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Biochemical characterization of two non-specific acid phosphatases from Cucurbitaceae (*Lagenaria siceraria*) edible seeds exhibiting phytasic activity

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1 SUMMARY

Investigation on the enzymatic potential of oleaginous cucurbit (*Lagenaria siceraria* round-fruited cultivar) seeds led to purification of two acid phosphatases named R*Ls*AP1 and R*Ls*AP2. Properties of these enzymes were examined in order to explore their potential in biotechnology applications. A four steps procedure including, anion and cation exchange, size exclusion and hydrophobic interaction chromatography were used for purification. The enzymes had native molecular weights of approximately 70 and 55 kDa, respectively and functioned both as dimeric structures. The two phosphatases displayed acidic and mesophilic activities by using *para*-nitrophenylphosphate as a substrate. Their activities were enhanced by Mg^{2+}, K^{+} and ethylene diamine tetraacetic acid (EDTA). Substrate specificity indicated that the two enzymes hydrolyzed a broad range of phosphorylated substrates mainly consisting of adenosine-5'-triphosphate (ATP) and adenosine-5'-diphosphate (ADP) hydrolysis. Furthermore, the purified acid phosphatase R*Ls*AP1 exhibited an interesting phytasic activity. These results suggest that the two purified acid phosphatases might play an important role in energy transfer, releasing of inorganic phosphate and in reducing the rate of phytate, an antinutrient contained in this plant seeds. R*Ls*AP1 could find potential use in both human food and animal feed.

2 INTRODUCTION

Phosphatases are enzymes that are generally classified as alkaline or acid based solely upon whether their optimal activity is above or below pH 7.0 (Vincent *et al.*, 1992; Duff *et al.*, 1994). Plant alkaline phosphatases have been characterized as having specific metabolic roles whereas most of acid phosphatases have been shown to display broad specificity over a variety of phosphorylated substrates *in vitro* (Penheiter *et al.*, 1997; Yan *et al.*, 2001). Consequently, the physiological role of acid phosphatases in cells is not well understood, partly because they are widely distributed in nature and appear to be ubiquitous, exhibiting minimal substrate

specificity (Duff *et al*., 1994). Acid phosphatases are implicated in the release, transport and recycling of inorganic phosphate (Yoneyama *et al*., 2004). Elsewhere, Hoehamer *et al.* (2005) have reported on the hydrolysis of some organophosphate insecticides by *Spirodela oligorrhiza* (aquatic plant) acid phosphatase. This study indicated that the hydrolysis of neurotoxic agents was enzyme-mediated. Plant acid phosphatases have been also reported to play a role in defence against herbivorous insects (Liu *et al*., 2005). In seeds and seedlings, the established physiological function of acid phosphatases was to provide inorganic phosphate to the growing plant during germination. Besides this main function, many phosphate esters of sugar and other phosphorylated substrates stored in seeds and seedlings also need to be hydrolyzed during germination and growth by requiring a high phosphatasic activity (Gahan & McLean, 1969; Schultz & Jensen, 1981; Akiyama & Suzuki, 1981). Indeed, these observations led Gonnety et al. (2007) to purify and characterize an acid phosphatase from germinating peanut (*Arachis hypogaea*) seeds that exhibited phytasic activities.
Phytate acts in a broad pH range as a high negatively charged ion and has therefore a tremendous affinity for food component with positive charge(s) such as minerals, trace elements and proteins (Cheryan, 1980; Konietzny & Greiner, 2002). This interaction does not affect only nutritional consequences, but also affects yield and quality of food ingredients such as starch, corn steep liquor or plant protein isolate. Up to now, phytases (Greiner & Konietzny, 2006) or phosphatases exhibiting a phytasic activity (Hamada *et al*., 2004) have been, if not solely, used as animal feed additive in diets largely for swine and poultry, and to some extent for fish. Indeed, the decline in feed phytate results in an increase in the absorption of proteins, amino acids and in mineral bioavailability.
As concern plant acid phosphatases, their expression is mediated by a variety of environmental and developmental factors (Duff *et al*., 1994) and is also species-specific. Many authors have purified and characterized acid phosphatases from various sources as roots (Panara *et al*., 1990), leaves (Staswick *et al*., 1994), bulbs (Guo & Pesacreta, 1997), tubers (Kouadio *et al*., 2006), seedlings (Gonnety *et al*., 2006) and seeds (Konan *et al*., 2008). To date, in spite of the genetic, nutritional and agronomic potentials of *Lagenaria siceraria* which were previously reported (Zoro Bi *et al*., 2006; Loukou *et al*., 2007; Achu *et al*., 2008), the enzymatic potential of their seeds has not yet been explored. These seeds were found to be rich in nutrients (Enujiugha & Ayodele-Oni, 2003), namely proteins (36 ± 2.17%) and fats (45.89 ± 4.73%). They were also described as an important staple food of a high economic value (Bisognin, 2002; Sanjur *et al*., 2002). Moreover, this under-researched crop contributes to the diet of many resource-poor consumers and at the same time generates income for small-holder farmers in developing countries, particularly in Africa (Esfeld *et al*., 2009).
In search of new sources of acid phosphatases, oleaginous cucurbit (*Lagenaria siceraria*) seeds were investigated. The present report could much contribute to the development of this neglected crop.

