

COMPARATIVE STUDIES ON THE ACID PROTEINASE ACTIVITIES IN THE DIGESTIVE FLUIDS OF *NEPENTHES*, *CEPHALOTUS*, *DIONAEA*, AND *DROSERA*

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Introduction

There are various kinds of carnivorous plants in nature, and most of them excrete acidic digestive fluids which contain digestive enzymes, especially acidic proteinases, to digest trapped insects and other prey for nutrition (Juniper *et al.* 1989). Previous inhibitor studies (Lobareva *et al.* 1973; Takahashi *et al.* 1974; Tökés *et al.* 1974) indicated that the acid proteinase nepenthesin from *Nepenthes* belongs to the aspartic proteinase family. Recently, we purified two nepenthesins from the digestive fluid of *N. distillatoria*, investigated their molecular and enzymatic properties, and elucidated their primary structures by cDNA cloning of nepenthesins from *N. gracilis* (Athauda *et al.* 1998, 2002, 2004; Takahashi *et al.* 2005). The peptide bond cleavage specificity of nepenthesin was investigated using *N. alata* pitcher fluid (Ann *et al.* 2002), partially purified nepenthesin from a mixture of several *Nepenthes* species (Amagase *et al.* 1969) and from *N. macfarlanei* (Tökés *et al.* 1974), and fully purified nepenthesin from *N. distillatoria* (Athauda *et al.* 2004; Takahashi *et al.* 2005). In addition, some enzymatic properties of the acid proteinase activities in the crude digestive fluids of *Nepenthes* sp. (Lüttge, 1964) and *Dionaea muscipula* (Scala *et al.* 1969; Robins & Juniper 1980) and a partially purified acid proteinase from *Drosera peltata* (Amagase *et al.* 1972a, 1972b) were reported. Except for these studies, however, not many studies have been performed on the enzymatic properties of these acid proteinases. Therefore, further studies are necessary to understand more extensively the nature of the acid proteinases in the digestive fluids of carnivorous plants. Since the digestion takes place in the crude digestive fluid, it is thought to be important to characterize the acid proteinase activity as a whole as well as to purify and characterize the individual proteinases. Thus, we have investigated in the present study some enzymatic properties of the acid proteinase activities in the crude digestive fluids of typical carnivorous plants including two pitcher plants, *Nepenthes alata* and *Cephalotus follicularis*, one plant with a snap-trap, *Dionaea muscipula* (Venus flytrap), and one plant with a mucilage trap, *Drosera capensis* (sundew), in a comparative manner. The results demonstrated significant differences among them, presumably reflecting the phylogenetic diversity of these carnivorous plants.

Materials and Methods

The crude digestive fluids of *Nepenthes alata* and *Cephalotus follicularis* were collected in the Daishoen plantation (Numazu). *Dionaea muscipula* and *Drosera capensis* were obtained from the Daishoen plantation and grown at the botanical garden of Tokyo University of Pharmacy and Life Sciences to obtain their digestive fluids. The digestive fluid of *Dionaea* was collected 3 to 4 days after giving a piece (about 3.5 × 3.5 × 2.3 mm) of boiled egg white, thereby inducing closure of the two lobes by stimulating the trigger hairs. The digestive fluid of *Drosera* was collected by soaking ten leaves successively (1 leaf for 1 min at a time) in 800 µl of 0.1 M sodium acetate buffer, pH 4.0, in a test tube to wash out the digestive fluid through up-and-down strokes. The pH was determined using a glass electrode in a Horiba pH-meter. The samples were kept frozen at -20°C

until use. Porcine pepsin A, bovine hemoglobin, the B chain of oxidized bovine insulin and leucyl-4-methylcoumaryl-7-amide (Leu-MCA) and arginyl-4-methylcoumaryl-7-amide (Arg-MCA) were obtained from Sigma and pepstatin A from Peptide Institute, Osaka. Other reagents used were of analytical grade.

