peanut seed at the 5th day of germination. At least, two acid phosphatases were purified by successive chromatography separations on DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-100 HR, and Phenyl-Sepharose HP to apparent homogeneity from five days old cotyledon of peanut after germination. These isoenzymes, designated peanut cotyledon acid phosphatase 1 and 2 (PCAP 1 and PCAP 2), had native molecular weights of approximately 27.5 and 24 kDa by gel permeation, respectively. SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) of PCAP 1 and PCAP 2 resolved a single protein band (each) that migrated to approximately 27 and 29 kDa, respectively. Thus, these acid phosphatases likely function as a monomer. The two isoenzymes had a similar optimum temperature (55°C), two closely optima pH (5.6 and 5.0), and appeared to be stable in the presence of some detergents such as Triton X-100, Nonidet P-40, Taurocholic acid sodium salt, Polyoxyethylene-9-lauryl ether as well as Mg^{2+}, Sr^{2+}, Fe^{3+} and Ba^{2+}. Substrate specificity indicated that PCAP 1 and PCAP 2 hydrolyzed a broad range of phosphorylated substrates. However, natural substrates such as ADP, ATP and phenylphosphate had the highest rate of hydrolysis for the two isoenzymes.
Keywords*: Acid phosphatase; Arachis hypogaea; Cotyledon; Germination; Peanut*

INTRODUCTION

Acid phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) catalyze the hydrolysis of a broad and overlapping range of phosphomonesters with an optimum pH below 7.0 (Vincent *et al.*, 1992). The physiological role of these enzymes in cells is not well understood, partly because acid phosphatases are widely distributed in nature and appear to be ubiquitous, exhibiting minimal substrate specificity (Duff *et al.*, 1994). However, acid phosphatases have been implicated in the release, transport, and recycling of inorganic phosphate (Yoneyama *et al.*, 2004**)**. Recently, Hoehamer *et al.* (2005) reported the hydrolysis of some organophosphate insecticides by *Spirodela oligorrhiza* (an aquatic plant) acid phosphatase. This study indicated that the hydrolysis of nerve agents was enzyme-mediated. Thus, the enzyme responsible for *in vivo* transformation of the nerve agents could be an unspecific phosphatase with functional capabilities similar to the *S. oligorrhiza* acid phosphatase (Hoehamer *et al.*, 2005). In seeds and seedlings, the physiological function of acid phosphatases is to provide inorganic phosphate to the growing plant during germination. Furthermore, many different phosphate esters of sugars and substrates stored in the seed and seedling also need to be hydrolyzed during germination and growth (Gahan and McLean, 1969; Schultz and Jensen, 1981; Akiyama and Suzuki, 1981). Plant acid phosphatase expression is mediated by a variety of environmental and developmental factors (Duff *et al.*, 1994) also is species-specific. 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). To date, few works concerning the

Correspondence to:* **Sebastien Niamke, Ph.D.
Tel: +225 07 846409; Fax: +225 20 304303
E-mail: niamkes@yahoo.fr

purification of acid phosphatase (Basha, 1984) and lysophosphatidic acid phosphatase (Shekhar *et al.*, 2002) from peanut seeds have been reported.

In the present study, we report the purification, characterization and detailed comparison of two distinct acid phosphatases isoenzymes from cotyledons of germinated peanut seed. These data should provide some basic knowledge for additional studies on the tropical peanut seed agriculture development.

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-5´-diphosphate (ADP), adenosine-5´-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD); 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-100 HR and Phenyl-Sepharose HP were purchased from Pharmacia Biotech. Standard proteins were obtained from Bio-Rad. All the other reagents used were of analytical grade.

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

Enzyme extraction: Peanut cotyledons (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 7750 ×*g* for 30 min. The supernatant filtered through cotton was used as the crude extract and conserved at 4°C.

Enzyme assay: The standard acid phosphatase (PCAP 1 or PCAP 2) assay was performed in a total volume of 250 µl, containing 100 mM sodium acetate buffer (pH 5.0), substrate (*p*NPP, 5 mM) and the enzyme crude extract (25 µl). The reaction mixture was incubated at 37°C for 10 min, then 2 ml of Na_2CO_3 (2% w/v) was added to stop the reaction. The optical density of the samples was measured at 410 nm and *p*NP as the standard using a spectrophotometer. Under the above experimental conditions, one unit of enzyme activity was defined as releasing 1 µmol of *p*NP per min. Specific activity was defined as the units of enzyme activity per mg of protein.

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

Enzyme purification: All purification steps were carried out in the cold room. The crude extract from peanut cotyledons (five days old) was loaded onto a DEAE-Sepharose CL-6B column (2.6 × 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 (0.1; 0.3; 0.5 and 1M) NaCl, in 20 mM sodium acetate buffer, pH 5.0 (flow rate 1 ml/min, 3.0 ml fractions).

