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Characterisation of purple acid phosphatase from breadfruit (*Artocarpus communis*) seeds

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ABSTRACT
Objectives: To investigate the biochemical properties, physiological role and possible application of a purple acid phosphatase isolated from breadfruit (*Artocarpus communis*) seeds.
Methodology and results: An acid phosphatase was purified 13.7-fold to apparent homogeneity from breadfruit (*Artocarpus communis*) seeds, using successive chromatographies on DEAE-Sepharose Fast-flow, Sephacryl S-100 HR, and phenyl Sepharose 6 Fast-flow. The enzyme was a purple acid phosphatase (λ_{max} = 510 nm) that appeared to be a monomeric protein with molecular weight of approximately 27.3 kDa by SDS PAGE. The purple acid phosphatase was optimally active at pH 5.5 and 55°C, but strongly inhibited by molybdate, vanadate, phosphate and zinc. Moreover it did not require divalent cations for catalysis. This phosphatase showed wide substrate specificity with high affinity for sodium pyrophosphate, suggesting that pyrophosphate could be its potential physiological substrate. The enzyme also hydrolyzed sodium phytate.
Potential application of finding: The purified enzyme could be applied in the mineral fertilizers industry or as a food additive for monogastric animals, e.g. pigs, in farming systems.

Key words: purple acid phosphatase, *Artocarpus communis*, breadfruit, pyrophosphatase activity.

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INTRODUCTION

The hydrolysis of phosphomonoesters in biological systems is an important process that is linked to energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways (Kerovuo *et al*., 2000). The biological hydrolysis of a wide variety of phosphomonoesters is catalysed by phosphatases. Acid phosphatases (EC 3.1.3.2) catalyse the hydrolysis of inorganic phosphate (Pi) from a broad range of phosphate monoesters and anhydrides with a pH optimum of 4 - 7 (Vincent *et al*., 1992; Duff *et al*., 1994). They are believed to function in the production, transport, and recycling of Pi, which is a crucial macronutrient for cellular metabolism and energy transduction processes (Duff *et al*., 1994).

Purple acid phosphatases represent a distinct category of non-specific acid phosphatases containing a binuclear metal-ion complex at their active site (Vincent *et al*., 1992; Schenk *et al*., 1999). They are distinguished from other phosphatases by their characteristic pink or purple colour (due to the presence of a phenolate to metal charge transfer complex), as well as insensitivity to L-tartrate inhibition. Plant purple acid phosphatases contain a Fe(III)–Zn(II) or Fe(III)–Mn(II) binuclear metal centre, whereas mammalian purple acid phosphatases have a Fe(III)–Fe(II) unit in their active site (Schenk *et*

al., 1999). In mammals, biological roles for purple acid phosphatases have been suggested in iron transport (Ketcham *et al*., 1985), bone resorption by osteoclasts (Reinholt *et al*., 1990), dephosphorylation of erythrocyte phosphoproteins (Schindelmeiser *et al*., 1987), and the production of hydroxyradicals and reactive oxygen species (Kaija *et al*., 2002). In plants, a role in the release of phosphate from organophosphates has been proposed for purple acid phosphatase from *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum*) cell cultures (Bozzo *et al*., 2002). Purple acid phosphatases are widely distributed in plants, and have been found in seeds (Olczak *et al*., 1997), seedlings (Vasko *et al*., 2006) and tubers (Shenck *et al*., 2001; Kusudo *et al*., 2003). To our knowledge, there are no reports concerning the purification and characterization of purple acid phosphatase from breadfruit seed.

The objective of this study was to isolate acid phosphatase from breadfruit (*Artocarpus communis*) seeds, biochemically characterize the enzyme and determine its kinetic properties in order to provide data which may help in understanding the physiological role played by this enzyme.

MATERIALS AND METHODS

Chemicals: *para*-Nitrophenylphosphate (*p*NPP), inorganic pyrophosphate (PPi), adenosine-2'-3'-cyclomonophosphate, adenosine- 5'- triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-1-phosphate , fructose-6-phosphate, phenylphosphate and sodium phytate were obtained from Sigma-Aldrich (Germany). DEAE-Sepharose Fast-flow, Sephacryl S-200 HR, Sephacryl S-100 HR and Phenyl-Sepharose 6 Fast-flow gels were purchased from Pharmacia Biotech (Sweden). Standard proteins were obtained from Bio-Rad (France). All other reagents were of analytical grade.

Enzyme source and preparation of crude extract: Breadfruit (*Artocarpus communis*) seeds used as enzyme source were obtained locally in Côte d'Ivoire. Seeds (20 g) were rinsed in cold distilled water and dried with blotting paper. They were ground in a mixer (Moulinex optiblend 2000) in 40 ml of 20 mM sodium acetate buffer (pH 5.5) 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 was filtered through cotton and conserved at 4°C until use as the crude extract.