The proteinase activities of the digestive fluids of *Nepenthes*, *Cephalotus*, and *Dionaea* were determined with bovine hemoglobin as a substrate at pH 2.0 as described (Athauda *et al.* 1989), and expressed in pepsin equivalent using porcine pepsin A (Sigma) as a standard. The proteinase activity of the digestive fluid of *Drosera capensis* in the hemoglobin assay was markedly low as compared with those of the other three. Therefore oxidized insulin B chain was used as a substrate and the digestion products were analyzed by HPLC. The assay mixture was composed of 1-40 μ l of enzyme solution, 10 μ l of oxidized insulin B chain (1 mg/ml), 50 μ l of 0.1 M sodium acetate buffer, pH 4.0, an appropriate volume (0-39 μ l) of water in a total volume of 100 μ l, and the digestion was performed at 37°C for 1 h and terminated by the addition of 100 μ l of 0.5 M H₃BO₃-KCl/NaOH buffer, pH 9.8. A 100- μ l aliquot from the reaction mixture was analyzed by reverse-phase HPLC using a Shimadzu LC10A system on a Tosoh ODS-120T column (4.6 \times 250 mm) eluted with an acetonitrile gradient (0 to 60%) in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min and monitored at 215 nm. The activity was estimated from the decrease in the amount of the substrate, and expressed in pepsin equivalent determined using porcine pepsin A as a standard under the same assay conditions at pH 4.0.

Aminopeptidase activities were determined with Leu-MCA and Arg-MCA as substrates. The assay mixture contained 5 μ l (*Nepenthes*), 200 μ l (*Cephalotus*), or 10 μ l (*Dionaea*) of enzyme solution, 5 μ l of 10 mM substrate in dimethylsulfoxide, 310 μ l of 0.1 M citric acid-Na₂HPO₄ buffer, pH 3.0-6.0, and an appropriate volume (0-195 μ l) of water in a total volume of 515 μ l. The digestion was performed at 37°C for 30 min and stopped by the addition of 2.5 ml of 5% trichloroacetic acid. The amount of 7-amino-4-methylcoumarin liberated was measured in a Hitachi spectrofluorometer with excitation at 380 nm and emission at 460 nm.

Native electrophoresis followed by activity staining was performed as follows. An appropriate portion (20 μ l) of each pitcher fluid was submitted to native polyacrylamide gel electrophoresis using 10% 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 essentially as described (Furihata *et al.* 1972).

To investigate the substrate specificity, oxidized insulin B chain was used as a substrate. The reaction mixture contained 10 μ l (*Nepenthes*), 40 μ l (*Cephalotus*), or 3 μ l (*Dionaea*) of the digestive fluid containing about 1 pmol enzyme as porcine pepsin equivalent, 10 μ l of oxidized insulin B chain (1 mg/ml), 50 μ l of 0.1 M sodium formate buffer, pH 3.0, and an appropriate volume (0-37 μ l) of water in a total volume of 100 μ l. The digestion was performed at 37°C for 3 h, and the resulting peptides were analyzed using an HPLC apparatus (1100 series, Agilent Technology) with a TSKgel ODS-120T column (2.2 \times 150 mm) connected to an LCTM-DUO mass spectrometer (ThermoQuest). Amino acid sequences of the peptides produced were determined from the mass spectra of the original and fragmented ions by using Xcalibur Bioworks 1.0 software installed in the apparatus as described (Nishii *et al.* 2002).

Results and Discussion

The collected fluid samples of *Nepenthes alata*, *Cephalotus follicularis*, and *Dionaea muscipula* had a pH of 2.9, 2.9, and 3.9, respectively, and were shown to contain approximately 10 pmol, 2 pmol, and 40 pmol, respectively, of acid proteinase in pepsin equivalent per 100 μ l as determined with hemoglobin as a substrate. On the other hand, the digestive fluid of *Drosera capensis* had a pH of about 2.5, and the washate of ten leaves with 800 μ l of the pH 4.0 buffer contained approximately 100 pmol of acid proteinase in pepsin equivalent per 100 μ l as determined with oxidized insulin B chain as a substrate.

