PURIFICATION AND CHARACTERIZATION OF ACID PROTEINASES FROM *NEPENTHES DISTILLATORIA* L.

A THESIS PRESENTED

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ABSTRACT

Purification and characterization of acid proteinases from *Nepenthes distillatoria* L.

Plant aspartic proteinases so far have received much less attention in contrast to well-characterized mammalian, fungal and viral aspartic proteinases. They are widely dispersed in the plant kingdom and have been detected in seeds, leaves and flowers of different plants as well as in the digestive fluid of some insectivorous species. Insectivorous plant *Nepenthes distillatoria* is growing in the lowland wet zone of Sri Lanka and is a good source of proteolytic enzymes. Proteolytic enzymes are interesting not only from the point of view of plant physiology but also from the point of view of structure - function relationship and molecular evolution of aspartic proteinases. The objective of this study was to isolate, purify and characterize acid proteinases from the crude juice of the pitcher of *N. distillatoria*, which could be useful for future studies on plant physiology, structure - function relationship and molecular evolution of aspartic proteinases.

In this study, two acid proteinases, *Nepenthes* major and minor proteinases present in the crude juice of pitchers of *N. distillatoria* were purified to near homogeneity. Purification steps used were DEAE cellulose chromatography, sephacryl S-200 chromatography, pepstatin-sepharose chromatography and mono Q chromatography. Enzymes after purification were analyzed using SDS-PAGE to confirm the purity and to determine their molecular weights. Enzymatic properties of purified proteinases such as

time dependency, enzyme concentration dependency, pH dependency, temperature dependency, stability at different temperature and pH, effect of proteinase inhibitors were studied. Partial amino terminal amino acid sequences of both proteinases were determined and compared with reported sequences of other known plant aspartic proteinases such as rice, barley and cardoon.

Antibodies to both enzymes were produced by immunizing rabbits with purified enzymes. Antibodies were purified by ammonium sulphate saturation and affinity chromatography on protein - A sepharose. Histochemical staining using both antibodies was performed using transverse sections of fresh *Nepenthes* pitchers obtained under freezing conditions. Proteolytic action of *Nepenthes* major proteinase at different pH levels was investigated on natural proteins.

Purification fold and yield obtained after mono Q chromatography step were 59 times and 26.1 % with major proteinase and 44 times and 15.6% with minor proteinase. Based on the characteristics, it is suggested that both proteinases have similar properties. Purified enzymes are likely to be aspartic proteinases as reflected by the complete inhibition of proteolytic activity by 0.1mM pepstatin. Both proteinases were inhibited by diazoacetyl-DL norleucine methyl ester (DAN) and the pattern of inhibition is completely different with that of porcine pepsin suggesting that they are non-pepsin type aspartic proteinases. Molecular weights of major and minor enzymes are 43 kDa and 35 kDa as per SDS-PAGE separation. Purified enzymes have an optimum pH of 3.0 with 2% denatured hemoglobin as substrate. Optimum temperatures for activity of major and minor enzymes are 55 ^{0}C and 45 ^{0}C respectively. Both enzymes show a remarkable stability at higher

temperatures (50 ^{0}C) and at a wide pH range (pH 2-10) compared to porcine pepsin. Low homology of both major and minor proteinases with the amino acid sequences of known aspartic proteinases suggest the unique structural features of *Nepenthes* proteinases.

Immunohistochemical staining suggest that both enzymes are produced by the cells located in the inner wall of the lower 1/3 part of the pitcher. Proteolytic action of *Nepenthes* major acid proteinase on dhal and other proteins at acidic as well as neutral pH levels was remarkable.

Further studies on the three dimensional structure of the enzymes is recommended to precisely relate the unique properties of *Nepenthes* major and minor proteinases to their structure.