Pooled unbound acid phosphatase activity was loaded onto a CM-Sepharose CL-6B column (2.6 × 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 sulfate was slowly added to give a final concentration of 4.2 M (80% saturation). The pellet obtained after centrifugation (6,000 rpm) was suspended in 1 ml of 20 mM sodium acetate buffer pH 5.0 and applied to Sephacryl -100 HR column (1.6 × 65) equilibrated with the same buffer. Fractions of 1ml were collected at a flow rate of 0.25 ml/min and those containing acid phosphatase activity were pooled. Each phosphatase activity obtained was satured to a final concentration of 1.7 M sodium thiosulfate and applied to a Phenyl-Sepharose HP column (1.4 × 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 thio-

sulfate concentration (from 0.9 to 0.0 M) in the same sodium acetate buffer at a flow rate of 0.33 ml/min. Fractions of 1 ml were collected. The pooled active fractions, dialyzed overnight against 20 mM sodium acetate buffer, pH 5.0, 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. For 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 weight determination: The purified enzymes were 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 weight. Molecular weight standards used were β-amylase (200,000 Da), BSA (66,000 Da), ovalbumin (45,000 Da) and cytochrome C (12,400 Da).

Temperature and pH optimum: The effect of pH on enzyme activitiy was determined by measuring the hydrolysis of *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 (100 mM) from pH 3.6 to 5.6 and sodium citrate (100 mM) from pH 4.6 to 6.0. The pH value of each buffer were determined at 25°C.

The effect of temperature on each acid phosphatase activity was performed in 100 mM acetate buffer pH 5.0 or pH 5.6 according to the isoenzyme 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 ranging from 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 phosphatase activity.

The thermal inactivation of the enzymes was determined at 37 and 55°C for a period of 10 to 120 min. The enzymes were incubated in 100 mM acetate buffer at pH 5.0 for PCAP 2 and pH 5.6 for PCAP 1. Aliquots were withdrawn at intervals and immediately cooled. For thermal denaturation tests, aliquots of each 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, were expressed as percentage activity of zero-time control of untreated enzyme.

Substrate specificity and kinetic parameters: Substrate specification of PCAP 1 and PCAP 2 were determined by incubating each enzyme with various phosphorylated substrates (10 mM) at 37°C in 100 mM sodium acetate buffer at pH 5.6 and 5.0, respectively for 30 min except sodium phytate which was incubated at 50°C for 2h. 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) of PCAP 1 and PCAP 2 were determined in 100 mM sodium acetate buffer at pH 5.6 and 5.0, respectively at 37°C. The hydrolysis of *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 (0.5 to 6 mM) 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 (5mM) was added to the medium and incubated at 37°C for 10 min. The residual activity was assayed under 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 phosphatase activity (0.4 units/mg) was obtained at the 5 days after germination (Fig. 1). The acid phosphatasic activity decreased progressively and the activity was around 0.13 units/mg at 13 days after germination.

Purification: The results of the purification of acid phosphatases from peanut cotyledons are summarized in Table 1. Two isoenzymes were purified from the crude extract.

One major peak of acid phosphatase activity was resolved on the DEAE-Sepharose CL-6B column, after washing the column with 20 mM sodium acetate buffer (pH 5.0) and was designated peanut cotyledon acid phosphatase (PCAP).

The PCAP fractions were subjected to cation-exchange chromatography on CM-Sepharose CL-6B column. A single peak of PCAP activity was eluted at 0.3 M NaCl. After this step, the pooled fractions were loaded onto a gel filtration chromatography on Sephacryl S-100 HR column. Two peaks showing acid phosphatase activity were resolved and were designated PCAP 1 and PCAP 2, respectively. These two acid phosphatase activities were ultimately purified using hydrophobic interaction on phenyl-Sepharose HP gel. PCAP 1 was eluted at 0.5 M sodium thiosulfate and PCAP 2 at 0.7 M sodium thiosulfate with 57.5 fold and 98.8 fold, respectively.

Each acid phosphatase isoenzyme (PCAP 1 and PCAP 2) showed a single protein band on native-polyacrylamide gel electrophoresis staining with Coomassie brillant blue R-250 (Fig. 2).