Figure 1 shows the activity staining after native polyacrylamide gel electrophoresis (PAGE) of the crude digestive fluids. Two broad bands are seen for the *Nepenthes* sample; the major, slow-moving band should correspond to nepenthesin I and the minor, fast-moving band to nepenthesin II. These broad bands may contain more than one acid proteinase isozyme as shown for nepenthesins I and II from *N. gracilis* and *N. distillatoria* (Athauda *et al.* 2004; Takahashi *et al.* 2005). The *Dionaea* sample gave a single broad band moving almost in parallel with nepenthesin I, but the band corresponding to nepenthesin II was hardly seen. The major band may contain more than one acid proteinase component. Indeed, it was reported previously that two major bands were detected on native PAGE of the digestive fluid of *Dionaea muscipula* (Robins & Juniper 1980). In the case of *Cephalotus*, there are two bands comparable with the nepenthesin bands, but the major band corresponding to nepenthesin I had higher mobility toward cathode. Thus, the electrophoretic patterns are similar but not identical among the three samples. The analysis of the *Drosera* sample gave no clear band presumably due to the low sensitivity of the enzyme toward hemoglobin (data not shown).

Figure 2 shows the pH profiles of the enzymatic activities. Both the *Nepenthes* and *Cephalotus* samples had the pH optimum at around 2.5, whereas the *Dionaea* and *Drosera* samples showed the pH optimum at 3.0 and 3.5, respectively. The result with the *Nepenthes* sample is similar to the pH profiles of nepenthesins I and II from *N. distillatoria* having an optimum at pH 2.6 and that of the crude fluid from *N. distillatoria* with an optimum at pH 2.8 (Athauda *et al.* 2004; Takahashi *et al.* 2005). As compared with the pH profile of the *Cephalotus* sample, those of the other three had broader pH profiles, which might mean that they are composed of more than one enzyme component with fairly different pH optima. As for the *Dionaea* digestive fluid, the pH-activity profile with casein as a substrate was reported to show two peaks at pH 4.0 and 5.0 (Robins & Juniper 1980) or one sharp peak at pH 5.3 (Scala *et al.* 1969). Several pH optima (Chandler & Anderson 1976) or an irregular pH profile (Clancy & Coffey 1977) with casein as a substrate were reported for the digestive fluids of *Drosera* species. The reason for the differences between the present results and those reported previously is not clear at present, but might be at least partly due to the difference in the assay conditions, especially in the substrate used.

The temperature dependence of activity of each sample is shown in Figure 3a. The *Nepenthes* sample showed a temperature-activity profile with a maximum at about 57°C. This is similar to that of nepenthesin I from *N. distillatoria* which has a maximum at 55°C, but the maximum temperature was higher than that of the crude fluid from *N. distillatoria* with a maximum at 50°C (Athauda *et al.* 2004; Takahashi *et al.* 2005). The results are roughly similar to that reported for the digestive fluid of *N. khasiana* (Lüttge 1964). On the other hand, the *Cephalotus*, *Dionaea*, and *Drosera* samples showed a temperature-activity profile with a maximum at 47°C, 47°C, and 42°C, respectively, which are apparently similar to that of nepenthesin II from *N. distillatoria* (Athauda *et al.* 2004; Takahashi *et al.* 2005). The profile of the *Dionaea* sample has a shoulder at around 60°C, which might indicate the presence of more than one component with fairly different temperature dependence of activity.

The results of temperature-stability experiments are shown in Figure 3b, in which each crude sample was incubated at different temperatures for 1 h, then the remaining activity was determined. As for the *Nepenthes* sample, the activity was stable up to around 53°C, then started to decrease and was lost completely at around 80°C. This result was similar to those obtained with nepenthesin I and the crude fluid from *N. distillatoria* (Athauda *et al.* 2004; Takahashi *et al.* 2005). A similar result was obtained with the

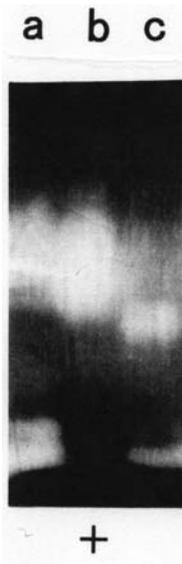


Figure 1:
Native polyacrylamide gel electrophoresis with proteinase activity staining of the digestive fluids of carnivorous plants. (a) *Nepenthes alata*, (b) *Dionaea muscipula*, (c) *Cephalotus follicularis*.