3 MATERIAL AND METHODS

3.1 Enzymatic source and enzymes extraction: Seeds of *Lagenaria siceraria* round-fruited cultivar were obtained from the collection of the University of Abobo-Adjamé (Abidjan, Côte d'Ivoire). To obtain sufficient number of seeds, *L. siceraria* round-fruited cultivar was grown during the rainy season (April to July) in 2009 at the experimental farm of the University (5°23N, 4°00W, and 7 m above sea level). After three months, the fruits were harvest and split using a stainless steel kitchen knife and the seeds removed for crude extract preparation. Cucurbit seeds (10 g)

were ground using a blender in 20 ml sodium chloride solution 0.9% (w/v). The homogenate was subjected to sonication using a TRANSSONIC T_{420} for 10 min and then centrifuged at 10,000 revolutions for 30 min at 4 °C. The supernatant filtered through cotton wool was used as the crude seeds extract.

3.2 Chemicals: Phosphorylated substrates such as *para*-nitrophenylphosphate (*p*NPP), phenylphosphate, sodium pyrophosphate, adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP), adenosine-5'-triphosphate (ATP), α-nicotinamide adenine dinucleotide (α-NAD); β-nicotinamide adenine dinucleotide (β-NAD), adenosine-2-3'-cyclomonophosphate; glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-1-phosphate (F-1-P), fructose- 6-phosphate (F-6-P), sodium phytate and *para*-nitrophenol (*p*NP) were purchased from Sigma-Aldrich. DEAE-Sepharose Fast Flow, CM-Sepharose CL-6B, Sephacryl S-100 HR and Phenyl-Sepharose 6 Fast Flow were provided from Pharmacia Biotech. Bovine serum albumin (BSA) was obtained from Fluka Biochemika. Standard molecular weights proteins were provided from Bio Rad. All the other reagents used were of analytical grade.

3.3 Enzymes purification: All the purification procedure was carried out in a cold room. The crude enzyme extract of *Lagenaria siceraria* round-fruited cultivar seeds was loaded onto a DEAE-Sepharose Fast Flow column (2.5 × 4.5) equilibrated with 20 mM sodium acetate buffer pH 5.6. The column was washed at a flow rate of 3 ml/min with two bed volumes of equilibration buffer to remove unbound proteins. Bound proteins were then eluted with a stepwise salt gradient (0.1, 0.2, 0.4 and 1 M) of NaCl in 20 mM sodium acetate buffer pH 5.6 and, fractions of 3 ml were collected. Two peaks of phosphatase activity were obtained. On the one hand, unbound acid phosphatase activity (Peak 1) and on the other hand, bound acid phosphatase activity (Peak 2). Each pooled active fraction was subjected to ammonium sulphate precipitation at 80% final saturation. After centrifugation at 10,000 revolutions for 30 min, each precipitate was dissolved in 1 ml of 20 mM sodium acetate buffer (pH 5.6). Then, the resulting solutions were separately loaded onto a Sephacryl S-100 HR column (capacity, 1.5 cm × 67 cm; flow rate, 0.2 ml/min; fractions, 1 ml) previously equilibrated with the same buffer and, active fractions were pooled. On the one hand, pooled acid phosphatase activity resulting from Peak 1 was loaded onto a CM-Sepharose CL-6B column (2.6 × 4.0) equilibrated with 20 mM sodium acetate buffer pH 5.6. The column was washed with the same buffer at a flow rate of 1 ml/min. Acid phosphatase activity was eluted with a stepwise salt gradient (0.2, 0.4 and 1 M) of NaCl in 20 mM sodium acetate buffer pH 5.6 and, fractions of 2 ml were collected. To the pooled active fractions, solid sodium thiosulphate was slowly added to give a final concentration of 1.7 M and the resulting enzyme solution was subsequently applied on a Phenyl Sepharose 6 Fast Flow column (1.5 cm × 3.2 cm) previously equilibrated with 20 mM sodium acetate buffer pH 5.6 containing 1.7 M of sodium thiosulphate. The column was washed at a flow rate of 1 ml/min with a reverse stepwise salt-gradient of sodium thiosulphate (from 1.7 to 0 M) incorporated in the same sodium acetate buffer. 1 mL fractions were collected. The pooled active fractions were dialyzed overnight at 4 °C against 20 mM sodium acetate buffer pH 5.6 and constituted the purified enzyme solution. The second acid phosphatase activity (Peak 2) resulting from DEAE-Sepharose Fast Flow and Sephacryl S-100 HR chromatographies was saturated to 1.7 M final concentration of sodium thiosulphate and loaded onto the Phenyl-Sepharose 6 Fast Flow column according to the same procedure described above. Finally, the pooled active fractions were also dialyzed against 20 mM sodium acetate buffer (pH 5.6) and stored at 4 °C for assays.

3.4 Enzymes assay: The acid phosphatases activity was performed in a total volume of 250 µl, containing 100 mM sodium acetate buffer (pH 5.6), substrate (*p*NPP, 5 mM) and the enzyme solution (25 µl). 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 5. *para*-Nitrophenol (*p*NP) was used as the standard. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per min under the assay conditions. The

specific activity was expressed as unit of activity per mg of protein.

3.5 Proteins estimation: Protein concentrations and elution profiles from chromatographic columns were determined by the Folin method (Lowry *et al*., 1951). Bovine serum albumin (BSA) was used as the standard protein.