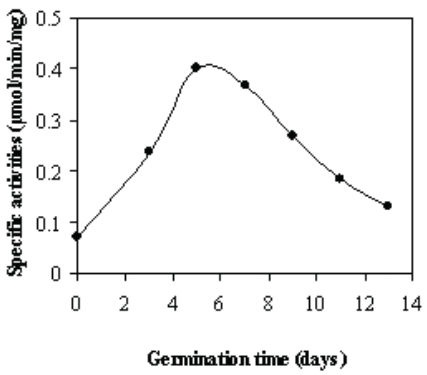

Figure 1. Evolution of acid phosphatase activity during peanut seed germination, using *p*NPP as substrate. The experiments were performed in triplicate and the values given are the averages.

Molecular weight estimation: After SDS-PAGE analysis under reducing conditions, PCAP 1 and PCAP 2 showed a single protein band for each protein (Fig. 3). Their molecular weights were estimated to 27 and 29 kDa, respectively.

Gel permeation chromatography with TSK QC-PAK GFC 200 column showed that purified enzymes PCAP 1 and PCAP 2 had molecular weight of 27.5 and 24 kDa, respectively. These results suggest that the purified enzymes had monomeric structures

Effect of pH and temperature: The effect of pH and temperature on the two acid phosphatase activities is shown in Table 2, Figure 4, 5, 6 and 7. The pH optima for PCAP 1 and PCAP 2 were 5.6 and 5.0, respectively (Fig. 4). At 37°C, PCAP 1 was stable over a pH range 5.0 to 6.0, while PCAP 2 exhibited its stability over a pH range from 4.6 to 5.6 (Table 2).The two enzymes were most active at 55°C (Fig. 5).

The thermal inactivation studies indicated that at their pH optima, the two acid phosphatases remained fully stable for 120 min at 37°C (data not shown). But at 55°C (optimum temperature) PCAP 1 and PCAP 2 were less stable and lost 93 and 75% of their hydrolyt-

Table 1. Purification of PCAP 1 and PCAP 2 from cotyledon of germinating peanut seed. Values given are the averages of at least three experiments.

Purification steps	Total protein (mg)	Total activity (Units)[a]	Specific activity (Units/mg)	Yield (%)	Purification factor
Crude extract	314.2	125.7	0.4	100	1
DEAE-Sepharose CL-6B					
PCAP	74	75	1	59.6	2.5
CM-Sepharose CL-6B					
PCAP	27.9	70.8	2.5	56.3	6.3
Sephacryl-S 100 HR					
PCAP 1	5.3	19.3	3.6	15.3	9
PCAP 2	4.5	22.1	4.9	17.6	12.3
Phenyl-Sepharose HP					
PCAP 1	0.3	6.9	23	5.5	57.5
PCAP 2	0.2	7.9	39.5	6.3	98.8

[a] One units equals 1 mmol of pNP release per min

Figure 2. Native-PAGE analysis of the purified enzymes PCAP 1 and PCAP 2 from peanut seed. The samples were loaded onto a 12% gel. Lane 1, crude extract of peanut cotyledon; lane 2, PCAP 1; lane 3, PCAP 2.

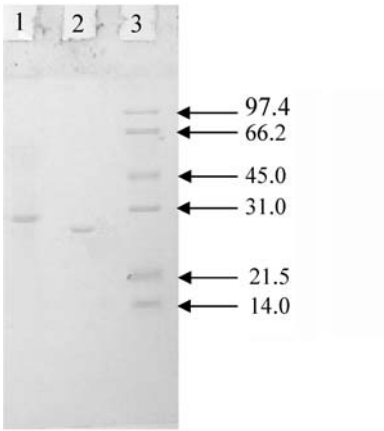

Figure 3. SDS-PAGE analysis of the purified acid phosphatases from peanut seed. The samples were loaded onto a 12% gel. Lane 1, PCAP 2; lane 2, PCAP 1; lane 3, molecular weight marker. Numbers on the right indicate the molecular mass (kDa) of the makers.

Table 2. Some physicochemical characteristics of PCAP 1 and PCAP 2 from cotyledons of germinating peanut seed. Values given are the average of at least three experiments.

Physicochemical properties	Values	
	PCAP 1	PCAP 2
Optimum temperature (°C)	55	55
Optimum pH	5.6	5.0
pH stability	5.0-6.0	4.6-5.6
Molecular weight (kDa)	-	-
SDS-PAGE	27	29
Gel filtration	27.5	24
Q_{10}	1.75	1.38
Activation energy (kJ/mol)	42.45	27.19

Table 3. Substrate specificity of purified PCAP 1 and PCAP 2 from cotyledons of germinating peanut seed. Values given are the average of at least three experiments.

