

VARIATIONS IN THE CONTENT AND ISOZYMIC COMPOSITION OF
NEPENTHESIN IN THE PITCHER FLUIDS
AMONG *NEPENTHES* SPECIES

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Introduction

Carnivorous plants are of great evolutionary interest in their function to catch prey (mainly insects) to digest their proteins as a nitrogen source (Juniper *et al.* 1989; Frazier 2000). Among them, *Nepenthes* species utilize pitchers which contain digestive fluid with the acid proteinase nepenthesin (Amagase *et al.* 1969; Woessner 2004). Previous inhibitor studies (Lobareva *et al.* 1973; Takahashi *et al.* 1974; Tökés *et al.* 1974) indicated that nepenthesin belongs to the aspartic proteinase family. Recently, two nepenthesins have been purified to homogeneity and characterized from *N. distillatoria*, and their primary structures were elucidated by cloning of the cDNAs from the pitcher of *N. gracilis* (Athauda *et al.* 1998, 2002, 2004; Takahashi *et al.* 2005). Cloning of aspartic proteinases from the pitcher tissue of *N. alata* has also been reported recently (Ann *et al.* 2002a); however, these enzymes appear to belong to vacuolar aspartic proteinases. The peptide bond cleavage specificity of nepenthesin has been investigated using *Nepenthes* pitcher fluid (Ann *et al.* 2002b), partially purified nepenthesin (Amagase *et al.* 1969; Tökés *et al.* 1974) and fully purified nepenthesin (Athauda *et al.* 2004; Takahashi *et al.* 2005). The results of these investigations appear to be rather variable; this may be partly due to the differences in the substrates and *Nepenthes* species used and enzyme purity. *Nepenthes* is known to include around 70 different species (Juniper *et al.* 1989), and so far it is not clear how much quantities and how many kinds of such proteinases are generally present in the pitcher fluids of various *Nepenthes* species and how much they are different from each other in structural and enzymatic properties. The present study was performed as a preliminary attempt to shed some light on these issues. First, we assayed the acid proteinase activity in the pitcher fluids of various *Nepenthes* species to obtain information on the enzyme contents among them. Second, we analyzed the isozymic composition by using native polyacrylamide gel electrophoresis combined with activity staining. The results indicate that there are significant variations in the content of nepenthesin in the pitcher fluids among *Nepenthes* species but that the proteolytic activity is largely due to two groups of nepenthesins.

Materials and Methods

The *Nepenthes* species and hybrids examined were grown as free as possible from the access of insects, and pitcher fluids were collected within a few days before and after the lid opening. The proteinase activity was determined at pH 2.0 using bovine hemoglobin as a substrate as described (Athauda *et al.* 1989), and expressed in pepsin equivalent using porcine

pepsin (Sigma) as a standard. The pH 2.0 was chosen to compare the activity with that of porcine pepsin obtained under the standard pepsin assay conditions; most workers agree on a single pH optimum for *Nepenthes* secretion around 2.2, very similar to that of pepsin (Juniper *et al.* 1989). Electrophoresis and activity staining were performed as follows. An appropriate portion (20µl) of each pitcher fluid was submitted to native polyacrylamide gel electrophoresis using 7.5% acrylamide gel and Tris-glycine buffer, pH 8.7, and then proteinase activity was examined by activity staining with hemoglobin as a substrate at pH 1.7 and 7.3 as described (Furihata *et al.* 1972). The pH value of each pitcher fluid was measured using a glass electrode in a Horiba pH-meter.

Results and Discussion

Table 1 shows the pH values and the acid proteinase activity of the pitcher fluids of 17 different *Nepenthes* species and hybrids examined. The pH values varied from 2.6 to 4.7, and the proteolytic activity per ml of the pitcher fluid ranged from 0 to 52µg as expressed in pepsin equivalents.. Thus, there are marked variations in the content of the acid proteinase among the species examined. The total proteolytic activity in the pitcher fluid varied from 0 to 105 µg, partly reflecting the differences in pitcher size. It is notable that the open pitcher fluids always contained a substantial level of acid proteinase activity whereas no activity was detected with at least three of the ten samples of the unopen pitchers examined. These results suggest that before the lid opening, in at least some cases, the acid fluid is secreted first into the pitcher, followed later by the secretion of the enzyme.

Figure 1 shows the results of activity staining after native polyacrylamide gel electrophoresis of the pitcher fluids from 15 different *Nepenthes* species. When the activity was stained at

Table 1: Acid proteinase activities in the pitcher fluids of various *Nepenthes* species

No.	<i>Nepenthes</i> species	No. of pitchers used ^a	pH	Activity ^b per ml	Activity ^b per pitcher
1	<i>N. × mixta</i>	1 (o)	4.7	7.5	52
2	<i>N. × coccinea</i>	6 (u/o)	4.4	6.9	7
3	<i>N. × superba</i>	1 (u)	4.3	8.9	83
		2 (u/o)	4.7	9.3	33
4	<i>N. thorelii</i>	3 (u)	2.8	52	36
		4 (o)	4.3	35	62
5	<i>N. rafflesiana</i>	1 (u)	3.9	0	0
6	<i>N. alata × merrilliana</i>	2 (u)	4.3	0	0
7	<i>N. hybrid</i> ^c	1 (o)	3.9	1.0	17
8	<i>N. mirabilis</i>	1 (u)	3.7	0	0
		2 (o)	2.8	5.1	28
9	<i>N. × intermedia</i>	1 (u)	4.1	0.4	1
10	<i>N. thorelii × N. × coccinea</i>	1 (u)	3.8	1.6	10
		1 (o)	2.6	9.1	101
11	<i>N. dicksoniana × dyeriana</i>	3 (u)	4.4	14	35
12	<i>N. × henryana</i>	1 (u)	3.8	0.8	2
13	<i>N. alata</i>	2 (o)	4.1	3.0	6
14	<i>N. maxima</i>	1 (u)	3.9	26	24
15	<i>N. × wrigleyana</i>	1 (o)	3.8	6.7	27
16	<i>N. ventricosa</i>	2 (o)	4.4	41	43
17	<i>N. thorelii × maxima</i>	1 (o)	4.2	28	105

^aFor those samples where two or more pitchers were used, the pitcher fluids were combined, then used for the pH and activity measurements. The states of pitchers are shown in parentheses: u, unopen pitcher; o, open pitcher; u/o, a mixture of unopen and open pitchers.

