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Full Length Research Paper

Partial purification and biochemical characterization of acid phosphatase from germinated mung bean (*Vigna radiata*) seeds

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Mung bean (*Vigna radiata*) is one of the important crops of the North Eastern Region of India. In the present study, acid phosphatase enzyme was isolated and partially purified from germinated local mung bean seeds. The sequential partial purification process was performed using ammonium sulphate precipitation method. The crude enzyme having a specific activity of 0.50 U/mg was purified using 30 to 70% ammonium sulphate precipitation method. The acid phosphatase was purified by 2.6 fold with a yield of 58.9% and specific activity of 1.3 U/mg. One prominent band was obtained on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which confirmed the purity of the enzyme. Molecular weight of the enzyme was estimated as 34.5 kDa. The enzyme activity was measured at different incubation time, pH, temperature and substrate concentration. The activity increased slowly from 10 to 70 min of incubation. The maximum activity was obtained at 70 min, thereafter the activity decreased gradually. The enzyme was found to be active over a wide range of temperature (30 to 80°C) and maximum activity was observed at 70°C. The optimal pH value of the enzyme activity was found to be 5.2. There was a corresponding increase in the rate of reaction with the increase in the substrate concentration from 0.1 to 0.8 mM and a linear relationship was obtained at 2 to 8 mM. Both K_m and V_{max} value were calculated as 0.416 mM and 1.33 μ moles/min, respectively.

Key words: Acid phosphatase, mung bean, *Vigna radiata*, enzyme purification, enzyme characterization.

INTRODUCTION

Acid phosphatases (E.C.3.1.3.2) are group of important enzyme that catalyzed the hydrolysis of a number of phosphomonoester and phosphoprotein but not the phosphodiester bond. Hydrolysis of phosphomonoesters by phosphatase in biological system is considered as an important process. This process links to energy metabolism, metabolic regulation and wide variety of cellular transduction pathways (Allan et al., 1994). Phosphatases have been classified as acid phosphatase and alkaline phosphatase according to optimum pH required for their catalytic activity (Barret-Lanned et al., 1982; Shahbazkia et al., 2009). They are produced by

both prokaryotes and eukaryotes and are presumed to convert organic phosphorous into available phosphorous (Eshanpour and Amini, 2003; Amlabu et al., 2009) during active cellular growth (Carswell et al., 1997). Under phosphorous deficiency condition during abiotic stress (water, salt and osmotic), the enzyme is induced to maintain certain level of inorganic phosphate inside the cell (Olmos and Hellin, 1997). Acid phosphatase from various sources catalyzes many substrates such as 3-phosphoglycerate (Randal and Tolbert, 1971), PEP (Duff et al., 1989), phytate (Gibson and Ullah, 1988) and Trp-phosphorylated proteins (Gellatly et al., 1994). They are ubiquitous and found to be localized in vacuole, cytoplasm and cell wall of *Phaseolous vulgaris* (Cashikar and Rao, 1995), cell wall of *Pisum sativum* (Olmos and Hellin, 1997), root caps of rice (Chen et al., 1992) phloem of *Nicotaina tabacum* (Bentwood and Cronshaw, 1975)

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and xylem (Charvat and Easu, 1974). Acid phosphatase present in cell wall play an important role in mobilizing organic phosphates in the soil during germination (Cashikar and Rao, 1995). The mechanism which regulates acid phosphatase distribution and activity is unclear although their abundance may be regulated by the level of phosphorous or phosphate in the environment and their activity is clearly influenced by local polyelectrolytes (Tu et al., 1990). Acid phosphatase expresses its isozymes in many plants such as soyabean seeds (Ferreira et al., 1998), *Vigna sinensis* (Biswas and Cundiff, 1991), tea leaves (Baker and Tadakazu, 1973) and lentil seeds (Bose and Taneja, 1998).

In this work, some properties of partially purified acid phosphatase from germinated mung bean seeds are demonstrated as a step towards understanding its phosphorous metabolism during germination.

MATERIALS AND METHODS

The chemicals and reagents for the enzyme assay and SDS-PAGE analysis were purchased from Himedia Bioscience, Merck Bioscience and Lonza, USA. All chemicals used in the present study were of analytical grade. The electrophoresis apparatus and gel imaging system were procured from Merck Bioscience and Bio Rad, respectively. Each experiment was replicated thrice to increase the precision in the result.