3.6 Polyacrylamide gel electrophoresis (PAGE): Electrophoresis was carried out by using Laemmli (1970) method on 10% (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 in a sample-buffer without 2-mercaptoethanol and SDS. Silver staining was used to localize protein bands (Blum et al. 1987). The standard molecular weights (Bio-Rad) comprising myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45.0 kDa) were used.

3.7 Native molecular weights determination: Purified enzymes were applied to gel filtration on a Sephacryl S-200 HR column (0.8 cm × 35 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.6) to estimate the native molecular weights. Elution was done at a flow rate of 0.2 mL/min and fractions of 0.5 ml were collected. Standard molecular weights (SIGMA) used for calibration were β-amylase from sweet potato (206 kDa), BSA (66 kDa), ovalbumin from egg white (45 kDa) and cellulase from *Aspergillus niger* (26 kDa).

3.8 pH and temperature optima: The effect of pH on the enzymes activity was determined by performing the hydrolysis of *para*-nitrophenylphosphate in a series of buffers (100 mM) at various pH values ranging from 3.0 to 6.2. Buffers used were sodium acetate from pH 3.6 to 5.6 and sodium citrate from pH 3.0 to 6.2. pH values of each buffer were determined at 25 °C. The effect of temperature on phosphatase activities was performed in 100 mM sodium acetate buffer (appropriate pHs) over a temperature range of 30 to 80°C using *p*NPP (2.5 mM) as substrate under the enzyme assay conditions.

3.9 pH and temperature stabilities: The pH stability of each phosphatase was studied in a pH range of 3.0 to 6.2 with 100 mM buffers. Buffers used were the same as in pH and temperature optima study (above). After 2 h preincubation at 25 °C (room temperature), residual phosphatase activities were measured at 37 °C for 10 min by adding substrate *para*-nitrophenylphosphate. The thermal inactivation was determined at 37 °C and at each enzyme optimum temperature. Enzymes in appropriate buffers (pHs) were exposed to each temperature for up to 120 min. Aliquots were withdrawn at intervals (10 min) and immediately cooled. Concerning thermal denaturation tests, aliquots of each enzyme solution were preheated at different temperatures ranging from 30 to 80 °C for 15 min. Residual activities, determined in the three cases at 37 °C under the enzyme assay conditions, were expressed as percentage activity of zero-time control of untreated enzymes.

3.10 Effect of some chemical agents: To determine the effect of various compounds (cations, detergents, sulphidryl specific and reducing agents) as possible activators or inhibitors of the purified phosphatases, each enzyme solution was preincubated at 37 °C for 30 min with the compounds and then, the activity was assayed under the enzyme assay conditions. Residual activities were expressed as percentage refers to a control without chemical agents.

3.11 Substrate specificity and kinetic parameters determination: Substrate specificity of the two acid phosphatases was determined by incubating the enzymes with various phosphorylated substrates (10 mM) at 37 °C in 100 mM sodium acetate buffer (pH 5.6) for 30 min. However, when sodium phytate is used as substrate, incubation was done at 50 °C for 2 h. The hydrolysis of these substrates was determined by titration of the released inorganic phosphate according to Heinonen and Lahti (1981) method. Kinetic parameters (K_M, V_{max} and V_{max}/K_M) of the two acid phosphatases were determined in 100 mM sodium acetate buffer (pH 5.6) at 37 °C. The hydrolysis of *p*NPP was quantified on the basis of the released *p*NP as in the standard enzyme assays. As regards the other substrates, their hydrolysis was measured by quantifying the released inorganic phosphate according to Heinonen and Lahti (1981) method. K_M and V_{max} were determined from a Lineweaver-Burk (1934) plot by using different

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concentrations (from 0 to 5 mM) of phosphorylated substrates.

4 RESULTS

4.1 Enzymes purification: The results on the purification of acid phosphatases from *Lagenaria siceraria* round-fruited cultivar seeds are summarized in table 1.

Table 1: Purification procedure of acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds

Purification steps	Total protein (mg)	Total activity (UI)	Specific activity (UI/mg)	Yield (%)	Purification (fold)
Crude extract	233.80	159.50	0.68	100.00	1.00
DEAE-Sepharose Fast-flow					
R*Ls*AP1	55.23	71.45	1.29	44.80	1.90
R*Ls*AP2	31.40	52.00	1.65	32.60	2.43
$(NH_4)_2SO_4$ 80%					
R*Ls*AP1	18.67	44.70	2.39	28.02	3.51
R*Ls*AP2	10.20	27.00	2.64	16.90	3.90
Sephacryl-S 100 HR					
R*Ls*AP1	1.80	24.27	13.48	15.21	19.82
R*Ls*AP2	0.40	8.37	20.92	5.20	30.80
CM-Sepharose CL-6B					
R*Ls*AP1	0.51	9.10	17.84	5.70	26.24
Phenyl-Sepharose 6 Fast Flow					
R*Ls*AP1	0.06	3.27	54.50	2.05	80.15
R*Ls*AP2	0.03	1.90	63.33	1.20	93.14

Note. UI = 1µmol of *p*NP per min.