Phosphatase assay: Under the standard test conditions, phosphatase activity was measured at 37°C for 10 min in 100 mM acetate buffer (pH 5.5) containing 1.5 mM *para*-Nitrophenylphosphate. After pre-warming the mixture at 37°C for 5 min, the reaction was initiated by adding 50 µl (6 µg of protein) of enzyme solution. The final volume was 250 µl and the reaction was stopped by adding 2 ml of sodium carbonate (2 %, w/v). Absorbances were measured at 410 nm using a spectrophotometer (SHIMADZU) using *para*-Nitrophenol (*p*NP) as the standard. Under the above experimental conditions, one unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of *p*NP per minute. Specific activity was expressed as units per mg of protein (U/mg of protein).

When substrates other than *p*NPP were used in the assay, the liberated inorganic phosphate was determined by the method of Heinonen and Lahti (1981) with KH_2PO_4 as standard.

Protein estimation: Protein elution profiles from chromatographic columns and the concentration of purified enzyme were determined according to Lowry *et al*. (1951). Bovine serum albumin (BSA) was used as the standard protein.

Enzyme purification: The entire purification procedure was carried out in cold room (25°C). The crude extract (10 ml) was loaded onto a DEAE-Sepharose Fast-flow column (2.3 cm x 8.5 cm) that had been equilibrated with 20 mM acetate buffer (pH 5.5). Unbound proteins were removed by washing the gel with two bed volumes of equilibration buffer. Bound proteins were then eluted with a stepwise gradient (0, 0.2, 0.4, 0.6 and 1 M) NaCl in 20 mM acetate buffer (pH 5.5). Fractions (3 ml) were collected at a flow rate of 6 ml/min and assayed for enzyme activity. The active fractions were pooled and saturated overnight by 80 % ammonium sulphate in a cold room. After centrifugation at 6,000 rpm for 30 min, the pellet was dissolved in 1 ml of 20 mM acetate buffer (pH 5.5). The enzyme solution was loaded directly into a Sephacryl S-100 HR column (1.6 cm x 64 cm) equilibrated with the same buffer. Proteins were eluted at a flow rate of 0.25 ml/min. Fractions of 1 ml were collected and active fractions were pooled. The pooled fractions from the previous step was saturated to a final concentration of 1.7 M ammonium sulphate and applied on a Phenyl-

Sepharose 6 Fast-flow column (1.4 cm x 5.0 cm) previously equilibrated with 20 mM acetate buffer (pH 5.5) containing 1.7 M ammonium sulphate. The column was washed with equilibration buffer and the proteins retained were then eluted with a reverse stepwise gradient (1.7, 0.6, 0.4, 0.2 and 0 M) ammonium sulphate in 20 mM acetate buffer (pH 5.5). Fractions of 1 ml were collected at a flow rate of 0.3 ml/min and active fractions were pooled. The pooled fractions were dialysed against 20 mM acetate buffer (pH 5.5) overnight in a cold room and constituted the purified enzyme.

Polyacrylamide gel electrophoresis (PAGE): Electrophoresis was carried out by the method of Laemmli (1970) 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. In non-denaturing conditions, samples were mixed just before running with sample buffer without 2-mercaptoethanol and SDS. Proteins were stained with silver nitrate according to the method of Blum *et al*. (1987). The standard molecular weights (Bio-Rad) comprising myosine (200 kDa), beta-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45 kDa) were used.

Visible absorbance spectrum: The visible absorption spectrum was obtained using a solution containing 2 mg/ml of purified phosphatase, in sodium acetate buffer, at various wavelengths ranging from 330 to 600 nm.

Native molecular weight determination: The purified enzyme was applied to gel filtration on Sephacryl S-200 HR column (1.6 cm x 64 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.5) to estimate the molecular weight. Standard molecular weights (Sigma) used to calibrate the gel were beta-amylase (206 kDa), bovine serum albumin (66 kDa), amyloglucosidase (63 kDa), ovalbumin (45 kDa) and cellulase (26 kDa). Fractions of 1 ml were collected at a flow rate of 0.25 ml/min. The void volume of column was estimated with blue dextran (2000 kDa).