Seed

Mung bean seeds were collected from farmer's field (Imphal West, Manipur, India), dried under shade and kept in airtight desiccators for experimentation. Mung bean seeds (10 g) were soaked in 0.1% (v/v) sodium hypochlorite solution for 10 min and washed two times with distilled water. The seeds were soaked in a Petri plate containing 20 ml of sterile distilled water and incubated at 28°C for 24 h to induce germination.

Isolation of acid phosphatase

The germinated mung bean seeds were crushed with pre-chilled mortar and pestle and mixed uniformly in 25 ml precooled extraction buffer (0.1M citrate buffer pH 5.2, 40 mM EDTA). The homogenate was filtered through four layer of muslin cloth and centrifuged at 10000 rpm for 30 min at 4°C. The filtrate was used for partial purification of acid phosphatase.

Partial purification

The supernatant was purified by dissolving 0.166 g ammonium sulphate per ml of enzyme extract to bring the final concentration of 30%. The mixture was kept on ice for 30 min and centrifuged (REMI C-24 cooling centrifuge) at 10000 rpm for 30 min at 4°C. The precipitate was removed and supernatant was again brought to 70% final concentration by addition 0.253 g ammonium sulphate per ml of extract. The mixture was kept on ice for 30 min and centrifuged at 12000 rpm for 30 min. The pellet was dissolved in 2 ml extraction buffer and dialyzed against the same buffer with constant stirring for overnight. It was used for acid phosphatase activity assay.

Protein determination

Protein concentration was measured throughout the purification process by the method given by Lowry et al. (1951) using BSA as standard protein.

Enzyme assay

The enzyme assay was performed using p-nitrophenyl phosphate (p-NPP) as substrate with three replications. For the assay, 1 ml substrate (6 mM p-NPP, 10 mM MgCl₂, 100 mM citrate buffer of pH 5.2) was taken in a test tube and pre-incubated at 30°C for 10 min. The reaction was started after addition 0.1 mL of the enzyme extract and terminated after 10 min by addition of 4.5 mL of 0.1M NaOH. The released p-nitrophenol was read at 405 nm in a spectrophotometer (Thermo Scientific GENESYS 10 UV) against the reagent control. For the reagent control, the enzyme was added after addition of NaOH. The absorbance values correspond to amount of p-NPP released by the enzyme. To calculate the enzyme activities, a standard curve of 0.14 to 0.71 μmole p-nitrophenol was constructed. Here, one unit (1U) of enzyme activity is defined as micromoles of p-nitrophenol released per minute at 30°C under assay conditions.

SDS-PAGE analysis

The purity of the enzyme was examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was carried out in 12% separating gel containing 4 ml of 30% Acrisis (29:1), 2.5 ml of 1.5M Tris-HCl (pH 8.8), 8 μl of TEMED and 3 ml distilled water and 93 μl of 10% Ammonium persulphate and 4% stacking gel containing 0.566 ml 30% Acrisis, 0.416 ml 1.5M Tris-HCl (pH 6.8), 2.26 ml distilled water, 3 μl TEMED, 33 μl 10% Ammonium persulphate. 10 μl of samples (100 μg protein) were loaded into the wells along with loading buffer (Merck Bioscience). Standard molecular weight protein marker (Lonza, USA) ranging from 175 to 9 kDa was used for the study. The gel was stained with Ezee blue gel stainer (Merk Bioscience). Gel image was captured by using Bio Rad ChemiDoc XRS+ System and molecular weight was calculated by using ImageLab software version 4.0 (Bio Rad).

Effect of time in enzyme activity

The effect of incubation time on enzyme activity was monitored by incubating the enzyme reaction mixture in different time period (10 to 80 min).

Effect of pH and temperature on enzyme activity

Optimum pH for enzyme activity was determined by incubating the enzyme substrate mixture in 0.1 M citrate buffer of different pH; 3.5 to 6.0. Temperature stability was determined by incubating the enzyme substrate mixture in 30 to 90°C under assay condition.

Effect of substrate concentration on enzyme activity

For studying the effect of substrate p-nitrophenyl phosphate (p-NPP) concentration on enzyme activity, 0.1 ml of enzyme extract was added to 1ml citrate buffer containing various concentrations ranging from 0.1 to 12 mM. Catalytic parameter such as K_m and V_{max} were determined from Lineweaver-Burk plot.