Two isoenzymes were purified from the crude extract. Purification profiles (data not shown) involved four chromatographic steps for the first isoenzyme and three steps for the second one. Two peaks of acid phosphatase activity named R*Ls*AP1 and R*Ls*AP2 were resolved on the anion-exchange (DEAE-Sepharose Fast Flow) chromatography used as the first step of purification. Active proteins were eluted respectively at 0 and 0.2 M of NaCl. After precipitation in 80% ammonium sulphate, R*Ls*AP1 and R*Ls*AP2 pooled fractions were separately loaded onto a gel filtration chromatography by using a Sephacryl S-100 HR column. One peak showing an acid phosphatase activity was resolved for both of the two activities. R*Ls*AP1 activity was in addition subjected to a cation-exchange chromatography on a CM-Sepharose CL-6B column and, a single peak of bounded acid phosphatase activity was eluted with 0.2 M of NaCl. After these steps, the two acid phosphatase (R*Ls*AP1 and R*Ls*AP2) activities were subsequently purified by using an ultimate hydrophobic chromatography on a phenyl-Sepharose 6 Fast Flow column. The active proteins were eluted with 0.10 and 0.80 M of sodium thiosulphate, respectively (data not shown). Finally, R*Ls*AP1 and R*Ls*AP2 were purified with overall yields of 2.05 and 1.20% and enriched about 76.33 and 93.10 fold, respectively (Table 1). Each isoenzyme showed a single protein band by silver staining on native polyacrylamide gel electrophoresis (figure 1).

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Figure 1: Native-PAGE analysis of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds. The samples were loaded on to a 10% gel. Lane 1, crude extract; Lane 2, R*Ls*AP1; Lane 3, R*Ls*AP2.

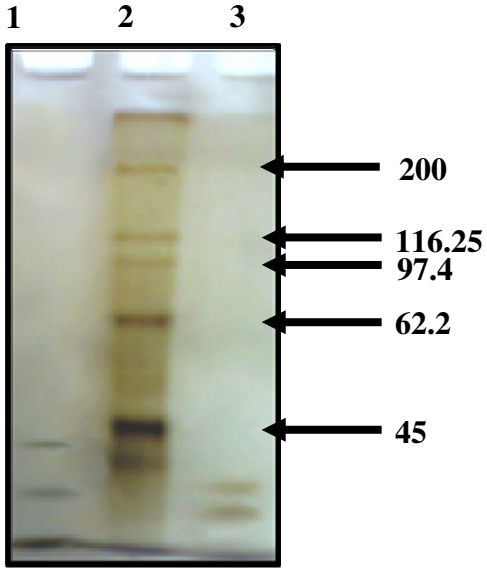

Figure 2: SDS-PAGE analysis of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds. The samples were loaded on to a 10% gel.
Lane 1, R*Ls*AP1; Lane 2, Molecular weight markers; Lane 3, R*Ls*AP2. Numbers on the right indicate the molecular weights (kDa) of protein markers.

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Table 2: Some physicochemical properties of the purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds

Physicochemical properties	Values	
	R*Ls*AP1	R*Ls*AP2
Optimum temperature (°C)	60	55
Optimum pH	5.6	5.6
pH stability	4.8 – 6.2	4.6 – 6.2
Q_{10}	1.9	1.7
Activation energy (kJ/mol)	58.3	49.3
Molecular weight (kDa)		
SDS-PAGE	67	52
Gel filtration	70	55

4.2 Molecular weights estimation: After SDS-PAGE analysis under reducing conditions, each phosphatase showed two protein bands. Their relative molecular weights were estimated to be 67 kDa for R*Ls*AP1 and 52 kDa for R*Ls*AP2 (figure 2). Molecular weights of the native enzymes, as determined by gel filtration, were approximately 70 kDa (R*Ls*AP1) and 55 kDa (R*Ls*AP2) (Table 2).

4.3 Effect of pH and temperature: R*Ls*AP1 and R*Ls*AP2 hydrolytic activities were maximal in sodium acetate buffer (pH 5.6) at 60 and 55 °C, respectively (figures 3, 4). At 37 °C, the purified enzymes showed best stability over pH values ranging from 4.6 to 6.2 (Table 2) conserving at least more than 80% of total activities. Values of temperature coefficients (Q_{10}) calculated were found to be around 1.8. From Arrhenius plot, values of 58.3 and 49.3 kJ/mol were calculated for the activation energy of R*Ls*AP1 and R*Ls*AP2, respectively (Table 2). The thermal inactivation study indicated that at 37 °C, in sodium acetate buffer (pH 5.6), the purified enzymes remained fully stable for 120 min (figure 5). However, at their optimum temperatures, the two phosphatases were less stable with half-lives of around 50 and 40 min for R*Ls*AP1 and R*Ls*AP2, respectively (figure 5). The thermal denaturation (figure 6) showed that the purified enzymes retained 100% of their activities to temperatures up to their optima. Above, their activities declined progressively as the temperature increased. Enzymes were completely inactivated at 80 °C (figure 6).