Temperature and pH optima: The effect of pH on enzyme activity was determined by measuring the hydrolysis of *p*NPP in a series of buffers at various pH values ranging from pH 3.0 to 6.0. The buffers used were sodium acetate buffer (100 mM) from pH 3.6 to 5.5 and sodium citrate buffer (100 mM) from pH 3.0 to 6.0. The pH values of each buffer were determined at 25°C. The effect of temperature on phosphatase activity was observed in 100 mM citrate buffer pH 5.5 over a temperature range of 30 to 80°C using 5 mM *para*-nitrophenyl-phosphate under the standard test conditions.

pH and temperature stabilities: The pH stability of the enzyme was studied in pH range 3.0 to 6.0 in 100 mM citrate buffer and temperature optima (determined as described above). After 1 h preincubation at 37°C, aliquots were taken and immediately assayed for residual phosphatasic activity. The thermal stability of the enzyme was determined at 37 and 55°C after exposure to each temperature for time periods of 15 to 150 min. The enzyme was incubated in 100 mM citrate buffer (pH 5.5). Aliquots were withdrawn at intervals and immediately cooled in ice-cold water. For the thermal denaturation tests, 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 the untreated enzyme.

Substrate specificity and kinetic parameters: The substrate specificity of the acid phosphatase was determined by incubating the enzyme with various phosphorylated substrates (10 mM) at 37°C in 100 mM sodium citrate buffer (pH 5.5) for 20 min, except sodium phytate which was incubated at 55°C for 2 h. The liberated inorganic phosphate was determined by the Heinonen and Lahti (1981) method.

The kinetic parameters (K_M, V_{max} and V_{max}/K_M) were determined in 100 mM citrate buffer (pH 5.5) at 37°C. The hydrolysis of *para*-nitrophenyl-phosphate was quantified on the basis of released *para*-nitrophenol as in the standard enzyme assay. The hydrolysis of other substrates was quantified by determination of released inorganic phosphate according Heinonen and Lahiti (1981). K_M and V_{max} were determined from Lineweaver and Burk (1934) plot using different concentrations (0.5 to 5 mM) of *para*-nitrophenyl-phosphate, pyrophosphate and adenosine-5'-triphosphate.

Effect of chemical agents: To determine the effect of various compounds (metal ions, detergents and dithiol-reducing agents) as possible activators or inhibitors of the purified phosphatase, the enzymatic solution was preincubated at 37°C for 20 min with the compounds and the activity assayed. The substrate *p*NPP (5 mM) was added to the medium and incubated at 37°C for 10 min. The results were expressed as percentage of activity based on the control without chemical agents.

RESULTS

The purification protocol of the acid phosphatase from Breadfruit (*Artocarpus communis*) seeds involved three steps of chromatography (table 1; Fig. 1 [a, b, c]). The phosphatase resolved a single peak on DEAE-Sepharose Fast-flow column at 0.2 M NaCl concentration in 20 mM sodium acetate buffer (pH 5.5) (Fig. 1a). When the peak of phosphatase activity that resolved on the preceding chromatography was precipitated in 80% ammonium sulphate and applied to a Sephacryl S-100 HR gel, one peak showing phosphatase activity was eluted (Fig. 1b). This phosphatase activity was ultimately purified using hydrophobic interaction chromatography on phenyl-Sepharose 6 Fast-flow gel and the enzyme eluted at 0.2 M ammonium sulphate (Fig. 1c). The final phosphatase exhibited a pink colour. The physical basis for this colour is apparent from its visible absorption spectrum, peaking at about 510 nm (Fig. 2). The enzyme showed a single protein band after native polyacrylamide gel electrophoresis and staining with silver nitrate (Fig. 3a).

After SDS-PAGE analysis under reducing conditions, the acid phosphatase showed a single protein band and its relative molecular weight was estimated to be 27.3 kDa (Fig. 3b). The relative molecular weight of the native enzyme, as determined by gel filtration Sephacryl S-200 HR column, was approximately 28.2 kDa.

Table 1: Purification procedure of purple acid phosphatase from breadfruit seeds.

Purification steps	Total protein (mg)	Total activity [a](Units)	Specific activity (Units/mg)	Yield (%)	Purification factor
Crude extract	412	226.9	0.55	100	1
DEAE-Sepharose Fast-flow	52.4	68.1	1.3	30	2.4
Sephacryl-S 100 HR	2.4	42.9	18.1	18.9	32.9
Sephacryl-S 100 HR	2.4	42.9	18.1	18.9	32.9
Phenyl-Sepharose 6 Fast-flow	0.6	31.2	51.7	13.7	94
Phenyl-Sepharose 6 Fast-flow	0.6	31.2	51.7	13.7	94

[a]One unit equals 1 µmol of *p*NP release per min.

Table 2: Some physicochemical characteristics of purple acid phosphatase from breadfruit seeds.