Table 1. Summary of the partial purification results of acid phosphatase from germinated mung bean seeds.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Percent recovery
Crude	441	223	0.5	1.0	100.0
30 to 70% Ammonium sulphate precipitate	101	131	1.3	2.6	58.9

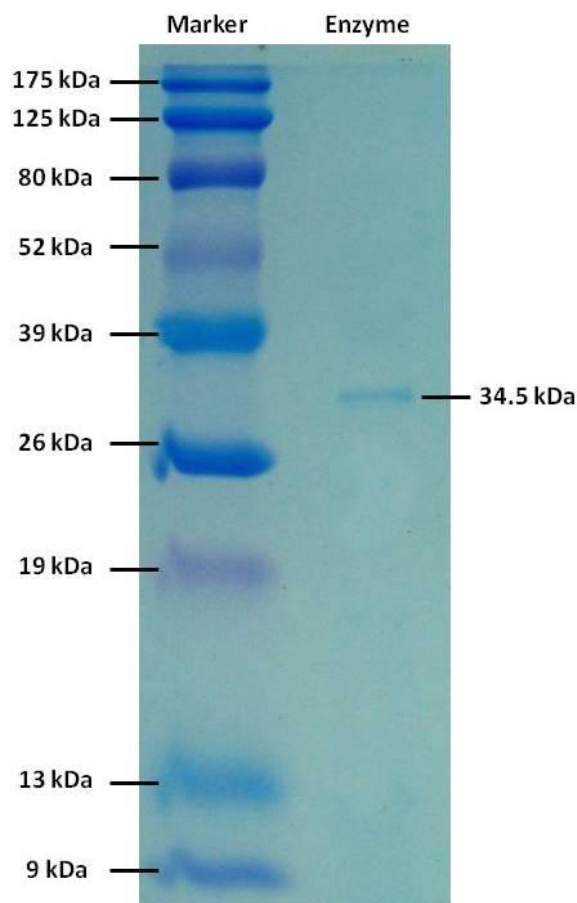


Figure 1. Purity analysis and determination of molecular weight of partially purified acid phosphatase on SDS-PAGE. Lane 1, Molecular weight protein marker; lane 2, purified enzyme.

RESULTS

Purification of acid phosphatase

The crude enzyme having a specific activity of 0.50 U/mg was partially purified using ammonium sulphate precipitation method. Purification results of the enzyme are given in Table 1. The acid phosphatase was purified 2.6 fold with a yield of 58.9%. The specific activity of the purified enzyme was found as 1.3 U/mg. The results show that the protein content decreased with the

purification step, whereas, the specific activity increased. One prominent band was obtained on SDS-PAGE which confirmed the purity of the enzyme. Molecular weight of the enzyme was estimated as 34.5 kDa on SDS-PAGE gel (Figure 1).

Effect of incubation time on acid phosphatase activity

Acid phosphatase activity was measured at different periods of incubation (10 to 80 min). The activity increased slowly from 10 to 70 min. The maximum activity was obtained at 70 min and after that, the activity decreased gradually (Figure 2).

Effect of temperature on acid phosphatase activity

Acid phosphatase was found to be active over a wide range of temperature (30 to 70°C). Maximum enzyme activity was observed at 70°C (Figure 3) and thereafter the enzyme activity decreased noticeably but not inactivated completely even at 80°C. The results indicate the thermostable nature of acid phosphatase isolated from germinated mung bean seeds.

Effect of pH on acid phosphatase activity

Acid phosphatase activity was recorded at different pH ranging from 3.5 to 6.0. The enzyme activity increased from pH 3.5 to 5.2 and optimal activity was recorded at pH 5.2 (Figure 4).

Effect of substrate concentration on acid phosphatase activity

Acid phosphatase activity was measured at different substrate (p-NPP) concentration from 0.1 mM to 12 mM. The result (Figure 5) showed that for increase in the substrate concentration from 0.1 to 0.8 mM, there was a corresponding increase in the rate of reaction and a linear relationship was obtained at 2 to 8 mM. By plotting the reciprocal of the reaction rate (V^{-1}) versus the reciprocal of substrate concentration (S^{-1}), a straight line curve was obtained. According to Lineweaver-Burk