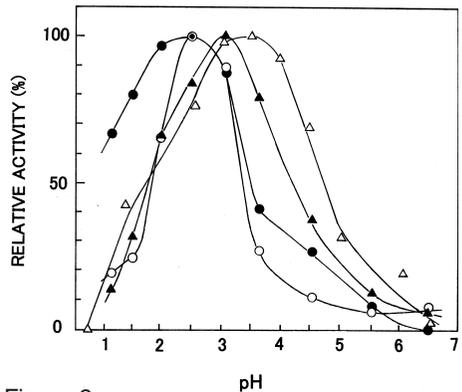


Figure 2:
pH dependence of the proteinase activities of the digestive fluids of carnivorous plants. Closed circle, *Nepenthes alata*; open circle, *Cephalotus follicularis*; closed triangle, *Dionaea muscipula*; open triangle, *Drosera capensis*. The same symbols are used in Figures 3-5. Buffers used were 0.1 M HCl/KCl buffers (pH 1.1 to 2.0) and 0.1 M citric acid/Na₂HPO₄ buffers (pH 2.5 to 6.5) except for *Drosera* for which 0.1 M HCl/KCl buffers (pH 0.7 to 1.4), 0.1 M glycine/HCl buffers (pH 2.6 to 3.5), 0.1 M potassium acetate/HCl buffers (pH 4.0 to 5.4) and 0.1 M KH₂PO₄/NaOH buffers (pH 6.1 to 6.6) were used.

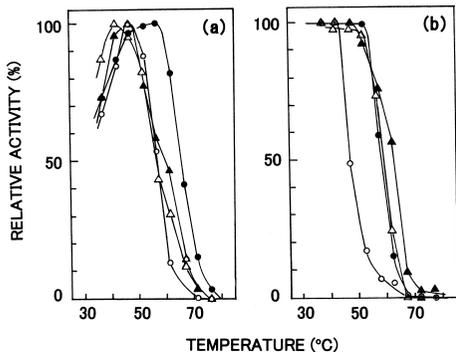


Figure 3:

Effects of temperature on the proteinase activities and stabilities of the digestive fluids of carnivorous plants. (a) The activity was measured at various temperatures at pH 2.0 except for the *Drosera* sample which was assayed at pH 4.0. (b) Each sample was incubated at various temperatures and pH 2.9 (*Nepenthes* and *Cephalotus*), 3.9 (*Dionaea*), or 4.0 (*Drosera*) for 1 h at an enzyme concentration of approximately 5-10 pmol pepsin equivalents per 200 μ l and then assayed as above.

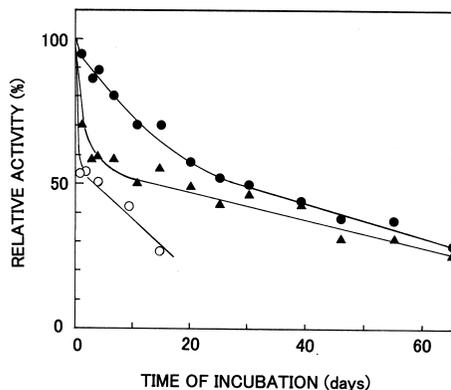


Figure 4:

Stabilities of the proteinase activities of the digestive fluids of carnivorous plants on long incubation. The activity was measured at 37°C after incubation of each sample for various periods at 37°C and pH 2.9 (*Nepenthes* and *Cephalotus*) or 3.9 (*Dionaea*) at an enzyme concentration of approximately 5-10 pmol pepsin equivalents/200 μ l. The experiment with *Drosera* was not performed.

Drosera sample. On the other hand, the activity of the *Cephalotus* sample was less stable. It was stable up to 42°C, where it started to decrease to zero at around 70°C. Interestingly, the *Dionaea* sample was apparently more stable than the *Nepenthes* sample at above 55°C. Thus, the Tm values were approximately 63°C, 59°C, 58°C, and 47°C, respectively, for the *Dionaea*, *Drosera*, *Nepenthes*, and *Cephalotus* samples. The temperature-stability profile of the *Dionaea* sample was different from those of the other three. The activity was stable at around 50°C, but above this temperature the inactivation appeared to proceed in two steps, suggesting the presence of at least two enzyme components different in heat stability. These results are consistent with the temperature dependences of the activities shown in Figure 3a.