Physicochemical properties	Values
Optimum temperature (°C)	55
Optimum pH	5.5
pH stability	5-6
Molecular weight (kDa)	
SDS-PAGE	27.3
Gel filtration	28.2
Activation energy (kJ/mol)	49.6
Q_{10}	1.63

With regard to the effect of pH and temperature on the purified acid phosphatase, assays in the presence of *para*-nitrophenyl phosphate exhibited high activity at pH 5.5 and 55°C (table 2). At 37°C, the phosphatase presented best stability over pH values ranging from 5.0 to 6.0, conserving at least more than 90 % of total activity. This enzyme was more active in sodium citrate buffer than other buffers used.

The temperature coefficient (Q_{10}) for the enzyme as calculated between 40 and 50°C was around 1.6. The apparent activation energy was estimated at pH 5.5 from the slope of log V-versus 1/T. The data showed excellent linearity from 30 to 45°C. The Arrhenius activation energy for the hydrolysis of *para*-nitrophenyl phosphate was 49.6 kJ/mol (table 2). The thermal inactivation study indicated that the acid phosphatase remained fully stable for 150 min at 37°C, while at 55°C (optimum temperature) it was less stable and lost 50% of its hydrolytic activity after 75 min of preincubation, corresponding to the half-life of the enzyme (Fig. 4a). As concerns thermal denaturation, the purified enzyme was fairly stable at temperature up to its optima (55°C). Above this temperature, the activity declined rapidly, as the temperature increased,

though the enzyme was not completely inactivated even at 80°C (Fig. 4b).

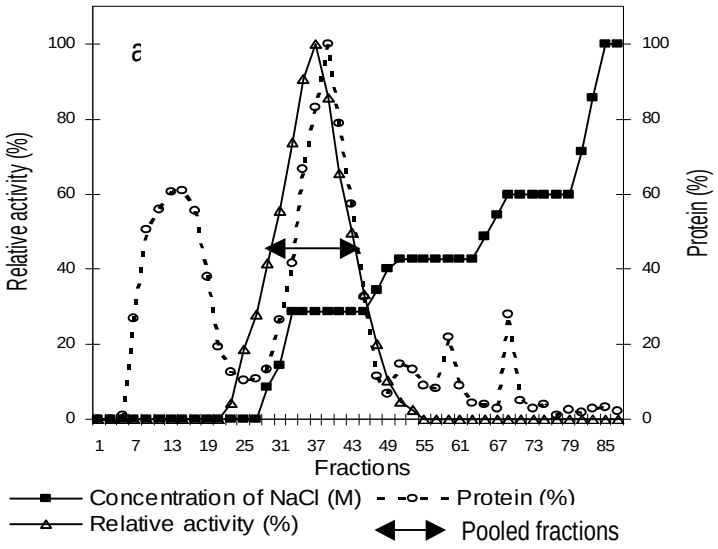

Figure 1a: Anion-exchange chromatography on DEAE-Sepharose Fast-flow.

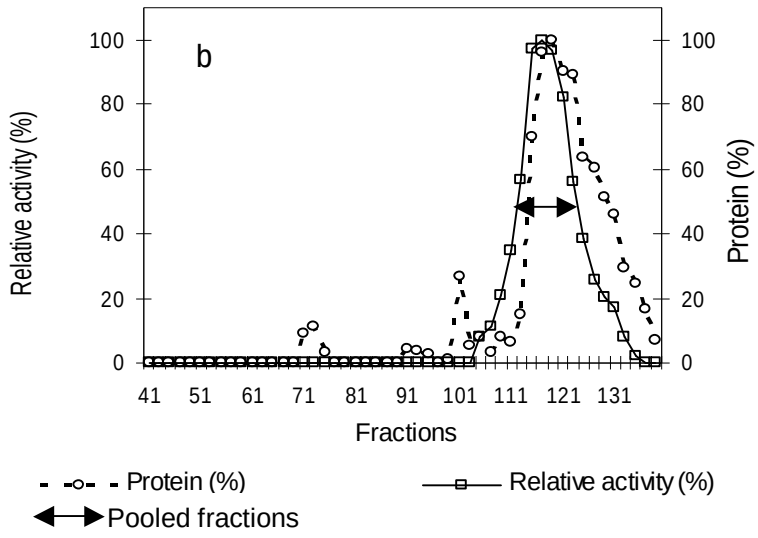

Figure 1b: Gel filtration chromatography on Sephacryl S-100 HR.