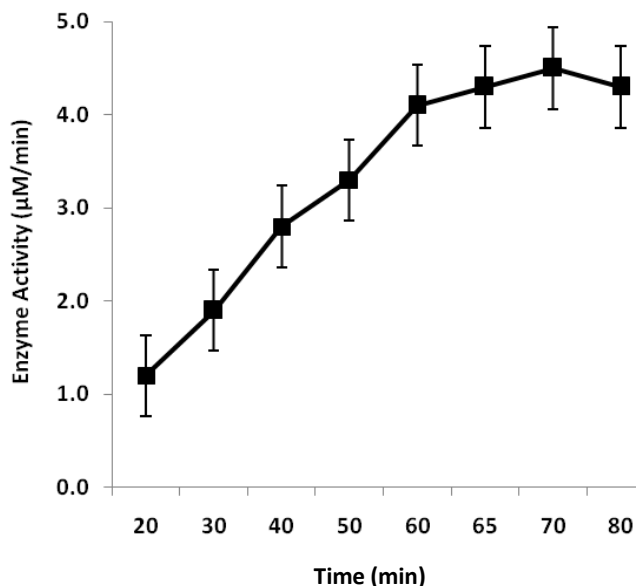


Figure 2. Effect of incubation time (10 to 80 min) on acid phosphatase activity.

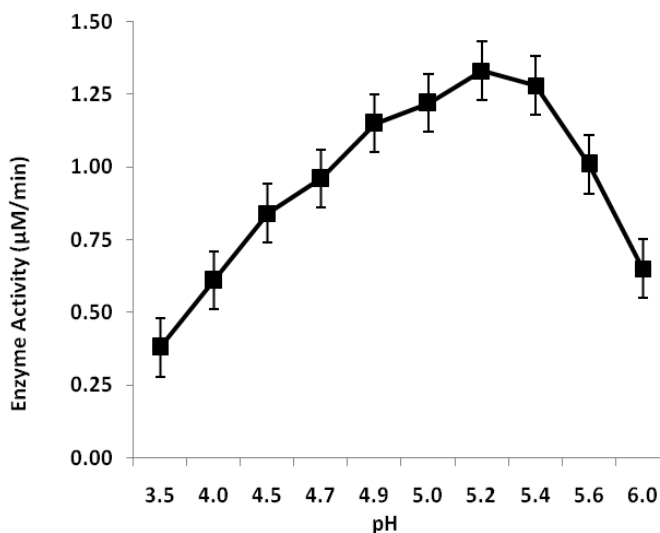


Figure 4. Effect of pH (3.5 to 6.0) on acid phosphatase activity.

hypothesis, both Michaelis-Menten Kinetics (K_m) and V_{max} value were calculated as 0.416 mM and 1.33 µmoles/min, respectively.

DISCUSSION

In the present study, the acid phosphatase from germinated mung bean seeds was isolated, partially purified with 30 to 70% ammonium sulphate precipitation

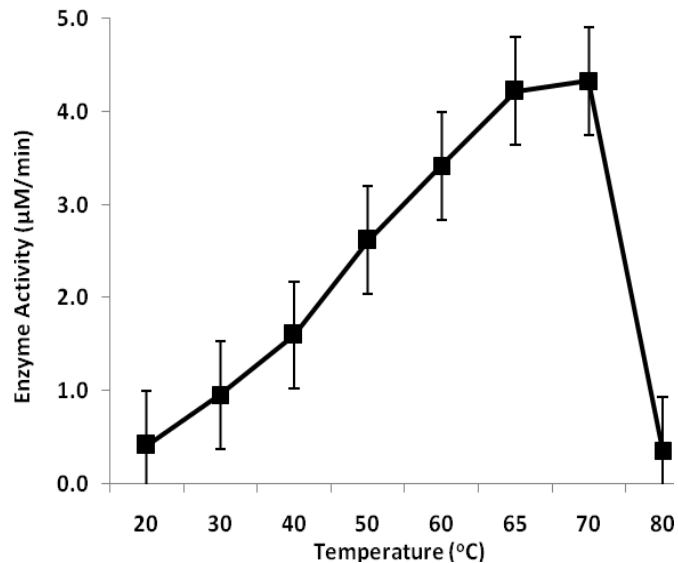


Figure 3. Effect of temperature (30 to 90°C) on acid phosphatase activity.

