Purification and kinetic characteristics of an acid phosphatase isoform from germinating pigeonpea seed

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Abstract: Using *p*-nitrophenolphosphate(*p*NPP) as substrate, one isoform of acid phosphatase from germinating pigeonpea seed, encoded as APase Ⅱ, was purified to 247 folds and the specific activity 51.8 U/mg protein through ammonium sulfate fractionation and three sequential DEAE-Sephadex A 25, Hydroxyapatite and Concanavalin A-Sepharose 4B column chromatography. The purified APⅡ was demonstrated by Native- and SDS-PAGE to be electrophoretic homogeneity and as a 33 kDa monomer. APase Ⅱ exhibited optimal pH at 5.0 and optimal temperature at 35 ℃ and strong stabilization at the pH ranging from 3.5 to 7.0 and at temperature below 55 ℃. APase Ⅱ showed a highest specificity against pyrophosphate, and was activated by K^+ and Mg^{2+}, while inhibited by Fe^{2+}, Mn^{2+}, $Mo_7O_{24}^-$, F^- as well as by organic acids including as tartrate, malate, isocitrate, oxalate, citrate, glycolate, glyoxylate and ascorbate.

Key words: pigeonpea; acid phosphatase; purification; kinetic characteristics

CLC Number: Q556.1 Document Code: A Article ID: 1000-3142(2008)03-0390-05

Acid phosphatases (EC 3.1.3.2), catalyzing the hydrolysis of a wide range of orthophosphate monoesters at pH optima below 6.0, are widely distributed in plants and localized in nearly all organisms and tissues. Plant acid phosphatases usually present in multiple forms, display different biochemical properties, and exhibit a broad specificity towards natural and synthetic phosphoric esters (Ferreira *et al.*, 1998). Plant acid phosphatases are involved in phosphorus mobilization and recycle and also up-regulated during salt or osmotic stress, seed germination, flowering, senescence and fruit ripening (Duff *et al.*, 1994; Dong *et al.*, 2005).

Pigeonpea plays an important role in sustainable agriculture development in arid district because of its high economic value and tolerance to extremely drought and nutrition deficiency (Ae *et al.*, 2000), and more and more researches have been focusing on its tolerance to abiotic stress. In our preliminary experiments, APase activity was found to increase sharply during the germinating period and when seedling was exposed to salt and drought stresses. In the present paper, APase protein was isolated and purified from germinated pigeon seed, its physiological and biochemical properties investigated and possible function discussed.

1 Material and methods

1.1 Plant material

300 gram of pigeonpea (*Cajanus cajan* cv. Guimu 2) seeds were surface-sterilized with 10% H_2O_2 for 5 min, rinsed with distilled water, and germinated in 25 ℃ incubator.

1.2 APase activity determination

APase activity determination was performed by

Received date: 2007-05-09 **Accepted date**: 2007-09-21

Foundation item: Supported by Postdoctoral Research Foundation of Guangxi Academy of Agriculture Sciences; Natural Science Foundation of College of Agricultural, Guangxi University

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determining the concentration of product(Pi) released as described by Duff *et al.* (1989), and one Unit was defined as 1 μmol Pi hydrolyzed from the corresponding substrates per minute. Protein concentration was determined as described by Bradford(1976), using Bovine serum albumin as standard.

1.3 Isolation and purification

1.3.1 Extraction of seed protein The germinated seeds were homogenized in the cold HAc-NaAc buffer (100 mmol/L, pH8.0, containing 2 mmol/L $MgCl_2$, 14 mmol/L mercaptoethanol and 2 mmol/L EDTA) in a ratio of 3 mL per gram. The homogenate was centrifuged at 10 000 g for 10 min, the pellet was re-extracted with the same buffer and procedure, and then supernatants were pooled and used as crude for purification.

1.3.2 Ammonium sulfate fractionation The crude was transferred into a beaker, and ammonium sulfate was added and stirred slowly to 20% saturation. After standing for 30 min at 4 ℃, the solution was centrifuged at 10 000 g for 10 min. The pellet was discarded and the supernatant transferred into another beaker, and then ammonium sulfate was added to the 60% saturation. After standing and centrifuging described as above, the supernatant was discarded, and the precipitant was suspended in Tris-HCl buffer(25 mmol/L, pH7.1, containing 2 mmol/L $MgCl_2$). The solution was dialysised with same buffer overnight until no SO_4^{2-} detectable with Ba^{2+} reagent, and concentrated with PEG-6000 to the minimum volume.