Substrates	Hydrolysis activity (%)	
	PCAP 1	PCAP 2
p-Nitrophenylphosphate	100	100
Phenylphosphate	85	98
Sodium pyrophosphate	70	55
Glucose-1-phosphate	0	4
Glucose-6-phosphate	0	10
Adenosine-5´-monophosphate	9	12
Adenosine-5´-diphosphate	82	79
Adenosine-5´-triphosphate	91	120
α-NAD [a]	0	0
Sodium phytate [b]	0	0

[a] Nicotinamide adenine dinucleotide; [b] Substrate incubated with the enzyme solution at 50°C for 2h.

ic activity after 120 min of preincubation, respectively. Their half-lifes were obtained at 38 and 60 min, respectively (Fig. 6).

The thermal denaturation was investigated by incubating the two enzymes at various temperatures for 15 min. The enzymes were fairly stable at temperature up to 55°C (Fig. 7). Above 55°C, their activities declined rapidly as the temperature increased.

Substrate specificity and kinetic properties: The results are summarized in Table 3. PCAP 1 and PCAP 2 hydrolyzed a broad range of phosphorylated substrates. The highest activity of PCAP 2 was observed with ATP and the synthetic substrate *p*NPP followed by phenylphosphate. PCAP 1 also hydrolyzed energetic substrates, and its highest activity was obtained with *p*NPP. The two enzymes did not hydrolyzed sodium phytate.

The kinectic parameters for the two acid phosphatases were studied using *p*NPP, phenylphosphate and ATP as substrates (Table 4). With the three substrates, PCAP 1 and PCAP 2 obeyed the Michaelis-Menten equation. The K_M and V_{max} values are reported in Table 4. The catalytic efficiency of the enzymes given by the V_{max}/K_M ratio is much higer for the synthetic substrate (*p*NPP) than for the natural substrates (Table 4).

Effect of metal ions, reducing and detergent agents: The influence of various metal ions and chelating agents on the purified enzymes PCAP 1 and PCAP 2 is presented in Table 5. The two enzymes showed different behaviours in the presence of these compounds. PCAP 1 was activated by Mg^{2+}, Fe^{3+}, Sr^{2+} and Ba^{2+}, while Zn^{2+}, Cu^{2+} and EDTA had an inhibitory effect (Table 5). The other ions had little effect or none. For PCAP 2, its activity was enhanced by all the ions tested and EDTA. However, Cu^{2+} and Zn^{2+} which had an

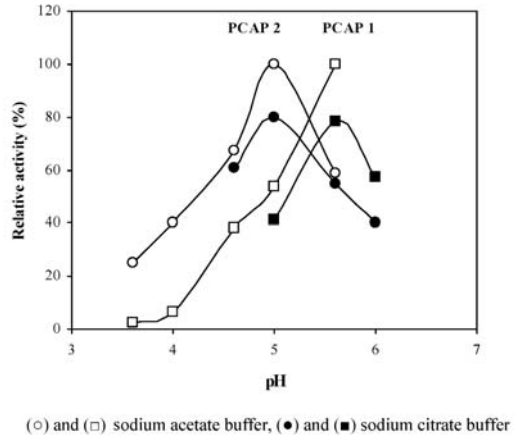

Figure 4. Effect of pH on the peanut cotyledon acid phosphatases (PCAP 1 and PCAP 2) activities. The experiments were carried out at 37°C with *p*-nitrophenylphosphate as substrate in series of buffers at various pH values ranging from pH 3.6 to 6.0. The buffers used were acetate buffer (100 mM) from pH 3.6 to 5.6; sodium citrate buffer (100 mM) from 4.6 to 6.0. Phosphatase activity was measured at 37°C under the standard test conditions. Values given are the average from at least three experiments.

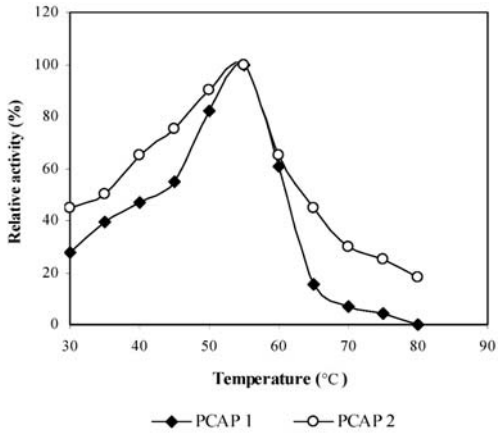

Figure 5. Effect of temperature on PCAP 1 and PCAP 2 activities. The experiments were carried out at the indicated temperature for 10 min in 100 mM acetate buffer pH 5.6 (for PCAP 1) and pH 5.0 (for PCAP 2) using *p*-nitrophenylphosphate as substrate under the standard test conditions. Values given are the average from at least three experiments.