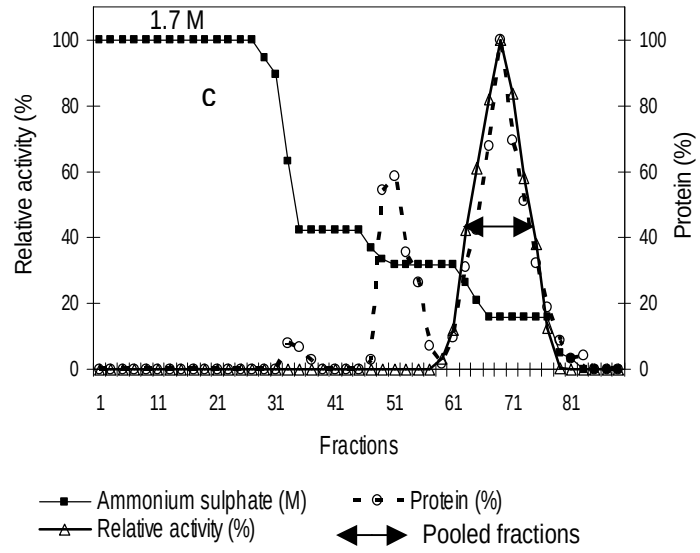

Figure 1c : Hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast-flow. The enzyme activity was measured in acetate buffer (pH 5.5) at 37 °C using *p*NPP as substrate.

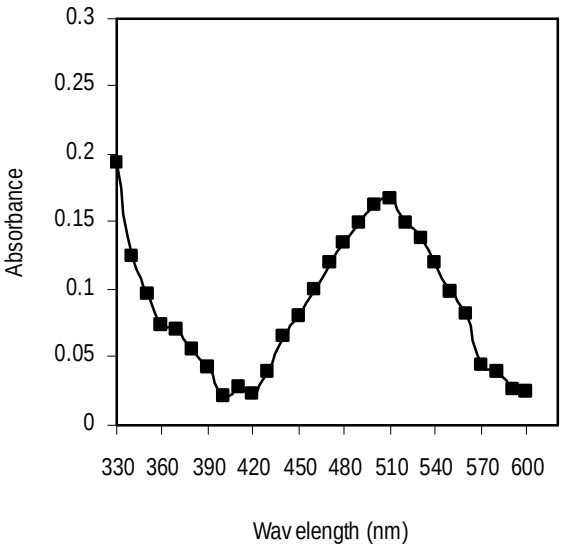

Figure 2: Visible absorbance spectrum of the purified acid phosphatase. The spectrum was obtained using a solution containing 2 mg/ml of acid phosphatase in acetate buffer.

The purified acid phosphatase hydrolyzed a broad range of phosphorylated substrates. The highest activity of the enzyme was observed with sodium pyrophosphate and Adenosine 5' triphosphate followed by the synthetic substrate *p*NPP. The enzyme also hydrolyzed sodium phytate (table 3).

The kinectic parameters for the acid phosphatase were studied using *p*NPP, sodium pyrophosphate and ATP as substrate (table 4). With the three substrates, the enzyme obeyed the Michaelis-Menten equation. The catalytic efficiency of the enzyme given by the V_{max}/K_M ratio was 3.7 x higher for sodium pyrophosphate than *p*NPP (table 4).

The result showed that most of cations tested did not affect the phosphatase activity (table 5). However, the enzyme was inhibited by the presence of Zn^{2+} in the medium. Except L-tartrate which had no effect, the other anions tested drastically inhibited the activity of the phosphatase from breadfruit seeds. With the exception of DL-dithiotreitol and β-mercaptoethanol which had no effect on the enzyme, all other reducing agents tested inhibited this enzyme (table 6). Apart from SDS which totally inhibited the activity of the acid phosphatase, the ionic and non-ionic detergents tested did not significantly affect enzyme activity (Table 7).

Table 3: Substrate specificity of purified purple acid phosphatase from breadfruit seeds. Values given are the averages of at least three experiments.

Substrat	Hydrolysis activity (%)
para-Nitrophenylphosphate	100
Phenylphosphate	87.1
Sodium pyrophosphate	164.3
Glucose-1-phosphate	0
Glucose-6-phosphate	0
Fructose-6-phosphate	0
Fructose-1-phosphate	0
α-nicotinamide adenosine diphosphate	47.6
Adenosine-2-3'-cyclomonophosphate	45.2
Adénosine-5'-triphosphate	132.1
Sodium phytate[a]	18

[a] Substrate incubated with the enzyme solution at 55°C for 2 h.

Table 4: Kinetic parameters of purified purple acid phosphatase from breadfruit seeds towards *p*NPP, ATP and pyrophosphate. (Values are averages of at least three experiments. The Michaelis constants (K_M) and the maximum velocities (V_{max}) are expressed as mM and units/mg protein, respectively.)