1.3.3 DEAE-Sepadex A 25 column chromatography The solution was loaded to DEAE-Sephadex A 25 column pre-equilibrated with Tris-HCl buffer(25 mmol/L, pH7.1, containing 2 mmol/L $MgCl_2$), and eluted with 400 mL of linear gradient buffer(0～0.5 mol/L KCl in the buffer) at the rate of 0.7 mL/min. Every 3mL of eluate was collected into a tube, and protein concentration and APase activity were detected, fractions containing higher APase activity were pooled and concentrated.

1.3.3 Hydroxyapatite column chromatography The concentrated protein from last step was loaded to hydroxyapatite column pre-equilibrated with HEPES-KOH buffer(5 mmol/L, pH7.0, containing 2 mmol/L $MgCl_2$), and then eluted with a linear gradient buffer (5～100 mmol/L KCl in the buffer) at the rate of 0.1 mL/min. Each fraction containing APase activity was pooled and concentrated.

1.3.4 Concanavalin A-Sepharose 4B column chromatography The solution from last step was loaded to Con A-Sepharose 4B column pretreated with HAc-NaAc buffer(0.1 mol/L, pH5.8, containing 1 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$, 0.5 mol/L NaCl and 1 mmol/L EDTA), and eluted with linear gradient buffer(0～0.5 mol/L glucose in the buffer) at the rate of 0.3 mL/min. Every 1 mL of eluate was collected into a tube, and protein concentration and APase activity were detected, fractions containing higher APase activity were pooled.

1.3.5 Identification of purity and estimation of molecular weight (MW) Proteins were run on native polyacrylamide gel electrophoresis (PAGE) (4% stacking gel, 7.5% resolving gel) and SDS-PAGE(4% stacking gel, 12.5% resolving gel) respectively, and then stained with coomassie brilliant blue as described by Guo (1999). MW was calculated referring to the plot of l g MW against Rf values of standard protein on SDS-PAGE gel.

2 Results

2.1 Purification of the APase

After DEAE-Sepadex A 25 column chromatography, two peaks with higher APase activities were detected. The first peak(encoded as APase Ⅰ) appeared from the 2nd to the 9th tubes, further purification and characterization of APase Ⅰ will be reported in another paper. The second peak(encoded as APase Ⅱ), which appeared from the 54th to the 64th tubes, was pooled and further purified through two sequential Hydroxyapatite and Concanavalin A-Sepharose 4B column chromatography. In totally, the enzyme was purified 247 fold, and the specific activity was 51.8 U/mg protein. Among all the procedures, Hydroxyapatite column was most effective, through which APase activity was increased about 10 folds(Table 1).

The purified APase Ⅱ appeared as a single band in

both non-denaturing PAGE(Fig. 1;A) and SDS-PAGE (Fig. 1;B), indicating our target protein was monomer and purified to electrophoretic homogeneity.

Table 1 Purification of the APase Ⅱ from pigeonpea seeds

Steps of purification	Total activity (U)	Total protein (mg)	Specific activity (U·mg^{-1})	Purification (fold)	Yield %
Crude	448.7	2126	0.21	1.00	100
Ammonium sulfate fractionation(20%~60%)	239	721	0.33	1..06	54
DEAE-Sephadex A 25 chromatography	117	37.3	3.13	11.6	26
Hydroxyapatite chromatography	17.8	0.56	31.7	117.4	4.0
Concanavalin A-Sepharose 4B chromatography	3.6	0.18	51.8	247	0.8

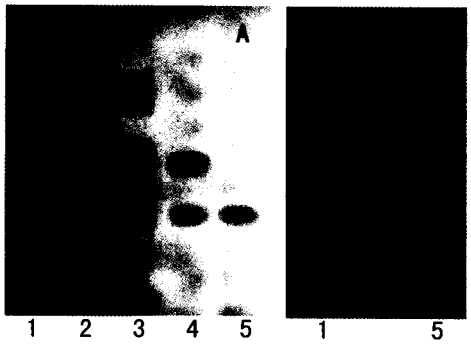

Fig. 1 Proteins on non-denatured PAGE gel(A) and SDS-PAGE(B)

1. proteins in crude; **2.** proteins after ammonium sulfate fractionation; **3.** proteins after DEAE- Sephadex A25 column; **4.** proteins after Hydroxyapatite column; **5.** protein after ConA-Sepharose 4B column.