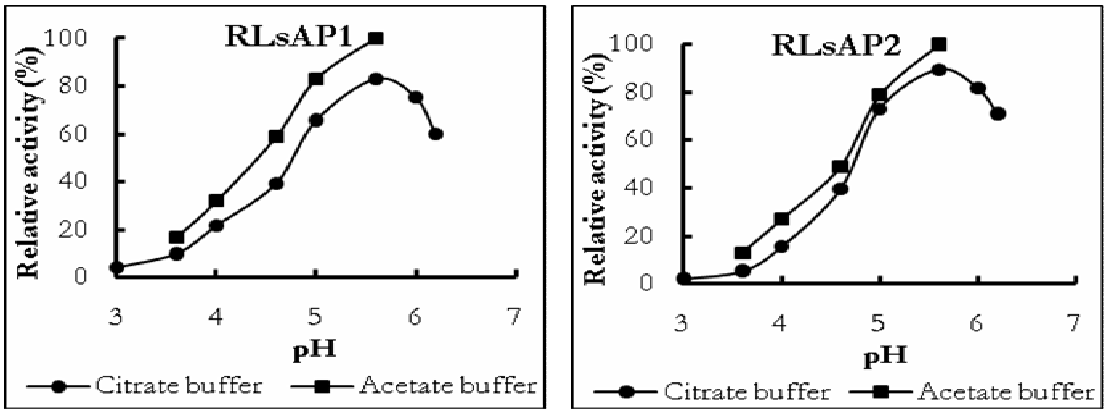

Figure 3: Figure 3: Effect of pH on the activity of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds. The experiments were carried out at 37 °C with *para*-nitrophenylphosphate as substrate in series of buffers at various pH values ranging from pH 3.0 to 6.2. The buffers used were sodium acetate buffer (100 mM) from pH 3.6 to 5.6; sodium citrate buffer (100 mM) from 3.0 to 6.2. Phosphatase activities were measured at 37 °C under the standard test conditions.

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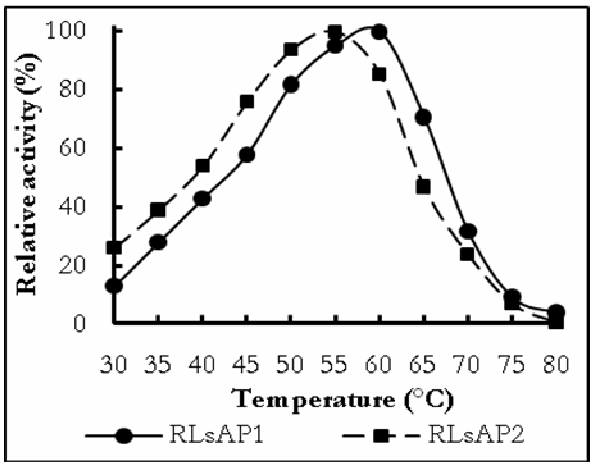

Figure 4: Effect of temperature on the activity of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds. The experiments were carried out at the indicated temperature for 10 min in 100 mM acetate buffer pH 5.6 by using *para*-nitrophenylphosphate as substrate under the standard test conditions.

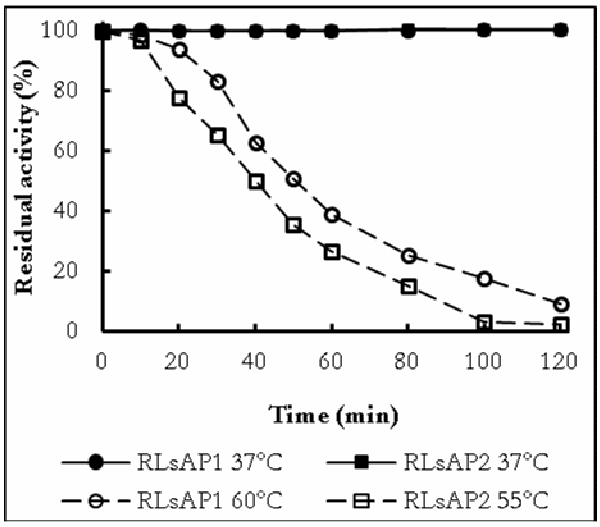

Figure 5: Thermal inactivation of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds. Each enzyme was separately preincubated at 37 °C and its optimum temperature, in 100 mM sodium acetate buffer (pH 5.6). At the indicated times, aliquots were withdrawn and the residual activity measured at 37 °C under the enzyme assay conditions.

4.4 Effect of metal ions, chelating, sulphidryl specific and reducing agents and detergents: The effect of some chemicals on the two acid phosphatase activities from *Lagenaria siceraria* round-fruited cultivar seeds was examined. As regards the influence of metal ions and EDTA (Table 3), common activators for the two enzymes were K^+, Mg^{2+} and EDTA while Zn^{2+} (1 mM) displayed a strong inhibitory effect (inhibition in the range of 90%). The other cations tested (Na^+, Ca^{2+} and Ba^{2+}) showed a slight stimulatory effect on R*Ls*AP1 and R*Ls*AP2 activities (Table 3).

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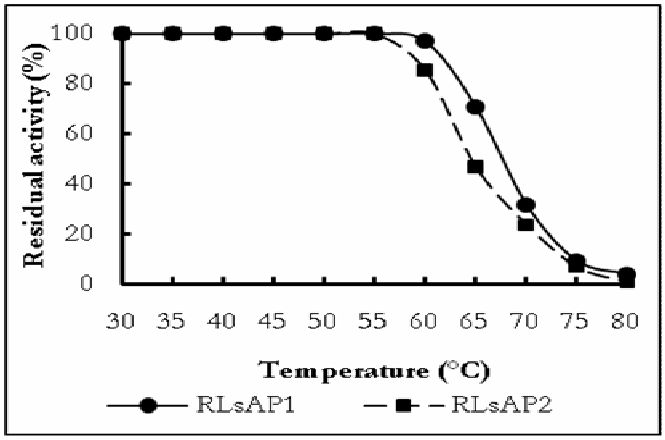

Figure 6: Thermal denaturation of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds. The experiments were carried out at temperatures ranging from 30 to 80 °C. The enzymes were preincubated at each temperature for 15 min and the residual activity measured at 37 °C under the enzyme assay conditions.