Substrates	K_M	V_{max}	V_{max}/K_M
*p*NPP	0.71	54.9	77.3
ATP	1.15	43.3	37.6
Sodium pyrophosphate	0.39	111.1	284.9

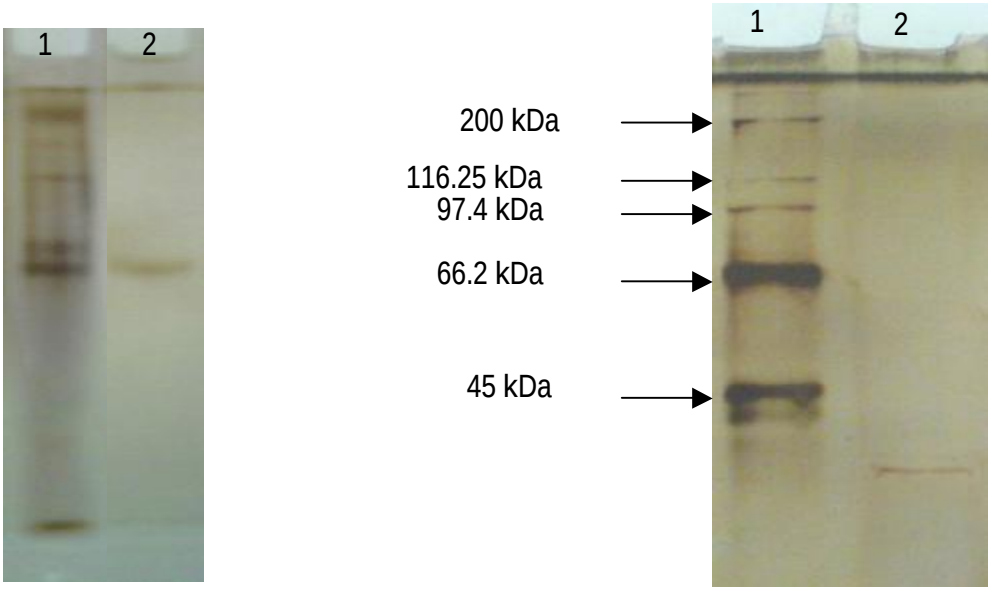

Figure 3a (left): Native-PAGE analysis of the purified acid phosphatase from breadfruit seeds. The sample was loaded onto a 10 % gel. Lane 1, crude extract of breadfruit seeds. Lane 2, purified purple acid phosphatase. Figure 3b (right): SDS-PAGE analysis of the purified acid phosphatase from breadfruit seeds. The sample was loaded onto a 10 % gel. Lane 1, molecular weight markers. Lane 2, purified acid phosphatase.

DISCUSSION

Although several studies have been reported on acid phosphatases from plant tissues (Duff *et al*., 1994; Gonnety *et al*., 2006), no information related to the purification of this enzyme from breadfruit (*Artocarpus communis*) seeds is available to date. Thus, in the present study an acid phosphatase was successfully purified by ion exchange (DEAE-Sepharose Fast-flow), gel filtration (Sephacryl S-100 HR) and hydrophobic interaction (Phenyl Sepharose 6 Fast-flow) chromatography from breadfruit seeds. The hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast-flow gel was crucial to separate the acid phosphatase from other proteins and impurities of the crude extract. The purification yield (13.7%) was higher compared to acid phosphatase from castor bean seeds (8%) (Granjeiro *et al*., 1999), buckwheat seeds (4%) (Greiner & Klauss-Dieter, 2002), *Lycopersicon esculentum* (11%) (Bozzo *et al*., 2004) and beet root (4.2%) (Eduardo & Gonzalez, 2004). PAGE followed by protein and acid phosphatase activity staining confirmed that the enzyme was purified to homogeneity.

The fact that acid phosphatase from breadfruit seeds exhibited an absorption maximum at 510 nm, displayed a pink colour in solution and was also insensitive to L-tartrate inhibition, ascertained that the enzyme is a purple acid phosphatase. Similar observations have been noticed when purifying purple acid phosphatases from sweet potato (Schenk *et al*., 1999). Moreover, most purple acid phosphatases isolated from soybean (Hegeman & Grabau, 2001), kidney beans (Cashikar *et al*., 1997) and from a wide range of other plants (Schenk *et al*., 2000) are characterized by a unique pink colour in solution due to the presence of binuclear Fe(III)-Zn(II) or Fe(III)-Mn(II) unit in their active site.