Figure 4 shows the changes of the acid proteinase activities upon longer incubation at 37°C. The activity of the *Nepenthes* sample decreased only very slowly on longer incubation; about 60% and 30% of the original activity were still retained after 30 days and 65 days of incubation, respectively. Thus, the activity of the crude digestive fluid of *N. alata* is considerably stable under the conditions used. However, under similar conditions, that of *N. distillatoria* retained nearly full activity after 30 days of incubation (Athauda *et al.* 2004; Takahashi *et al.* 2005). This difference might be due to the difference in the *Nepenthes* species used and/or the difference in the experimental conditions. In contrast to the *Nepenthes* sample, the activity of the *Cephalotus* sample was rather unstable; nearly 50% and 70% of the original activity were lost in 1 h and 15 h, respectively. On the other hand, the *Dionaea* sample appeared to be considerably more stable than the *Cephalotus* sample, but somewhat less stable than the *Nepenthes* sample in the early phase of incubation; about 40% and 25% of the original activity were lost in 3 h and 65 h, respectively. As can be seen from Figure 4, the profiles of the activity change of both *Cephalotus* and *Dionaea* samples appeared to be biphasic. This suggests that each of these samples contains a relatively unstable component and a more stable component. The latter component in *Dionaea* appears to be comparable in stability with the *Nepenthes* sample. The experiment with the *Drosera* sample has not yet been performed.

The effects of pepstatin A on the activity of each sample are shown in Figure 5. Under the conditions used, the activities of all samples appeared to be nearly half lost in the initial phase at a low pepstatin concentration (up to about 1 μ M). At higher concentration of pepstatin, the *Nepenthes* and *Cephalotus* samples were inhibited further, but more weakly, and nearly complete inhibition occurred with 120 μ M of the inhibitor. On the other hand, the activity of the

Dionaea sample was not inhibited completely; the activity was half inhibited at a low concentration of pepstatin A, but the remaining about 50% of the activity was almost insensitive to pepstatin A and remained uninhibited in the presence of 100 μM inhibitor. In contrast, the *Drosera* sample was inhibited completely at a pepstatin concentration of 5 μM .

These results indicate the following. First, the *Nepenthes* and *Cephalotus* samples each may contain two kinds of enzymes with a higher and a lower affinity to pepstatin. Indeed, nepenthesins I and II of *N. distillatoria* have been shown to have different affinity to pepstatin (Athauda *et al.* 2004; Takahashi *et al.* 2005). Second, the *Dionaea* sample may contain a pepstatin-insensitive acid proteinase in addition to the nepenthesin-like pepstatin-sensitive acid proteinase. This pepstatin-insensitive enzyme has not yet been identified, but might be similar to glutamic peptidases such as aspergilloglutamic peptidase (Huang *et al.* 2000; Yabuki *et al.* 2004; Sasaki *et al.* 2004) and scytalldoglutamic peptidase (Fujinaga *et al.* 2004; Kataoka *et al.* 2005) or serine-carboxyl peptidases such as physarolisin (Nishii *et al.* 2003a, 2003b) and pseudomonalisin (Wlodawer *et al.* 2001), each of which is known to be insensitive to pepstatin but has an acidic pH optimum. To our knowledge, such an enzyme as a glutamic peptidase or serine-carboxyl peptidase has not been found so far in plants. The occurrence of at least two different types of acid proteinases is consistent with the biphasic character of the *Dionaea* proteinase activity observed already. The pepstatin-insensitive activity in the *Dionaea* sample appeared to be less stable than the pepstatin-sensitive activity when the enzyme sample was stored at 4°C. In contrast to the present results, the acid proteinase activity in the *Dionaea* digestive fluid was previously reported to be insensitive to pepstatin (Robins & Juniper 1980). The reason for this difference is not certain at present, but might partly be due to the occurrence of two different types of acid proteinases. Third, the *Drosera* sample may contain only acid proteinases with a relatively higher pepstatin affinity.