2.2 Biochemical properties of purified enzyme

2.2.1 Molecular weight Referring to plot of logMW standard proteins versus relative migration distance, molecular weight of APase Ⅱ was estimated to be 33.1KDa(Fig. 2)

2.2.2 Optimal pH and pH stability Using *p*NPP as substrate, the enzyme were assayed in various reaction system with pH arrange from 3.5 to 8.0 to investigate the optimal pH; and the enzymes, after being stored in various buffers with pH arrange from 3.5 to 8.0 for 1 h at 4 ℃, were assayed in optimal reaction system to investigate the pH stability. APase Ⅱ exhibited an optimal pH of 5.0 and strong pH tolerance, APase Ⅱ lost less than 30% activity even stored in pH 3 or 7 buffer for 1 h(Fig. 3).

2.2.3 Optimal temperature and thermal stability Using *p*NPP as substrate, the enzyme was assayed at various temperatures to investigate the optimal temperature; and the enzymes, after being stored at various temperatures, ware assayed in optimal reaction system to investigate the thermal stability. APase Ⅱ exhibited an optimal temperature of 35 ℃, and was stable at temperatures up to 50 ℃, above which point the activity decreased sharply(Fig. 4).

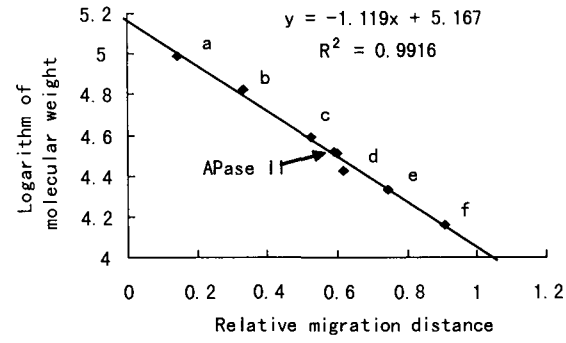

Fig. 2 Logarithm of MW versus relative migration distance(Rf) of proteins on SDS-PAGE gel

a. standard protein a is Phosphorylase b(97.4kDa); **b.** Bovine serum albumin (66.2kDa); **c.** Aldolase(39.2kDa); **d.** Triose phosphate isomerase (26.6kDa); **e.** Trypsin inhibitor(21.5kDa); **f.** Lysozyme (14.4kDa).

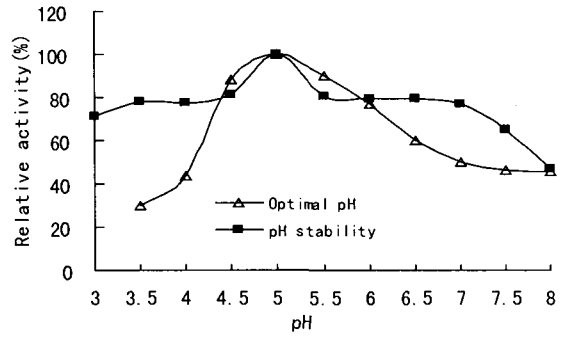

Fig. 3 Optimal pH and pH stability of APase Ⅱ

2.2.4 Substrate specificity In place of *p*NPP, a variety of natural phosphoric compounds including 3-phosphoglycerate(3-PGA), fructose-6-phosphate(F-6-P), phosphoenolpyruvate (PEP), adenosine diphosphate (ADP), phytate, fructose-1,6-diphosphate(F-1,6-P), glucose-6-phosphate (G-6-P), adenosine triphosphate (ATP) and pyrophosphate, all at a concentration of 2 mmol/L, were used as substrates to determine the APase Ⅱ activities, and relative activities as compared to *p*NPP were calculated.

APase Ⅱ showed relatively divergent substrate specificities, the highest specificity activity was recorded with pyrophosphate, followed by ATP, phytate and G-6-P, as 3.7-, 2.3-, 0.64-, 0.37-fold higher than with *p*NPP, respectively (Fig. 5).