SDS-PAGE of the purified protein showed that the purple acid phosphatase is homogenous with a subunit molecular weight of 27.3 kDa. On the other hand, the native enzyme had 28.3 kDa by gel filtration. Taken together these results indicated that the native functional enzyme was a monomer. This monomeric structure, contrasts this enzyme with the majority of plants' purple acid phosphatases studied, which were homodimers of 55 kDa subunits (Schenk *et al*., 1999; Vasko *et al*., 2006) and heterodimers (Bozzo *et al*., 2004). To our knowledge, purple acid phosphatase from breadfruit seeds is the second monomeric purple acid phosphatase to be characterized after the one from tomato (*Lycopersicon esculentum*) cell cultures (Bozzo *et al*., 2002).

In comparison to other molecular weights of purified plant acid phosphatases, breadfruit phosphatase was lower than those from castor bean seeds (60 kDa) (Granjeiro *et al*., 1999), buckwheat seeds (45 kDa) (Greiner & Klauss-Dieter, 2002),

Spirodela oligorrhiza (57 kDa) (Nakazato *et al*., 1998) and garlic seedling (58 kDa) (Yenigun & Guvenilir, 2003). Accordingly, purple acid phosphatase from breadfruit seeds appeared to be a low molecular weight acid phosphatase. Nevertheless, it had higher molecular weight than acid phosphatases from peanut seedlings (25.3, 22.4 and 24 kDa) (Gonnety *et al*., 2006).

Table 5: Effect of some cations, anions and chelating agent on the activity of purple acid phosphatase from breadfruit seeds. Values given are the averages of at least three experiments.

Reagent	Concentration (mM)	Relative activity (%)
Control	0	100
Metal ions		
	1	100
Na^+	5	102.4
	1	100
K^+	5	100
	1	102.4
Mg^{2+}	5	104.3
	1	103.1
Sr^{2+}	5	111.2
	1	97.9
Ca^{2+}	5	81.6
	1	50.5
Zn^{2+}	5	45.2
EDTA	1	93.8
	5	81.7
Anions		
	1	103.2
L-tartrate	5	97
	1	25.4
Phosphate	5	19.3
	1	17.1
Vanadate	5	11.4
	1	9.5
Molybdate	5	4.6

Using *p*NPP as substrate, the activity of breadfruit seeds purple acid phosphatase was maximal at pH 5.5, which is similar to other acid phosphatases from castor bean seed (Granjeiro *et al*., 1999), and tomato (*Lycopersicon esculentum*) cell cultures (Bozzo *et al*., 2002). The studied enzyme displayed better stability at pH ranging 5.0-6.0, which is largely consistent with that of other plant acid phosphatases (Haas *et al*., 1991; Ferreira *et al*., 1998).

Breadfruit seeds purple acid phosphatase was optimally active at 55°C. However, it was sensitive to temperature above 55°C and lost 100 % of its catalytic activity after 150 min. This attribute 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 fully lost its activity at 68°C after 10 min (Ullah & Gibson, 1988).

The purified enzyme hydrolyzed broad phosphorylated substrates to various degrees. Similar observations have been reported for acid phosphatase from sweet potato (Kusudo *et al*., 2003). Natural substrates such as PPi and ATP had the highest relative rate of hydrolysis, while the purple acid phosphatase did not hydrolyze phosphorylated sugars. In this regard, the enzyme probably was unable to participate in sugar metabolism. Sodium phytate hydrolysis may be a significant property of purple acid phosphatase from breadfruit seeds. It could cleave phosphate moieties from phytic acid (myo-inositol-hexakisphosphate) present in breadfruit seeds, 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 monogastric animals (Harland & Morris, 1995). Moreover, myo-inositol *via* its oxidation pathway is directed to cell wall polysaccharide biosynthesis (Loewus & Murthy, 2000), being important for cell wall elongation and growth. So, the physiological function of this enzyme appears to be more important for mammals that feed on breadfruit seeds.

Table 6: Effect of some reducing agents on the activity of purple acid phosphatase from breadfruit seeds. Values given are the averages of at least three experiments.

Reducing agent	Concentration (%; w/v)	Relative activity (%)
control		100
	0.1	104
DL-dithiothreitol	1	106.7
	0.1	90.8
*p*CMB [a]	1	81.6
	0.1	28.5
DTNB [b]	1	21.4
	0.1	96.9
L-cystein	1	94.9
	0.1 (v/v)	106.7
β-mercaptoethanol	1 (v/v)	97.3

a: *para*-chloromercuribenzoate de sodium; b: acide dinitro-2,2'-dithio-5,5'-dibenzoïque