Table 3: Effect of some cations and chelating agent on the activity of the purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds

Reagent	Concentration (mM)	Relative activity (%)	
		R*Ls*AP1	R*Ls*AP2
Control	0	100	100
Na^+	1	97.5 ± 3.7	105.0 ± 4.5
	5	108.5 ± 2.9	115.5 ± 4.1
K^+	1	106.0 ± 3.1	107.3 ± 3.9
	5	110.3 ± 4.3	120.0 ± 4.2
Mg^{2+}	1	102.8 ± 3.4	115.5 ± 4.5
	5	121.6 ± 4.3	118.7 ± 5.1
Ca^{2+}	1	109.0 ± 5.7	108.0 ± 4.7
	5	109.0 ± 3.9	109.0 ± 4.6
Ba^{2+}	1	111.3 ± 4.8	102.7 ± 2.5
	5	108.2 ± 5.3	107.3 ± 3.1
Zn^{2+}	1	11.5 ± 4.6	10.5 ± 2.8
	5	9.2 ± 1.2	4.6 ± 1.1
EDTA*	1	136.2 ± 2.3	136.1 ± 5.6
	5	139.0 ± 2.9	162.0 ± 6.1

Note. Values given are the averages of at least three experiments.
*Ethylene diamine tetraacetic acid

Sulphidryl specific and reducing agents tested were found to be inhibitory at different degrees on the enzymes activity (Table 4).

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Table 4: Effect of sulphidryl specific and reducing agents on the activity of the purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds

Chemicals	Concentration (% ; w/v)	Relative activity (%) R*Ls*AP1	R*Ls*AP2
Control	0	100	100
L-cysteine	0.1	88.2 ± 4.7	92.4 ± 3.5
	1	73.0 ± 3.9	84.0 ± 4,1
DL dithiothreitol	0.1	105.9 ± 2.9	94.9 ± 4.9
	1	93.0 ± 1.6	72.0 ± 3.5
*p*CMB*	0.1	65.3 ± 3.6	73.8 ± 4.1
	1	55.3 ± 2.6	71.7 ± 3.8
DTNB**	0.1	87.5 ± 4.5	80.3 ± 2.9
	1	63.4 ± 3.2	53.0 ± 3.0
β-Mercaptoethanol	0.1	79.4 ± 2.8	96.5 ± 2.9
	1	74.7 ± 2.3	69.0 ± 1.4

Note. Values given are the averages of at least three experiments
*sodium *para*chloromercuribenzoate.
**5,5'-dithio-2,2' dinitro -dibenzoïc acid.

Table 5: Effect of some detergents on the activity of the purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds

Detergents		Concentration	Relative activity (%) R*Ls*AP1	R*Ls*AP2
Control		0	100	100
Cationic	Tetradecyl Trimethyl Ammonium Bromide	0.1% (w/v)	89.0 ± 4.5	90.2 ± 5.1
		1% (w/v)	74.6 ± 4.8	56.4 ± 4.3
	Hexadecyl Trimethyl Ammonium Bromide	0.1% (w/v)	95.0 ± 5.5	83.3 ± 4,4
		1% (w/v)	68.8 ± 4.9	49.8 ± 2.9
Non ionic	Tween 80	0.1% (v/v)	98.8 ± 3.1	102.0 ± 1.2
		1% (v/v)	90.2 ± 4.8	104.8 ± 2.6
	Lubrol Wx	0.1% (v/v)	76.1 ± 3.7	93.2 ± 3.9
		1% (v/v)	84.2 ± 4.1	93.2 ± 4.5
	Triton X-100	0.1% (v/v)	77.5 ± 5.6	111.1 ± 4.4
		1% (v/v)	50.7 ± 4.2	102.1 ± 3.8
	Nonidet P-40	0.1% (v/v)	80.1 ± 3.8	113.2 ± 2.5
		1% (v/v)	41.2 ± 3.3	81.6 ± 1.4
	Sodium cholate	0.1% (w/v)	94.5 ± 3.2	91.5 ± 4.2
		1% (w/v)	41.5 ± 2.9	80.3 ± 4.1
Anionic	Polyoxyethylene 9 lauryl ether	0.1% (w/v)	86.0 ± 4.6	81.2 ± 3.5
		1% (w/v)	56.6 ± 2.9	74.8 ± 4.7
	Polyoxyethylene 10 oleyl ether	0.1% (w/v)	58.6 ± 3.3	90.6 ± 5.5
		1% (w/v)	49.1 ± 4.2	63.4 ± 3.7
	Sodium dodecyl sulphate	0.1% (w/v)	19.5 ±1.5	12.0 ±1.1
		1% (w/v)	16.5 ± 1.3	10.7 ± 0.7

Note. Values given are the averages of at least three experiments.

