Note

A Cysteine Endopeptidase (‘‘Dionain’’) Is Involved in the Digestive Fluid of Dionaea muscipula (Venus’s Fly-trap)

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The carnivorous plant Dionaea muscipula (Venus’s flytrap) secretes proteinases into the digestive fluid to digest prey proteins. In this study, we obtained evidence that the digestive fluid contains a cysteine endopeptidase, presumably belonging to the papain family, through inhibitor studies and partial amino acid sequencing of the major SDS–PAGE band protein. The name “dionain” is proposed for the enzyme.

Key words: cysteine endopeptidase; digestive fluid; Dionaea muscipula (Venus’s flytrap); dionain; partial amino acid sequence

Carnivorous plants secrete various endopeptidases extracellularly to digest prey proteins.1) These enzymes are interesting from the standpoint of structure-function relationship and molecular evolution. Until recently, however, no such peptidase had been completely purified and characterized. In 2004 we purified nepenthesins from the digestive fluid of Nepenthes, a pitcher-type carnivorous plant, and characterized them structurally and enzymatically.2,3) Through these studies, nepenthesin was shown to be a novel type of aspartic endopeptidase (MEROPS subfamily A1B) distinct from the pepsin type aspartic endopeptidases (MEROPS subfamily A1A). In a continuation of these studies, we have been attempting to characterize endopeptidases secreted by other carnivorous plants to digest prey proteins.4)

In the present study, we analyzed the digestive fluid of Dionaea muscipula (Venus’s flytrap), a snap-trap type carnivorous plant, through inhibitor studies using benzylxoycarbonyl-Phe-Arg 4-methyl-L-coumarylamide (Z-Phe-Arg-MCA), a synthetic substrate often used in the assay of certain cysteine peptidases such as cathepsins B and L,5,6) as a substrate, and partial amino acid sequencing. The results indicated that the digestive fluid contains a cysteine endopeptidase, presumably belonging to the papain family (MEROPS subfamily C1A).

D. muscipula was obtained from the Daishoen Plantation (Numazu, Japan). The digestive fluid was collected using boiled egg white as a prey protein, essentially as described previously.3) Z-Phe-Arg-MCA and trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64) were obtained from Peptide Institute (Osaka, Japan). Other reagents used were of analytical grade and were obtained from Wako Pure Chemical Industries (Tokyo), unless otherwise specified.

To measure the activity toward Z-Phe-Arg-MCA, the reaction mixture contained 20 μL of the digestive fluid, 5 μL of 2 mM Z-Phe-Arg-MCA in dimethyl sulfoxide, and 75 μL of 100 mM buffer at various pH values. The mixture was incubated at 37°C, the increase in fluorescence at 460 nm with excitation at 370 nm was measured at 5-min intervals for 55 min, and the activity was determined from the slope of the digestion curve. To measure the effects of other agents, a small volume of each reagent solution (e.g., 1 μL of 1 M diithiothreitol or DTT and 1 μL of 1 mM E-64) was added, and the mixture was preincubated at 37°C for 5 min before addition of the substrate.

Partial amino acid sequencing was performed as follows: A portion of the collected crude fluid was treated with trichloroacetic acid (TCA) to precipitate proteins. The protein fraction thus obtained was submitted to SDS–PAGE under reducing conditions with DTT in 10% polyacrylamide gel by the method of Laemmli,7) and the protein bands were detected by Coomasie Brilliant Blue staining. The major protein band was excised from the gel and destained with aqueous 50% methanol and 10% acetic acid. A portion of the sample was in-gel digested with asparaginyl endopeptidase (Asn-N, Roche) at pH 8.0 at 37°C for

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Abbreviations: Z-Phe-Arg-MCA, benzylxoycarbonyl-Phe-Arg 4-methyl-7-coumarylamide; DTT, diithiothreitol; E-64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; TCA, trichloroacetic acid
16 h, and analyzed as follows essentially as described elsewhere. The digest was submitted to HPLC on an Inertsil ODS-3 column (1 × 100 mm, GL-Sciences, Torrance, CA), using an acetonitrile gradient (0 to 10% in 10 min and 10 to 40% in 60 min) in 0.075% trifluoroacetic acid. The major band protein and some purified Asn-N peptides were sequenced with a Procise cLC protein sequencing system (Applied Biosystems, Foster City, CA).