The peptide bond cleavage specificities of the acid proteinases in the *Nepenthes*, *Cephalotus*, and *Dionaea* samples as examined with oxidized insulin B chain as a substrate are shown in Figures 6 through 8. In the *Nepenthes* sample, the major cleavages occurred at Leu¹⁵-Tyr¹⁶ and Phe²⁴-Phe²⁵, and moderate cleavages at Glu¹³-Ala¹⁴, Ala¹⁴-Leu¹⁵, Tyr¹⁶-Leu¹⁷, and Tyr²⁶-Thr²⁷. These results are roughly similar to those reported previously for the crude digestive fluid of *N. alata* (Ann *et al.* 2002) and the purified nepenthesin I from *N. distillatoria* (Athauda *et al.* 2004; Takahashi *et al.* 2005). In the latter case, Leu⁶-Cys(ox)⁷ bond was cleaved to a significant extent, which might be due to species or isozymic difference. In the *Cephalotus* sample, the major cleavages occurred at Leu¹⁵-Tyr¹⁶, Phe²⁴-Phe²⁵, and Lys²⁹-Ala³⁰, and moderate cleavages at Glu¹³-Ala¹⁴ and Ala¹⁴-Leu¹⁵. In the case of the *Dionaea* sample, the major cleavages occurred at Glu¹³-Ala¹⁴, Leu¹⁵-Tyr¹⁶, and Phe²⁴-Phe²⁵, and moderate cleavages at Ala¹⁴-Leu¹⁵, Tyr¹⁶-Leu¹⁷, Gly²³-Phe²⁴, and Lys²⁹-Ala³⁰. Thus, the major cleavages at Leu¹⁵-Tyr¹⁶ and Phe²⁴-Phe²⁵ were common, but the other cleavage sites varied significantly among the three samples. Especially, the *Cephalotus* and *Dionaea* samples differ from the *Nepenthes* sample in that Lys²⁹-Ala³⁰ and Glu¹³-Ala¹⁴, respectively, are also the major cleavage sites. These differences should reflect the difference in the cleavage specificity of the enzyme components in each sample.

Aminopeptidase activities in the acidic pH range were determined using Leu-MCA and Arg-MCA as substrates. Neither activity was detected with *Nepenthes* and *Cephalotus* in the pH range of 3-6 and 3-5, respectively. As for *Dionaea*, a trace activity toward Leu-MCA was detected but no activity toward Arg-MCA in the pH range of 3-6. Therefore, practically no aminopeptidase action is assumed to be involved in the cleavages of oxidized insulin B chain observed in the present study. On the other hand, the activities of carboxypeptidases were not analyzed in the present study; the possibility for the action of a carboxypeptidase(s) cannot be completely excluded, however, considering the reports of the occurrence of carboxypeptidase in the digestive fluids of *N. alata* (Ann *et al.* 2002) and *Dionaea* (Robins & Juniper 1980).

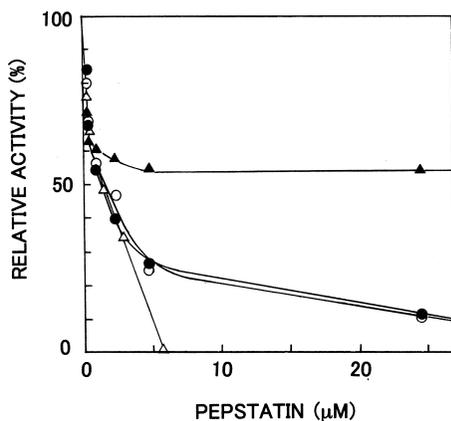


Figure 5: Effects of pepstatin A on the proteinase activities of the digestive fluids of carnivorous plants. The enzyme concentrations used were approximately 10-20 nM pepsin equivalents except for *Drosera* (400 nM), and the concentration of pepstatin was varied from 0 to 120 μM .