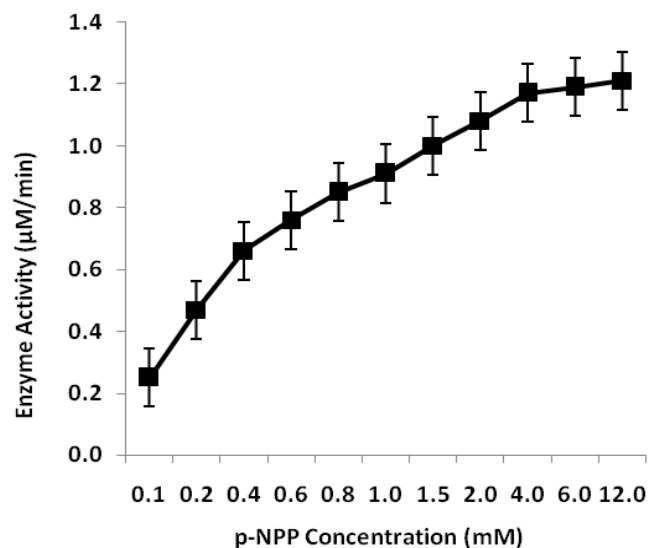


Figure 5. Effect of substrate (p-NPP) concentration (0.1 to 12.0 mM) on acid phosphatase activity.

and characterized in terms of molecular weight, incubation time, pH, temperature, substrate concentration and kinetic parameters. The value of purification fold and per cent recovery was higher than for acid phosphatase reported in *Vigna aconifolia* (Mohammed, 2010), in tobacco cell (Okuda et al., 1987) and in *Vigna sinensis* (Tapan et al., 1996). The maximum enzyme activity was obtained at 70 min of incubation. The value was higher than those reported by Okuda et al. (1987) in tobacco cell and by Mohammed (2010) in *Vigna aconifolia*. The

prolongation in the incubation time might be due to stability of enzyme in the reaction medium. The enzyme was also found to be thermostable with 70°C as its optimum temperature. Decrease in enzyme activity after 70°C might be due to irreversible denaturation. Cashikar et al. (1997) reported that acid phosphatase from Red kidney bean purple had optimal enzyme activity at 60°C. The acid phosphatase from soybean seeds was shown to be heat stable (Ullah and Gibson, 1988). However, the obtained temperature 70°C for acid phosphatase in germinated mung bean seed was higher than the value obtained for the enzyme in cotton seedling (Bhargava and Sacher, 1987) and in *Vigna aconifolia* (Mohammed, 2010). The enzyme showed optimal activity at pH 5.2. An optimum pH of 5.4 for acid phosphatase in maize endosperm was reported by Miernyk (1992); whereas, Biswas and Cundiff (1991) observed that pH 5.0 was optimum for acid phosphatase isolated from germinating seeds of *Vigna sinensis*. Moreover, the obtained pH 5.2 for acid phosphatase from germinated mung bean seed was lower than the values obtained for the enzyme from lupin seeds (Zhang and Duranti, 1995; Tapan et al., 1996), sunflower seed (Park and Vann-Etten, 1986), roots of *Trifolium* (Julie et al., 1999; Zhang and Macmanus, 2000) and cotton seedling (Bhargava and Sacher, 1987).

Generally, enzymes are sensitive to changes in pH. The pH can influence the enzyme activity in a number of ways. Firstly, it can change the ionization of the enzyme substrate complex. Secondly, it can change the ionization of various groups of the enzyme molecule which may affect the affinity of the enzyme for the substrate. Thirdly, it changes the ionization of the substrate to the enzyme. Fourthly, at extreme pH it can bring about changes in protein structure (Palmer, 1985). The present study also showed that acid phosphatase from germinated mung bean seeds had higher substrate (p-nitrophenyl phosphate) specificity. There was a corresponding increase in the rate of reaction with the increase in the substrate concentration from 0.1 to 0.8 mM and a linear relationship was obtained at 2 to 8 mM. According to Lineweaver-Burk hypothesis, the K_m and V_{max} value were calculated as 0.416 mM and 1.33 μ moles/min, respectively. The lower K_m value indicated that the enzyme has high affinity to the substrate. The K_m value of the acid phosphatase from germinated mung bean seed was lower than the K_m values reported by Mohammed (2010) in *Vigna aconifolia* and Miernyk (1992) in maize endosperm.

Conclusion

The acid phosphatase enzyme from the germinated mung bean seeds was found to be thermostable up to 70°C and most active at pH 5.2. The maximum enzyme activity was obtained at 70 min of incubation and the enzyme showed a linear relationship at 2 to 8 mM of the

substrate concentration in the assay condition. Hence, the characterization of acid phosphatase from germinating mung bean would be useful to study the basic kinetic activities of acid phosphatase enzyme in conversion of organic phosphorous into available inorganic phosphorous in energy transfer reaction and metabolic regulation that is involved in many different biological functions during germination.

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