Figure 1 shows the pH dependence of the peptidase activity in the digestive fluid toward Z-Phe-Arg-MCA. The activity was observed between pH 3.0 and 8.0, and was optimal at about pH 7.0. Activity was elevated more than 20 times in the presence of 10 mM DTT, and was inhibited completely with cysteine peptidase inhibitors such as 10 mM E-64 and 0.5 mM p-chloromercuribenzoate. No marked inhibition was observed for the other peptidase inhibitors, such as 10 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, and 10 mM pepstatin A. These results show that the present enzyme was a cysteine peptidase.

Figure 2 shows the SDS–PAGE patterns obtained under reduced conditions of the protein in the digestive fluid (lane 2) and its TCA precipitate fraction (lane 3). The major high molecular mass protein band was obtained at about 45 kDa. In addition, several lower molecular mass protein bands were seen, which were thought partially to represent degradation products of the prey egg-white proteins. The 45-kDa band protein in the TCA precipitate fraction was used in subsequent sequence studies.

Figure 3 shows the results of the sequence studies. In total, 69 residues were identified, including the N-terminal 13 residues of the protein and the 16, 19, and 21 residues of three selected Asn-N peptides, Asn-N #1, #2, and #3 respectively. A homology search in the Protein Data Bank revealed that the amino acid sequences thus obtained are most homologous with those of papain family peptidases (MEROPS subfamily CA1). Almost no sequence similarity was detected with the other classes of peptidases. The N-terminal 13 residue-sequence was 54%, and the sequences of Asn-N #1, #2, and #3 were 31, 26, and 48% respectively identical to the corresponding sequences in papain. Thus the overall identity was calculated to be 48% with the total 213 residues of papain. Although the sequences involving the active site Cys and His common to the papain family enzymes have not yet been identified, the results show that the 45-kDa protein is a cysteine endopeptidase, presumably belonging to the papain family. SDS–PAGE analysis suggested an apparently higher molecular mass for the present enzyme relative to papain (23.4 kDa). Previously, Scala et al. reported the mo-
lecular mass of the peptidase in the digestive fluid of *D. muscipula* to be approximately 40 kDa, as determined by Sephadex G-150 gel filtration. The molecular size may have been overestimated by some reason, for example, due to the presence of carbohydrate, or the enzyme might have a C-terminal extension like a potato papain-type cysteine peptidase (35.4 kDa) (accession no. CAB53515).

Scala *et al.*° and Robins and Juniper°° have reported that the major endopeptidase in the digestive fluid of *D. muscipula* might be a papain-like enzyme. However, this speculation was based mainly on the lack of activity toward pepsin, trypsin, and chymotrypsin substrates°°,°° and partial inhibition by iodoacetamide (about 22%).°° Therefore, the results obtained in the present study are thought to be the first definitive evidence indicating that a cysteine endopeptidase is present in the digestive fluid of *D. muscipula*. Considering its origin (*Dionaea*) and homology with papain, we propose the name “dionain” for this enzyme.

Previously we assumed that the digestive fluid of *D. muscipula* contains two endopeptidases, insensitive or sensitive to pepstatin A, based on partial inhibition with pepstatin A of the acid peptidase activity using hemoglobin as substrate.°° In the present study, dionain, a pepstatin-insensitive enzyme, was shown to be present in the digestive fluid. On the other hand, previous studies showed that the digestive fluid has endopeptidase activity with a pH optimum at 5.5 with congocoll°° and 3.0 with hemoglobin ° as substrate.°°° This activity is thought to be due partially to the pepstatin-sensitive enzyme, presumably a nepenthesin-type enzyme. Dionain is thought to work cooperatively with the pepstatin-sensitive enzyme for digestion of prey proteins at acidic to weakly acidic pH. It is interesting to note that *Dionaea* uses a different class of enzyme for prey digestion than *Nepenthes*, although they are phylogenetically close, belonging to the same order in the plant taxonomy. We are currently attempting to elucidate the molecular and enzymatic characteristics of dionain in more detail, including purification and determination of the complete amino acid sequence, and simultaneously to characterize the co-existing pepstatin-sensitive endopeptidase.

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**References**