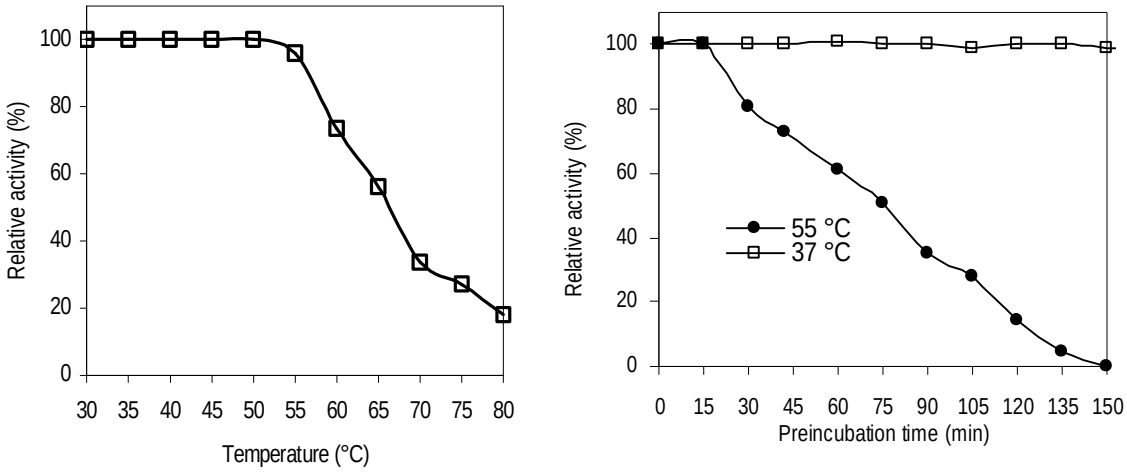

Figure 4a (right): Thermal inactivation of acid phosphatase from breadfruit seeds. The enzyme was preincubated at 37°C and 55°C in 100 mM sodium citrate buffer (pH 5.5). Figure 4b (left): Thermal denaturation of acid phosphatase from breadfruit seeds. The enzyme was preincubated at each temperature for 15 min and the remaining activity measured at 37°C under standard test conditions. Values are averages of at least three experiments.

The results of the kinetic study correlate well with substrate specificity and showed a Lineweaver-Burk plot. Among the substrates used, the purified purple acid phosphatase showed the highest catalytic efficiency (V_{max}/K_M) towards PPi. Judging from the V_{max}/K_M, the enzyme behaved as a plant pyrophosphatase similar to those from *Glycine max* seed (Dos Prazeres *et al*., 2004) and *Xanthosoma sp* tubers (Kouadio *et al*., 2006). Consequently, this enzyme would be playing an important role in cell anabolism, providing a thermodynamic sink for biosynthetic reactions such as protein, RNA, and DNA syntheses (Kornberg, 1962) in seed. The high activity towards PPi indicates the possibility of application of this purple acid phosphatase in the preparation of mineral fertilizers

The effects of metal cations, anions and chelating agent on the activity of purple acid phosphatase were also examined. The enzyme does not require metal ions. Similar results were observed for purple acid phosphatase (SAP1) from tomato (*Lycopersicon esculentum*) cell cultures (Bozzo *et al*., 2002). Besides, most divalent cations tested had no significant effect on the purified breadfruit seed phosphatase. However, the enzyme was potently inhibited by Zn^{2+}. Inhibition by Zn^{2+} was also observed for purple acid phosphatase isolated from red kidney bean (Cashikar *et al*., 1997) and for acid phosphatase from banana fruit (Turner & Plaxton, 2001).

As concerns anions, vanadate, phosphate and molybdate exhibited typical phosphatase inhibition. The inhibition by inorganic phosphate might represent a physiological regulation mechanism for this enzyme. The purple acid phosphatase was sensitive to reducing agents except DL- dithiothreitol and β-mercaptoethanol which did not affect enzyme activity. These chemicals thus must be eliminated after treatment of the substrate, when it needs to be reduced before hydrolysis by the enzyme.

This study showed that the phosphatase purified from breadfruit seeds was a purple acid phosphatase. The substrate specificity and kinetic properties indicated that pyrophosphate was a potential physiological substrate of the enzyme. The purified enzyme could be applied the mineral fertilizers industry or as a food additive for monogastric animals.

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Table 7: Effect of various detergents on the activity of purple acid phosphatase from breadfruit seeds. Values given are the averages of at least three experiments.

Detergents	Concentration (% ; w/v)	Relative activity (%)
Control		100
Anionic		
	0.1	0
SDS	1	0
Polyoxyethylene-9-	0.1	93.2
lauryl ether	1	91.8
Polyoxyetylene-10-	0.1	104
tridecyl ether	1	106.7
None ionic		
Triton X-100	0.1	105.4
	1	90.5
Tween 80	0.1	109.4
	1	101.3
Cationic		
Tetradecyl trimethyl	0.1	98.6
ammonium bromide	1	87.8
Hexadecyl trimethyl	0.1	109.9
ammonium bromide	1	87.8

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