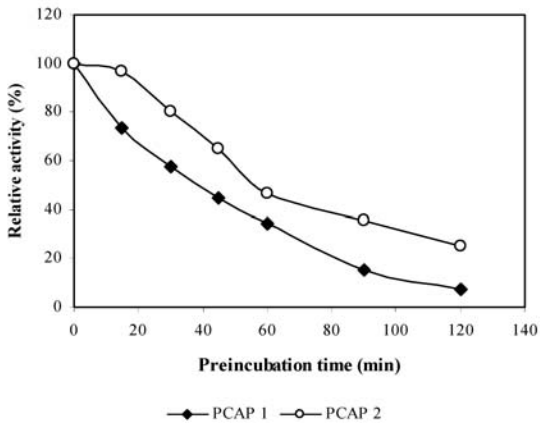

Figure 6. Thermal inactivation of the purified enzymes PCAP1 and PCAP 2 from peanut seed. The enzymes were preincubated at 55°C in 100 mM sodium acetate buffer (pH 5.6 or 5.0). At the indicated times, aliquots were withdrawn and the residual activity was measured at 37°C under the standard assay conditions.

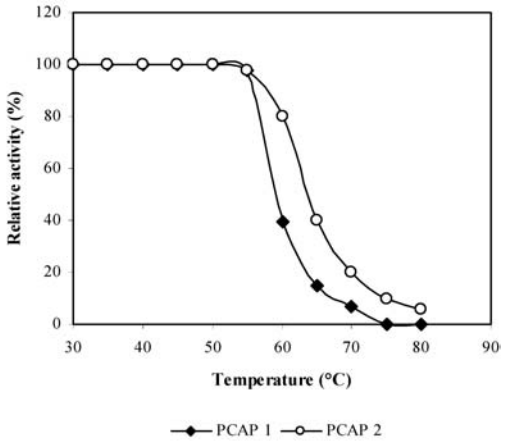

Figure 7. Thermal denaturation of the purified enzymes PCAP1 and PCAP 2 from peanut seed. The enzymes were preincubated at each temperature for 15 min. The remaining activity was measured at 37°C under the standard assay conditions.

activator effect at 1 mM, showed inhibitory effect at 5 mM. Thus, the common activator ions of the two acid phosphatases were Mg^{2+}, Sr^{2+}, Fe^{3+} and Ba^{2+}.

Table 6 shows the effect of reducing agents on the purified enzymes PCAP 1 and PCAP 2. With exception of DL-dithiothreitol which displayed little stimulatory effect on PCAP 1, all the other reducing agents tested were found to be inhibitory for the two acid phosphatases.

The influence of various detergents on the two acid phosphatase activities was studied (Table 7). All the detergents tested stimulated PCAP 1 and PCAP 2 with exception of cationic detergents and SDS. At 0.1% concentration, cationic detergents showed inhibition of PCAP 1 activity by 27 to 43%. SDS was a strong inhibitor of the two enzymes.

DISCUSSION

Germination induces the synthesis or activation of enzymes responsible for the degradation of seed 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 their activ-

Table 4. Kinetic parameters of purified PCAP 1 and PCAP 2 from cotyledons of germinating peanut seed.

Substrates	PCAP 1			PCAP 2		
	K_M	Vmax	Vmax/K_M	K_M	Vmax	Vmax/K_M
*p*NPP	0.67	25.25	37.68	0.51	50.25	98.52
phenylphosphate	0.56	11.17	19.94	1.92	20.66	10.76
ATP	0.53	14.14	26.67	0.64	18.34	28.34

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

Table 5. Effect of some metal ions and chelating agent on the activity of PCAP 1 and PCAP 2 from cotyledons of germinating peanut seed.

Reagent	Concentration (mM)	Relative activity (%)	
		PCAP 1	PCAP 2
Control	0	100	100
	1	100	101
Na^+	5	100	123
	1	100	102
K^+	5	100	116
	1	104	125
Mg^{2+}	5	151	130
	1	119	114
Sr^{2+}	5	147	115
	1	141	117
Fe^{3+}	5	213	146
	1	58	105
Ca^{2+}	5	38	123
	1	112	100
Ba^{2+}	5	134	112
	1	48	120
Cu^{2+}	5	25	91
	1	17	120
Zn^{2+}	5	15	94
	1	94.87	110
EDTA	5	91	150

Values given are the average of at least three experiments.

Table 6. Effect of reducing agents on the activity of PCAP 1 and PCAP 2 from cotyledons of germinating peanut seed.

Reducing agent	Concentration	Relative activity (%)	
		PCAP 1	PCAP 2
DL-Dithiothreitol	0.1 % (w/v)	111	100
*p*CMB [a]	0.1 % (w/v)	65	51
DTNB [b]	0.1 % (w/v)	10	8
L-cyteine	0.1 % (w/v)	87.61	40.89
β-mercaptoethanol	0.1 % (v/v)	77.88	85

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

ities 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 a 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. Taken together, these results suggest that temperature and species have an influence on seed germination. However, 3 to 9 days of germination seems to be corresponding to rapid cell growth and division regarding peanut cotyledons acid phosphatase activity (Hegeman and Grabau, 2001).