^bActivity was determined with hemoglobin as a substrate at pH 2.0 and denoted in terms of µg of porcine pepsin equivalents.

^cA hybrid with disputed parentage.

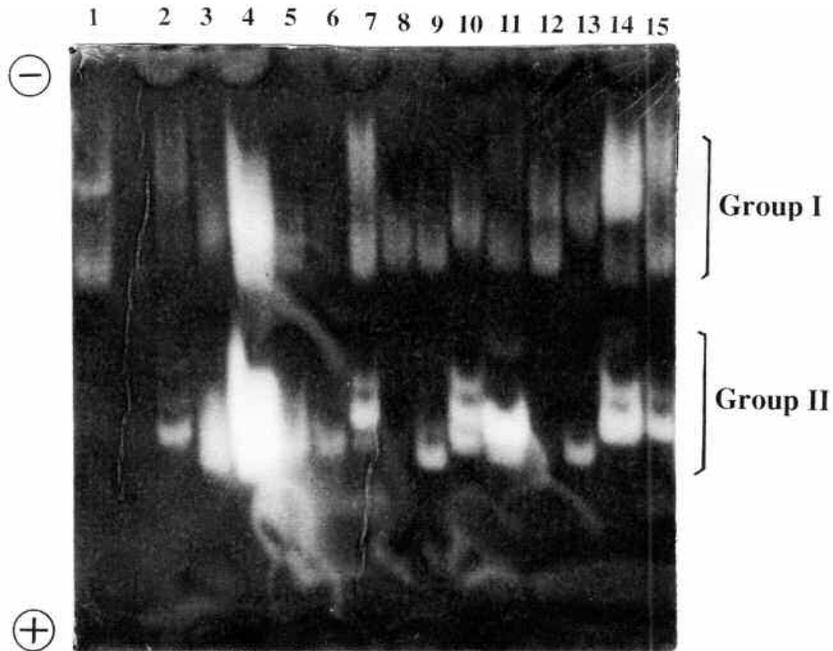


Figure 1: Activity staining of acid proteinases from the pitchers of *Nepenthes* spp. A portion (20 μ l) of each pitcher fluid was submitted to native polyacrylamide gel electrophoresis at pH 8.7, and the proteinase activity was stained by incubation of the gels with hemoglobin as a substrate at pH 1.7. The sample numbers correspond to those in Table 1. Pitcher fluids from unopen pitchers were used for sample Nos. 3-6, 8-12 and 14. The pitcher fluids of sample Nos. 16 and 17 were not analyzed.

pH 1.7 (Figure 1), the extents of staining were roughly comparable with the activity per each sample used. It is notable that activity was detected on the gel even with sample Nos. 5, 6 and 8 which showed practically no activity in the hemoglobin assay. This contradiction may be due to the difference in sensitivity of the two methods; however, the possibility that the bands obtained with sample Nos. 5, 6 and 8 may be due to artifacts cannot be completely excluded. Almost all species examined gave two or occasionally up to four acid proteinase bands, which seem to constitute two major groups (groups I and II) notably different in electrophoretic mobility, and hence in isoelectric point. Each sample showed one to two bands in the group I region and one to three bands in the group II region. This multiplicity is thought to reflect the isozymic variations of nepenthesin. On the other hand, when the activity staining was performed at pH 7.3, no proteinase activity was detected (data not shown), indicating that the pitchers may contain no endopeptidase capable of digesting hemoglobin other than the acid proteinase nepenthesin. A definite conclusion on this point, however, can be made only after a more detailed research.

Occurrence of the two groups of acid proteinases is consistent with the fact that two distinct types of acid proteinases with different isoelectric points (nepenthesins I and II) were found in and isolated and characterized from the pitcher fluids of *N. distillatoria* (Athauda *et al.* 1998, 2002, 2004; Takahashi *et al.* 2005), and that the corresponding two enzymes were structurally characterized by cloning and sequencing of the two cDNAs from the pitcher tissue of *N. gracilis* (Athauda *et al.* 2004; Takahashi *et al.* 2005). The isoelectric points of nepenthesins I and II from *N. gracilis* were calculated to be 3.94 and 3.09, respectively. Therefore, nepenthesins I and II are thought to correspond to the groups I and II acid proteinases, respectively. As for nepenthesin I from *N. gracilis*, two isozymic forms, nepenthesin Ia and Ib, were found, which have slight differences in amino acid sequence. Two isozymic forms with slight differences in amino acid sequence were also found in both nepenthesins I and II from *N. distillatoria*. Nepenthesin I is a

glycoprotein (Athauda *et al.* 1998), and therefore the multiple bands in the group I proteinases (see Figure 1) may be partly due to the heterogeneity in the carbohydrate moieties.

Although this study is a preliminary one using a limited number of *Nepenthes* samples, the results indicate that there is a significant variation in the content of the proteolytic activity in the pitcher fluid among the *Nepenthes* species, but that the proteolytic activity is largely due to the two groups of enzymes, nepenthesins I and II. The variations in the enzyme content may be in part related to the phylogenetic and habitat diversities of the species (Meimberg *et al.* 2001).

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