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The influence of various detergents on the two acid phosphatase activities was studied (Table 5). Except Triton X-100 (0.1%) and Nonidet P-40 (0.1%) which slightly improved R*Ls*AP2 activity up to 113.2 ± 2.5%, most of detergents tested were found to differently affect the enzymes activity at the same concentration (0.1%). Sharply inhibitions of around 80 or 90% were observed for the two enzyme activities in the presence of 0.1% sodium dodecyl sulphate (SDS) salt (Table 5).

4.5 Substrate specificity and kinetic properties: A variety of phosphorylated compounds were tested for their suitability to serve as substrates (Table 6). R*Ls*AP1 and R*Ls*AP2 hydrolyzed a broad range of these substrates at different rates. The highest activities observed for the two enzymes mainly consisted of the hydrolysis of synthetic substrates such as *p*NPP (100%) and phenylphosphate (74.8 ± 2.5%), and natural substrates as pyrophosphate (98.6 ± 4.6%), ATP (97.5 ± 4.6%) and ADP (89.6 ± 3.5%). Phosphorylated sugars and sodium phytate were to a lesser extent hydrolyzed (up to 43.1 ± 3.1% hydrolysis) by the purified enzymes (Table 6).

Table 6: Substrate specificity of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds

Substrates	Hydrolysis activity (%)	
	R*Ls*AP1	**R*Ls*AP2**
p-Nitrophenylphosphate	100.0 ± 1.0	100.0 ± 1.2
Phenylphosphate	65.0 ± 2.9	74.8 ± 2.5
Sodium pyrophosphate	98.6 ± 4.6	88.7 ± 2.3
Adenosine-2',3'-cyclomonophosphate	29.7 ±2.2	2.1 ± 0.4
Adenosine-5'-monophosphate	35.7 ± 2.5	42.5 ± 2.9
Adenosine-5'-diphosphate	89.6 ± 3.5	80.1 ± 3.1
Adenosine-5'-triphosphate	96.4 ± 3.3	97.5 ± 4.6
Glucose-1-phosphate	2.5 ± 0.7	22.7 ± 2.2
Glucose-6-phosphate	17.1 ± 1.3	43.1 ± 3.1
Fructose-1-phosphate	1.7 ± 0.2	10.0 ± 1.1
Fructose-6-phosphate	18.6 ± 1.4	16.3 ± 0.9
α-Nicotinamide adenine dinucleotide	4.2 ± 0.9	26.4 ± 1.7
β-Nicotinamide adenine dinucleotide	35.7 ± 2.3	1.9 ± 0.2
Sodium phytate	36.4 ± 2.9	2.5 ± 0.2

Note. Values given are the averages of at least three experiments.

Kinetic parameters of the two acid phosphatases were studied using *p*NPP, phenylphosphate, ATP, ADP and pyrophosphate as substrates (Table 7). With these substrates, the enzymes activity was as predicted by the Michaelis-Menten equation. K_M and V_{max} values, obtained by using a Lineweaver-Burk plot in the range of concentrations of 0 to 5.0 mM, are resumed in table 7. The catalytic efficiency of R*Ls*AP1 given by the V_{max}/K_M ratio is practically identical for the hydrolysis of ATP (92.60 ml/min/mg) and the synthetic substrate *p*NPP (91.58 ml/min/mg). By contrast with R*Ls*AP1, R*Ls*AP2 displayed its highest catalytic efficiency (125.79 ml/min/mg) towards the synthetic *p*NPP substrate (Table 7).

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Table 7: Kinetic parameters of purified acid phosphatases from the round-fruited cultivar of *Lagenaria siceraria* seeds towards different substrates

	R*Ls*AP1			**R*Ls*AP2**		
Substrates	K_M (mM)	V_{max} (µmol/min/mg)	V_{max}/K_M (mL/min/mg)	K_M (mM)	V_{max} (µmol/min/mg)	V_{max}/K_M (mL/min/mg)
*p*NPP	0.78	71.43	91.58	0.53	66.67	125.79
Phenylphosphate	0.94	52.63	56.00	0.97	33.33	34.36
ATP	1.20	111.11	92.60	1.89	65.20	34.50
ADP	0.58	41.50	71.55	1.85	52.63	28.45
Pyrophosphate	0.41	25.64	62.53	0.91	49.45	54.34

5 DISCUSSION

The anion-exchange chromatography on a DEAE-Sepharose Fast Flow column used as the first step of purification enabled phosphatase activity in the crude enzyme extract to be separated into two different isoforms (R*Ls*AP1 and R*Ls*AP2), while the hydrophobic interaction chromatography on a Phenyl-Sepharose 6 Fast Flow column led to the separation of each acid phosphatase from the other proteins and impurities. Similar results concerning Phenyl-Sepharose gel have been reported for the purification of acid phosphatases from coco yam (*Xanthosoma sp*) tubers (Kouadio *et al*., 2006), peanut (*Arachis hypogaea*) seedlings (Gonnety *et al*., 2006) and for the purification of other glycosidases (Faulet *et al*., 2006; Bédikou *et al*., 2009).

Regarding molecular properties of the purified enzymes, R*Ls*AP1 and R*Ls*AP2 they functioned as dimeric structures. In comparison with other purified plant acid phosphatases, their relative molecular weights were lower than those of peanut seeds (240 kDa) (Basha, 1984), tomato cell cultures (92 kDa) (Paul & Williamson, 1987), barley roots (79 kDa) (Panara *et al*., 1990) and potato tubers (100 kDa) (Gellatly *et al*., 1994). Nevertheless, they had higher molecular weights than those reported for the purple acid phosphatase (27.3 kDa) purified from breadfruit seeds (Konan *et al*., 2008) and for germinating peanut seeds acid phosphatase (24 kDa) (Gonnety *et al*., 2007).