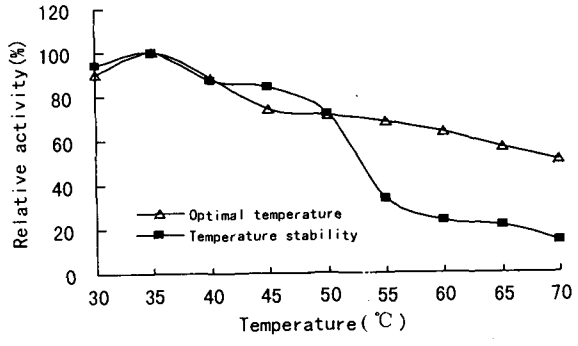

Fig. 4　Optimal temperature and heat stability of APase Ⅱ

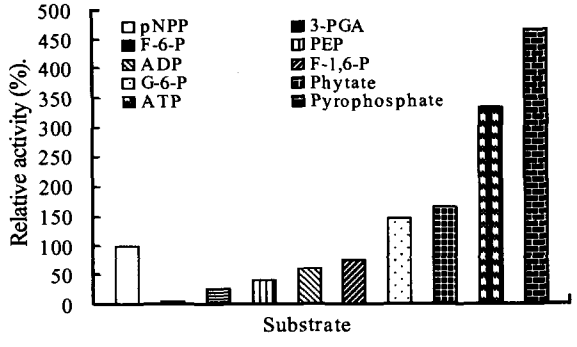

Fig. 5　Relative activity of APase Ⅱ against various substrates

2.2.5 Effects of ions on enzyme activity　In order to determine the effects of some ions as possible activators or inhibitors of APase Ⅱ, the enzyme was incubated with these compounds at concentration of 1 mmol/L and 5 mmol/L, respectively, and then the activity was determined. The activity of APase was activated by K^+, but inhibited by Fe^{2+}, Mn^{2+}, $Mo_7O_{24}^{6-}$ and F-. As to Zn^{2+} and Mg^{2+}, activatory effects were observed at low concentration of 1 mmol/L while inhibitory effects observed at high concentration of 5 mmol/L (Fig. 6).

2.2.6 Effects of organic acids on enzyme activity　In order to determine the effects of intermediate metabolites on APase Ⅱ, some organic acids including glycolate, glyoxylate, citrate, oxalate, tartrate, malate, ascorbate and isocitrate were added, respectively, to the reaction system to the ultimate concentration of 2 mmol/L, and then APase Ⅱ activities were determined. APase Ⅱ was inhibited by all organic acids used (Fig. 7).

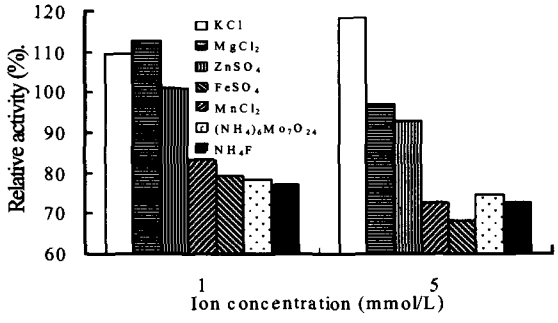

Fig. 6　Effects of ions on APase activity

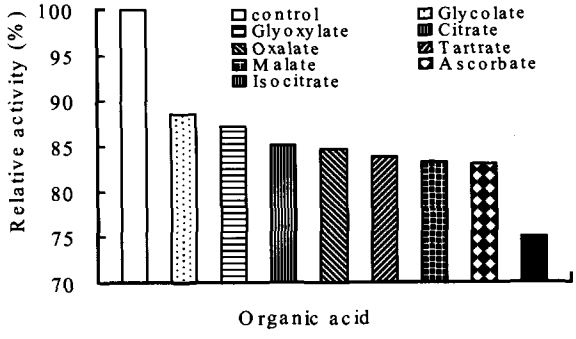

Fig. 7　Effects of organic acids on APase activity

3　Discussion

Although bodies of studies have been reported on acid phosphatases from plant tissues such as seeds (Ferreira *et al.*, 1998), leaves (Noel *et al.*, 2004; Tian, 2004), roots (Panara *et al.*, 1990), tubers (Gellatly *et al.*, 1994), and even exudates (Bozzo *et al.*, 2002), and their possible function have been assigned. Little information is available on APase purified from plant germinated seed, neither the possible function as relative to germination. To our knowledge, the present study represents the first detailed purification and characterization of an APase from pigeonpea and germinating seed.

APase generally exist as heterogeneities. In our experiment, two isoforms were isolated from germinated pigeonpea seed, the possibility could not be excluded that one or more other isoforms existed in the discarded supernatant after 60% ammonium sulfate fractionation.

The APase Ⅱ could bind with Concanavalin, indicating that it might be a kind of glycosylprotein. The

APase Ⅱ was a 33.1kDa monomeric protein with optimal pH5.0 and temperature 35 ℃, the properties are like those of the isoform AP3B from soybean seed(Ferreira *et al.*, 1998). The sharp loss of enzyme activity after treated with temperature above 50 ℃ might be due to the irreversible denaturation.

Like most of other plant APases reported, the APase Ⅱ was inhibited by molybdate and fluoride, and activated by magnesium. The APase Ⅱ was inhibited by tartrate, indicting that it might not be purple acid phosphatase, because the purple acid phosphatases are generally resistant to tartrate (Olczak *et al.*, 1997; Bozzo *et al.*, 2002).