Indeed, to understand the role played by acid phosphatase during germination of peanut seeds germination, two acid phosphatases from the crude extract of peanut cotyledons were purified to homogeneity by

Table 7. Effect of some detergents on the activity of PCAP 1 and PCAP 2 from cotyledons of germinating peanut seed.

Detergents	Concentration	Relative activity (% of control)	
		PCAP 1	PCAP 2
Anionic			
SDS	0.1 % (w/v)	0	56
Taurocholic acid sodium salt	1.0 % (w/v)	170	215
Polyoxyethylene-9-lauryl ether	1.0 % (w/v)	225	248
Non ionic			
Triton X-100	1.0 % (v/v)	147	225
Nonidet P 40	1.0 % (v/v)	170	238
Cationic			
Tretradecyl Trimethyl Ammonium Bromide	0.1 % (w/v)	57	226
Hexadecyl Trimethyl Ammonium Bromide	0.1 % (w/v)	73	230

Values given are the average of at least three experiments.

four chromatography processes and their properties were examined.

SDS-PAGE estimated the molecular weights of PCAP 1 and PCAP 2 to be 27 and 29 kDa, and by gel filtration chromatography, 27.5 and 24 kDa, respectively. Thus, the native structures of these enzymes were monomeric. However, the molecular weight of PCAP 2 determined by gel filtration (24.0 kDa), was somewhat lower than that obtained by SDS-PAGE analysis (29 kDa), probably due to specific interactions between glycosyl residues and gel filtration resin. In comparison to other molecular weights of purified plant acid phosphatases, molecular weight of peanut cotyledon acid phosphatases were lower than those from potato tuber (100 kDa) (Gellatly *et al.*, 1994), peanut seed (240 kDa) (Basha, 1984), tomato cell cultures (92 kDa) (Paul and Williamson, 1987), barley roots (79 kDa) (Panara *et al.*, 1990), wheat seedlings (35 kDa) (Chen and Tao, 1989) and soybean seeds (51, 58, 52 and 30 kDa) (Ferreira *et al.*, 1998). Therefore, peanut cotyledon acid phosphatases were low molecular weight acid phosphatases. However, these acid phosphatases had higher molecular weights than the purified acid phosphatase from bovine heart (18 kDa) (Zhang and Van-Etten, 1990).

The effect of pH on the two acid phosphatases showed that PCAP 1 and PCAP 2 were stable in a range of 5.0-6.0 and 4.6-5.6 with closely pH optima of 5.6 and 5.0, respectively. 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 two enzymes. The pH optima determined for these acid phosphatases were largely consistent with other pH optima (5.0-6.0) (Ferreira *et al.*, 1998; Duff *et al.*, 1989; Haas *et al.*, 1991).

The two acid phosphatases from peanut cotyledon were optimally active at 55°C. However, they were sensitive to temperature above 55°C and lost 75 to 93% of their catalytic activity after 120 min of preincubation. This behaviour seems to be common for 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 two 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 toward a single substrate. However, natural substrates such as phenylphosphate, ADP and ATP had the highest relative rate of hydrolysis, while sodium phytate was not hydrolyzed by the two enzymes. 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). The hydrolysis of phosphate esters is an important process in energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathway of plant cells (Vincent *et al.*, 1992). These observations show that purified acid phosphatases from peanut cotyledon play a peculiar role during seed germination. Thus, we suggest that these acid phosphatases seem to be involved in energy transfer and release of Pi and other reserve materials for the growing plant. For the two enzymes, kinetic study correlates well with substrate specificity and showed a Lineweaver-Burk plot. Among the substrates used, PCAP 1 and PCAP 2 showed the highest catalytic efficiency (V_{max}/K_M) toward *p*NPP. The K_M value (0.51 mM) of PCAP 2 was consistent with the K_M value (0.49 mM) of acid phosphatase AP1 from soybean seeds toward *p*NPP (Ferreira *et al.*, 1998). Judging from the Vmax/ K_M, ATP was considered as a best natural substrate for the two acid phosphatases. The relative high activity for ATP and ADP indicates the possibility to apply these enzymes to synchronous enzyme-reaction system, which needs energy resulting from the hydrolysis of these substrates.

The purified PCAP 1 and PCAP 2 were sensitive to ions at various degrees, depending on ion natures and isoenzymes. The requirement of metal 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).