In the present study, the hemoglobin-digestion method was not so useful for the assay of the *Drosera capensis* acid proteinase activity; instead, the oxidized insulin B chain was useful as a substrate for the assay as coupled with HPLC. Similar results were also obtained with *D. filiformis*. This may be due mainly to the difference in the substrate specificity of *Drosera* sp. from that of other carnivorous plants and remains to be clarified in a future study. During the course of the present studies, we have also examined the acid proteinase activities of the digestive fluids of some other carnivorous plants. So far, the digestive fluids of *Drosophyllum*

Figure 6: Cleavage specificity of the acid proteinase activity of the digestive fluid of *Nepenthes alata* on oxidized insulin B chain. (a) The chromatogram shows the HPLC pattern of the hydrolysis products. (b) The amino acid sequence of oxidized insulin B chain is given in the one-letter notation. C*, cysteic acid. Large, medium, and small closed arrowheads indicate the major, medium, and minor cleavages, respectively, and small open arrowhead, trace cleavage. The number for each peptide stands for the peak number, and that in parenthesis an approximate value for the relative yield of each peptide. The relative yield was calculated by dividing the peak height by the number of the peptide bonds, assuming the extent at the maximum cleavage site to be 100%.

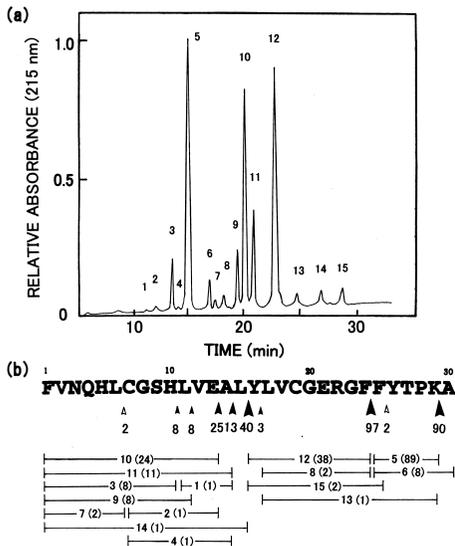
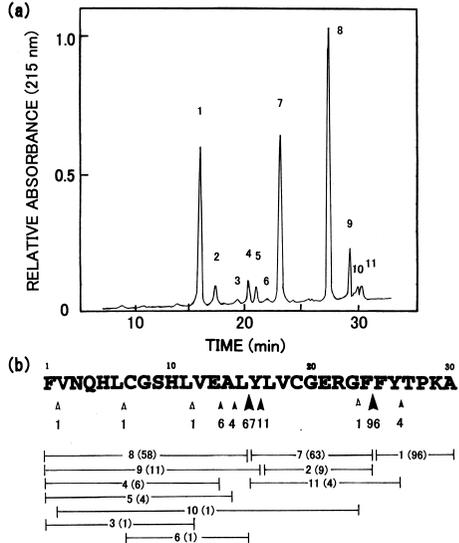


Figure 7: Cleavage specificity of the acid proteinase activity of the digestive fluid of *Cephalotus follicularis* on oxidized insulin B chain. The conditions are the same as those described in the legend to Figure 6.

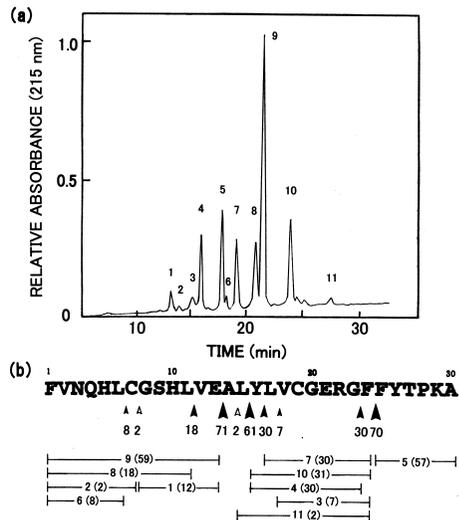


Figure 8: Cleavage specificity of the acid proteinase activity of the digestive fluid of *Dionaea muscipula* on oxidized insulin B chain. The conditions are the same as those described in the legend to Figure 6.

lusitanicum and *Byblis liniflora* showed high activity toward both hemoglobin and oxidized insulin B chain, whereas that of *Sarracenia purpurea* failed to give any activity toward hemoglobin. Further extensive studies along this line, as well as purification and characterization of individual enzymes, including many more carnivorous plants, are necessary for deeper understanding of the biochemistry and physiology of the acid proteinases in the digestive fluids of carnivorous plants.

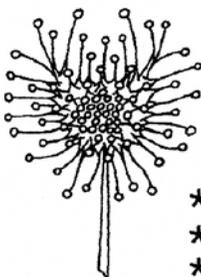
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