The purified acid phosphatase activities on *p*NPP were maximal in acidic region. This behaviour is in accordance with other acid phosphatases from castor bean seeds (Granjeiro *et al*., 1999) and tomato (*Lycopersicon esculentum*) cell cultures (Bozzo *et al*., 2002). In addition, the purified enzymes displayed better stability at pHs ranging from 4.6 to 6.2 which is largely consistent with that of other plant acid phosphatases (Haas *et al*., 1991; Ferreira *et al*., 1998). This stability is a good compromise for performing hydrolysis of natural substrates and biosynthesis reactions which required long-term incubations.

Acid phosphatases from *Lagenaria siceraria* seeds were mesophilic. These enzymes remained fully active at 37 °C for a long time (2h). However, half-lives at their optimum temperatures were approximately 40 and 50 min in optimum pH conditions. In this context, running biotechnological processes at moderate temperatures would be advantageous for these enzymes application.

Purified acid phosphatases from *Lagenaria siceraria* round-fruited cultivar seeds, namely R*Ls*AP1 and R*Ls*AP2 were sensitive to cations at various degrees, depending on ion kinds and isoenzymes. Indeed, K^+ and Mg^{2+} enhanced the two enzyme activities, while Zn^{2+} was found to be inhibitory. On the one hand, the stimulatory effect of Mg^{2+} has already been reported for various plant acid phosphatases (Turner & Plaxton, 2001; Bozzo *et al*., 2002). On the other hand, the inhibition by Zn^{2+} was also observed for the purple acid phosphatase isolated from red kidney beans (Cashikar *et al*., 1997) and for acid phosphatase from banana fruit (Turner & Plaxton, 2001). The fact that EDTA (cations chelator) displayed an activatory effect on the enzyme activities could suggest that R*Ls*AP1 and R*Ls*AP2 do not require metal ions at their active site to perform catalysis. The sensitivity of these enzymes to sulphidryl specific agents leads to assume that –SH groups participate in the two

enzymes catalysis. Since the purified acid phosphatases were inhibited by most of cationic, non-ionic, and anionic detergents tested, the concerning chemicals should be avoided in these enzyme solution preparations. However, it should be underlined that the behaviour of R*Ls*AP1 and R*Ls*AP2 towards most of detergents contrasts with those reported for peanut acid phosphatases (Shekar *et al*., 2002; Gonnety *et al*., 2007) which were activated by these chemicals.

The purified enzymes hydrolyzed a broad range of phosphorylated substrates at different degrees. Similar observations were reported for acid phosphatase from sweet potato (Kusudo *et al*., 2003). Among the natural substrates assayed, pyrophosphate, ATP and ADP showed the relatively highest rate of hydrolysis. A higher rate of ADP and ATP hydrolysis was also observed by using acid phosphatases from tobacco cells (Pan & Chen, 1988) and rice seedlings (Tso & Chen, 1997). The relatively high activity toward ATP indicates the possibility to apply these enzymes to synchronous enzyme-reaction system, which needs energy resulting from the hydrolysis of this substrate as reported by Gonnety *et al*. (2006). Indeed, 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 showed that acid phosphatases from *L. siceraria* round-fruited cultivar seeds play an important role during seeds ripening. Thus, these enzymes seemed to be involved in energy transfer, release of inorganic phosphate (Pi) and other reserve materials for the fruit and seeds ripening.

The rate of sodium phytate hydrolysis recorded by R*Ls*AP1 (36%) was higher than that reported for breadfruit purple acid phosphatase (18%) (Konan *et al*., 2008). Consequently, this enzyme could constitute an alternative biocatalyst in cleaving phosphate moieties from phytic acid (myo-inositol-hexakisphosphate), thereby generating myo-inositol, inorganic phosphate and some multivalent cations. It is well known that phytic acid (phytate) chelates multivalent cations and proteins, thereby rendering these biologically unavailable to the animal (Harland & Morris, 1995). Therefore, including adequate amounts of phytase in the diets for simple-stomached animals reduce the need for orthophosphate supplementation of the feed. Thus, the physiological function of this enzyme appears to be more important given that the enzymic conversion of phytic acid within seeds may be a rational alternative to improve the nutritional quality of *Lagenaria siceraria* seeds for humans and animals consumption. In addition, judging by the thermal stability, R*Ls*AP1 can compete with a commercial phytase (Phytase 5000) which is used in food industry for all kinds of processes in which raw material containing phytic acid (wheat, barley, rice, corn or soybean) is processed. This commercial enzyme is particularly active in the temperature range between 35 °C and 65 °C with an optimum at 50 °C. Therefore, R*Ls*AP1 could also find application in food industries as for breweries and distilleries.

5 CONCLUSION

From these results, it appears that the purified acid phosphatases from the neglected crop *Lagenaria siceraria* round-fruited cultivar seeds are of interest. The acid phosphatase R*Ls*AP1 which exhibited a significant phytasic activity could constitute an advantageous biocatalyst for bio-industrial applications. Thus, this crop deserves further investigations in search of new enzymes.

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