Indeed, for the two enzymes, Mg^{2+}, Fe^{3+}, Sr^{2+} and Ba^{2+} were found to be the common activator ions. The stimulatory effect of Mg^{2+} has also been shown for various plant acid phosphatases (Gellatly *et al.*, 1994; Turner and Plaxton, 2001; Duff *et al.*, 1991; Bozzo *et al.*, 2002). Zn^{2+} and Cu^{2+} were inhibitory for PCAP 1, while they activated PCAP 2. However, the level of Zn^{2+} and Cu^{2+} concentration concerning PCAP 2 activation should also be noted, because it seems critical for the enzyme activities. The concentration of these cations must not exceed 1 mM in reaction mixture. Similar 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 cotyledon acid phosphatases by giving best stability. Triton X-100 was an activator for the two 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 two acid phosphatases were sensitive to reducing agents, except dithiothreitol. These chemicals must be eliminated after treatment of substrate when this needs to be reduced before its hydrolysis by the enzymes.

Although, the two acid phosphatases purified from cotyledon of germinated peanut seeds had identical optimum temperature, their molecular weights, kinetic properties, substrates specificities and effect of various chemicals confirmed that these enzymes were different.

Acknowledgements

This work was supported by Ph.D. grant to the first author. The authors are grateful to professor Bernard Colas, Université de Nantes, France. Unité de Recherche sur la Biocatalyse, CNRS-UMR 6204 for assistance.

References

Akiyama T, Suzuki H (1981). Localization of acid phosphatases in aleurone layers of wheat seeds. *Pfanzenphysiol*, 101:131

Basha SM (1984). Purification and characterization of an acid phosphatase from peanut (*Arachis hypogaea*) seed. *Can J Bot.* 62: 385-391.

Biwas TK, Cundiff C (1991). Multiple forms of acid phosphatases in germinating seeds of *vigna sinensis. Phytochem*. 30:2119-2125.

Bozzo GG, Raghothama KG, Plaxton WC (2002). Purification and characterization of two secreted purple acid phosphatase isozymes from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures. *Eur J Biochem.* 269:6278-6286.

Chen HF, Tao M (1989). Purification and characterization of a phosphotyrosyl-protein phosphatase from wheat seedlings. *Biochim Biophys Acta.* 19:271-276.

Duff SMG, Plaxton WC, Lefebvre DD (1991). Phosphate-starvation response in plant cells: De novo synthesis and degradation of acid phosphatases. *Proc Natl Acad Sci USA.* 88:9538-9542.

Duff SMG, Lefebvre DD, Plaxton WC (1989). Purification and characterization of a phosphonoenol pyruvate phosphatase from *Brassica nigra* suspension cells. *Plant Physiol.* 90:734-741.

Duff SMG, Sarah G, Plaxton WC (1994). The role of acid phosphatases in plant phosphorus metabolism. *Plant Physiol.* 90:791-800.

Ferreira CV, Granjeiro JM, Taga EM, Aoyama H (1998) .Purification and characterization of multiples forms of soybean seed acid phosphatases. *Plant Physiol Biochem.* 36:487-494.

Gahan PB, McLean J (1969). Subcellular localization and possible functions of acid β-glycerophosphatase and naphtol esterases in plant cells. *Planta*, 89:126-136.

Gellatly KS, Moorehead GBG, Duff SMG, Lefebvre DD, Plaxton WC (1994). Purification and characterization of potato tuber acid phosphatase having significant phosphotyrosine activity. *Plant Physiol.* 106:223-232.

Granjeiro PA, Ferreira CV, Taga EM, Aoyama H (1999). Purification and kinetic properties of a castor bean seed acid phosphatase containing sulffhyldryl groups. *Plant physiol.* 107:151-158.

Guo J, Pesacreta JC (1997). Purification and characterization of an acid phosphatase from the bulb of *allium cepa* L. *J Plant Physiol.* 151:520-527.

Haas H, Redl B, Leitner E, Stoffler (1991). *Penicillium chrysogenum* extracellular acid phosphatase: purification and biochemical characterization. *Biochim Biophys Acta.* 1074:392-397.

Hegeman CE, Grabau EA (2001). A novel phytase with sequence similarity to purple acid phosphatases expressed in cotyledons of germinating soybean seedlings. *Plant Physiol.* 126:1598-1608.

Hoehamer CF, Mazur CS, Wolfe NL (2005). Purification and partial characterization of an acid phosphatase from *spirodela oligorrhiza* and its affinity for selected organophosphate pesticides. *J Agric Food Chem.* 53:90-97.

