PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ACID PHOSPHATASE FROM THE PITCHER FLUID OF *NEPENTHES DISTILLATORIA*

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Introduction

Carnivorous pitcher plants are known to secrete hydrolytic enzymes to digest trapped animals in pitchers, mainly insects, for uptake of nitrogen and phosphorous which are essential elements for plant growth and survival. *Nepenthes distillatoria,* locally known as "*bandura*" is a carnivorous plant endemic to Sri Lanka, and it inhabits areas with high rainfall and sunlight and containing soils that are water-saturated, acidic and deficient in nitrates or phosphates. A recent study on two aspartic proteases in *N. distillatoria* revealed that the proteases possess a remarkable stability at higher temperatures and over a wide range of pH[1]. Preliminary investigations on hydrolytic enzymes in the pitcher fluid showed the presence of acid phosphatases. Acid phosphatases act at an acidic pH optimum by hydrolyzing phosphoric acid monoesters into phosphate ions and molecules with free hydroxyl groups. In this study, we partially purified and characterized an acid phosphatase in the pitcher fluid of *N. distillatoria*.

Materials and Methods

Crude pitcher fluid was collected from open and unopened pitchers of *N. distillatoria* from Hakurugala forest patch at Ruwanwella. For the detection of acid phosphatase activity, the substrate p-nitrophenyl phosphate (pNPP) was used in sodium acetate buffer (pH 4.0). The reaction mixture contained 100µl of 0.5 mM pNPP, 100µl of 0.5 M sodium acetate at pH 4 .0 and 100µl of crude juice. After incubation at 37°c for 1.5 hours, 1 ml of sodium hydroxide was added to terminate the reaction and for the color development. Absorbance of paranitrophenolate released upon phosphatase action on pNPP was measured against a blank, at 405 nm. Control was treated in a similar manner except the crude juice was added after color development and just before measuring absorbance. All purification procedures were performed at 4°C. The collected pitcher fluid was filtered to remove the insoluble material and dialyzed against 0.01 M Tris-HCl at pH 7.5. Dialyzed fluid collected from open and unopened pitchers were fractionated on a DEAE cellulose column (2 cm x 55 cm) equilibrated with 0.01 M Tris-HCl buffer at pH 7.5. The column was eluted using a linear NaCl gradient from 0-1 M NaCl. Fractions with highest phosphatase activity were combined and the enzyme was further purified using a Sephacryl S-200 gel filtration column (3 cm x 65 cm). The purity of the enzyme was determined using SDS-PAGE. To characterize the

enzyme from open and unopened pitchers, the assay procedure was carried out by using buffers at varying pH (2-9) of 0.5 M concentrations and at varying temperatures from 30 – 80°C. The partially purified enzyme from open pitchers was incubated at pH 4.0 at 4°C, 25°C (room temperature), and 37°C for 2 weeks. Aliquots were drawn at 2 day intervals and the remaining activity was determined. The same procedure was carried out for a week with the partially purified enzyme from unopened pitchers. The phosphatase activity of the partially purified enzyme from open pitchers was evaluated with the presence or absence of 1 mM Zn^{2+}, Ca^{2+}, or Mg^{2+} ions. The enzyme assay was further carried out with varying concentrations of 0.1 to 2.5 mM Zn^{2+} and 0.1 to 6mM Mg^{2+}. SDS/PAGE was carried out on a 12% gel under reducing and non reducing conditions. SDS - PAGE analysis and gel filtration on the Sephacryl S-200 column were used to determine the molecular mass of the purified enzymes.

Results and Discussion

A single prominent peak was observed with anion exchange chromatography of both the open and unopened pitcher fluid at 0.484 M NaCl, confirming the presence of same acid phosphatase in both samples. The elution profile of gel filtration column further confirms this result since the enzyme eluted at the same position for both samples. Optimum temperature and the pH for the enzymatic activity were found to be 55°C and pH 4.5, respectively. Acid phosphatase was significantly stable at 4°C and room temperature. At 37°C, the stability decreased by approximately 25%. The relative high stability may be an adaptation to survive in its' original arid habitat. Acid phosphatases were found to be extraordinarily stable in a wide range of pH for two weeks. Maximum pH stability was observed at pH 5, that gave a remaining activity of 77%. The lowest stability was observed at pH 9 with a 49% of remaining activity. The enzyme was more stable at acidic pHs (from pH 2 to 5). At neutral pHs it was comparatively unstable. Enzymatic activity was affected by Mg^{2+} and Zn^{2+}, but not by Ca^{2+}. Twenty percent increment was the maximum enhancement observed and it occurred at 4 mM of Mg^{2+} concentration. Zn^{2+} inhibited the enzymatic activity by 24% at 1.5mM concentration. SDS-PAGE analysis showed a prominent single band around 50 kDa from purified enzyme preparations of both opened and unopened pitchers.

Conclusion

This study indicates that the acid phosphatase contained in pitcher fluid of *Nepenthes distillatoria* is of plant origin. A single endogenous acid phosphatase could be isolated, partially purified, and characterized from open pitcher fluid of *Nepenthes distillatoria.* The same endogenous acid phosphatase could be completely purified from unopened pitcher fluid. In DE 52 anion exchange chromatography, the enzyme from both open and unopened pitchers was eluted at the NaCl concentration of 0.484 M indicating the presence of similar enzymes in both fluids. In Sephacryl S-200 gel filtration chromatography, the enzymes were

eluted around the same fraction further supporting their similarity. Optimum temperature and pH for phosphatase activity was 55°C and pH 4.5 respectively. The isolated enzyme was moderately thermally stable. Moreover, it was remarkably stable over a broad pH range. Molecular weight of the enzyme is approximately 50 kDa. The enzyme activity was not severely affected by the metal ions and therefore it may be a non metallo phosphatase.

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