The APase Ⅱ had activity against all phosphate esters tester tested, indicating it was a multifunctional enzyme, but relatively high activity was against pyrophosphate, ATP, phytate, and G-6-P. Pyrophosphate is the byproduct during RNA, protein and saccharide synthesis, can be accumulated during the germinating process as reservoir of P and energy(Li *et al.*, 2004). Phytate is the major storage form of P in the plant seed. Therefore, the APase Ⅱ might play a role in phosphate mobilization and energy provision during germinating, but direct evidence in support of this assignment should be necessaries. Cloning and function identification of the APase Ⅱ gene might facilitate the understanding of its explicit physiological functions in relative with germinating.

参考文献:

Ae N, Arihara J, Okada K. 1990. Phosphorus uptake by pigeon pea and its role in cropping systems of the Indian subcontinent [J]. *Science*, **284**: 477－480

Bozzo G G, Raghothama F G, Plaxton W C. 2002. Purification and characteristics of two secreted purple acid phosphatase iozymes from phosphate starved tomato (*Lycopersicon esculentum*) cell cultures[J]. *Eur J Biochem*, **269**: 6 278－6 286

Bradford M M. 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding[J]. *Anal Biochem*, **72**: 248－254

Dong DF, Yang J, Jiang LG. 2005. Study on soybean phosphoenolpyruvate phosphatase(PEPP) Ⅲ. Purification and characterization of nonspecific acid phosphatase[J]. *Guihaia*, **25** (5): 472－476(in Chinese)

Duff S M G, Lefebvre D D, Plaxton W C. 1989. Purification and characterization of phosphoenopyruvate phosphatase from *Brassica nigra* suspension cells[J]. *Plant Physiol*, **90**: 734－741

Ferreira C V, Granjeiro J M, Taga E M, *et al.* 1998. Purification and characterization of multiple forms of soybean seed acid phosphatases[J]. *Plant Physiol Biochem*, **36**(7): 487－494

Gellatly K, Moorhead G B G, Duff S M G, *et al.* 1994. Purification and characterization of a potato tuber acid phosphatase having significant phosphotyrosine phosphatase activity [J]. *Plant Physiol*, **106**: 223－232

Guo RJ. 1999. Technique of protein electorphoresis[M]. Beijing: Science Press(in Chinese)

Li XY, Zeng RZ, Xiao XZ. 2004. Advances on the research of pyrophosphatase in plants[J]. *Life Sci Res*, **8**(4): 83－87(in Chinese)

Noel ATG, Marleni O, Carmen I. 2004. Partial purification and characterization of a non-specific acid phosphatase in leaves and root nodules of *Phaseolus vulgari*[J]. *Plant Physiol Biochem*, **42**: 585－591

Olczak M, Watorek W, Morawiecka B. 1997. Purification and characterization of acid phosphatase from yellow lupin(*Lupinus luteus*) seeds[J]. *Biochim Biophys Acta*, **1 341**: 14－25

Panara F, Pasqualini S, Antonielli M. 1990. Mutiple forms of barley root acid phosphatase: purification and some characteristics of the major cytoplasmic isoenzyme[J]. *Biochim Biophys Acta*, **1 037**: 73－78

Tian J. 2004. APase activity in soybean and common bean as related to P availability [D]. Doctor dissertation, Southern China Agricultural University: 60－88

萌发木豆种子中酸性磷酸酯酶的纯化和动力学特性

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摘 要：以对硝基苯磷酸为底物检测酶活性，通过20%～60%硫酸铵分部、DEAE-葡聚糖A25、羟基磷灰石、伴刀豆球蛋白－琼脂糖4B柱层析，从木豆萌发的种子中纯化到一个同工酶APase Ⅱ，酶最终纯化倍数为247倍，比活力达51.8 U/mg蛋白。非变性PAGE和SDS-PAGE表明所纯化的酶已经达到电泳纯，是一个分子量为33.1kDa的单体蛋白。APII的最适pH为5.0，最适温度为35 ℃，在pH3.5～7以及55 ℃以下稳定。该酶对焦磷酸有最大活性，受K^+和Mg^{2+}激活，受Fe^{2+}，Mn^{2+}，$Mo_7O_{24}^{6-}$，F-及酒石酸、苹果酸、异柠檬酸、草酸、柠檬酸、乙醇酸、乙醛酸和抗坏血酸等有机酸抑制。

关键词：木豆；酸性磷酸酯酶；纯化；动力学特性