Kamenan A (1984). Purification et étude physico-chimique de trois phosphatases acides de *Dioscorea cayenensis rotundata*, Thèse de doctorat d'Etat es- sciences physiques Faculté des sciences de l'Université d'Abidjan Cocody (Côte d' Ivoire) 134p.

Kouadio NEJP (2004). Contribution à l'étude du tubercule de taro *xanthosoma sp* variété «atoumbou oronô»: évolution de quelques paramètres biochimiques au cours de la conservation et purification et caractérisation de deux phosphatases acides, Thèse de doctorat 3ème cycle de l'UFR des sciences et technologie des aliments de l' Université d'Abobo-Adjamé (Côte d'Ivoire). 103p.

Kusudo T, Sakaki T, Inouye K (2003). Purification and characterization of purple acid phosphatase PAP1 from dry powder of sweet Potato. *Biosci Biotechnol Biochem.* 67:1609-1611.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London), 227:658-685.

Lowry OH, Rosebrough NJ, Farrra L, Randall RJ (1951). Protein measurement with Folin-phenol reagent. *J Biol Chem.* 193:265-275.

Mizuta S, Suda S (1980). A comparative study of multiple acid phosphatases in cellular fractions of bean hypocotyls. *Ann Bot.* 45:369-382.

Olczak M, Watorek W, Morawieka B (1997). Purification and characterization of acid phosphatase from yellow Lupin (*Lupinus luteus*) seeds. *Biochim Biophys Acta.* 1341:14-25.

Pan SM, Chen YR (1988). The effect of salt stress on acid phosphatase activity of Zea mays seedlings. *Bot Bull Acad Sin.* 29:33-38.

Pan SM, Huang YF, Chen YR (1987). Characterization and localization of acid phosphatase in the suspension cultured cells of *Nicotiana otophora*. *Taiwania*, 32:1-10.

Panara F, Pasqualini S, Antonielli M (1990). Multiple forms of barley root acid phosphatase: purification and some characteristics of the major cytoplasmic iso-enzyme. *Biochim Biophys Acta.* 1037:73-80.

Paul EM, Williamson VM (1987). Purification of acid phosphatase-1 from a nematode- resistant tomato cultivar. *Plant physiol.* 84:399-403.

Prazeres JN, Ferreira CV, Aoyama H (2004). Acid phosphatase activities during the germination of *Glycine max* seeds. *Plant*

physiol Biochem. 42:15-20.
Scandalois G (1974). Isozyme in development and differentiation. *Annu Rev Plant physiol.* 25:225-258.
Schultz P, Jensen WA (1981). Pre-fertilization ovule development in capsella: ultrastructure and ultracytochemical localization of acid phosphatase in the meiocyte. *Protoplasma.* 107:27-45.
Shekar S, Tumaney AW, Sreenivasa Rao TJV, Rajasekharan R (2002). Isolation of lysophosphatidic acid phosphatase from developing peanut cotyledons. *Plant Physiol.* 128:988-996.
Staswick PE, Papa C, Huang J, Rhee Y (1994). Purification of major soybean leaf acid phosphatase that is increased by seed-pod removal. *Plant physiol.* 104:49-57.
Taussky HH, Shorr E (1953). A microcolometric method for determination of organic phosphorus. *J Biol Chem.* 202:675-685.
Thomas TL (1993). Gene expression during plant embryogenesis and germination: an overview. *plant cell,* 5:1401-1410.
Tso SC, Chen YR (1997). Isolation and characterization of a group III isoenzyme of acid phosphatase from rice plants *Bot Bull Acad Sin,* 38:245-250.
Turner WL, Plaxton WC (2001). Purification and characterization of banana fruit acid phosphatase. *Planta,* 214:243-249.
Ullah AH, Gibson DM (1988). Purification and characterization of acid phosphatase from cotyledons of germinating soybean seeds. *Arch Biochem Biophys,* 260:514-520.
Vincent JB, Crowder MW, Averill BA (1992). Hydrolysis of phosphate monoester: a biological problem with multiple chemical solutions *Trends Biochem Sci,* 17:105-110.
Yenigun B, Guvenilir Y (2003). Partial purification and kinetic characterization of acid phosphatase from garlic seedling. *Appl Biotechnol,* 105:677-687.
Yoneyama T, Shiozawa M, Nakamura M, Suzuki T, Sagane Y, Katoh Y, Watanabe T, Ohyama T (2004). Characterization of a novel acid phosphatase from embryonic axes of kidney bean exhibiting vanadate-dependent chloroperoxidase activity. *J Biol Chem,* 279:37477-37484.
Zhang ZY, Van-Etten RL (1990). Purification and characterization of a low-molecular-weight acid phosphatase-A Phosphotyrosyl-protein phosphatase from bovine heart. *Arch Biochem Biophys,* 282:39-49.