The digestive fluid of *Drosera indica* contains a cysteine endopeptidase ("droserain") similar to dionain from *Dionaea muscipula*

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Introduction

Carnivorous plants are known to secrete various endopeptidases extracellularly to digest prey proteins. Previously we purified two isoforms of nepenthesin to homogeneity and elucidated their enzymatic and structural characteristics (Athauda *et al*. 1998, 2002, 2004; Takahashi *et al*. 2003, 2005). In a continuation of these studies, we have been attempting to characterize these and other endopeptidases secreted by carnivorous plants to digest prey proteins (Takahashi *et al*. 2009). Recently, we found and partially characterized a cysteine endopeptidase in the digestive fluid of *Dionaea muscipula* and named it "dionain" (Takahashi *et al*. 2011). In the present report, we describe the occurrence of a similar cysteine endopeptidase in the digestive fluid of *Drosera indica* and propose the name "droserain" to this enzyme. In this connection, we also propose the names of aspartic endopeptidases from the digestive fluids of relevant carnivorous plants.

Materials and Methods

The crude digestive fluid of wild specimens of *Drosera indica* was obtained in the Watarase retarding basin area, Tochigi. The digestive fluid was collected by soaking thirty leaves successively (1 leaf for 1 min at a time) in 10 ml of distilled water in a test tube to wash out the digestive fluid through up-and-down strokes. The diluted digestive fluid thus obtained was stored frozen until use. Benzyloxycarbonyl-Phe-Arg 4-methyl-7-coumarylamide (Z-Phe-Arg-MCA), a cysteine endopeptidase substrate, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) and pepstatin A were obtained from Peptide Institute, Osaka. Other reagents used were of analytical grade.

To measure the activity toward Z-Phe-Arg-MCA, the reaction mixture contained 20 µL of the diluted fluid, 5 µl of 2 mM Z-Phe-Arg-MCA in dimethyl sulfoxide, 75 µl of 100 mM buffer at various pH values, and ±10 mM dithiothreitol (DTT). The mixture was incubated at 37°C and the increase in fluorescence at 460 nm with excitation at 370 nm was measured at 5-min intervals for 60 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 DTT and 1 µl of 1 mM E-64) was

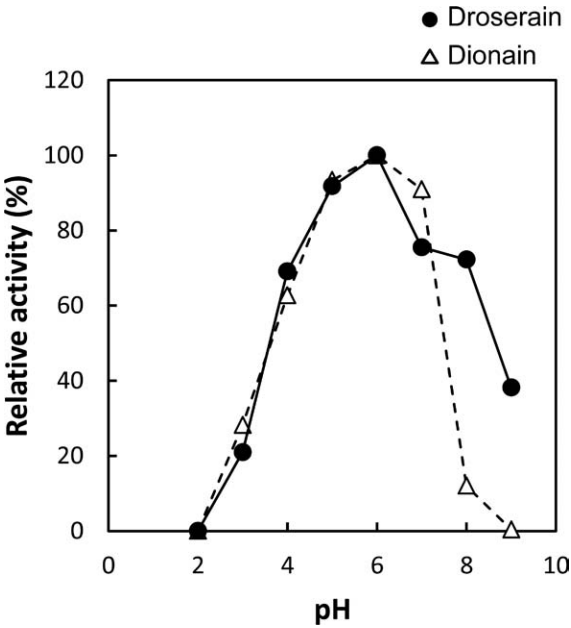

Figure 1: pH dependence of the activities toward Z-Phe-Arg-MCA of the digestive fluids of *Drosera indica* (droserain) and *Dionaea muscipula* (dionain) in the presence of 10 mM DTT. The activity at pH 6.0 was taken as 100%. The buffers (100 mM) used were KCl-HCl, pH 2.0, sodium citrate, pH 3.0-6.0, potassium phosphate, pH 6.0-8.0, and Tris-HCL, pH 8.0 and 9.0.

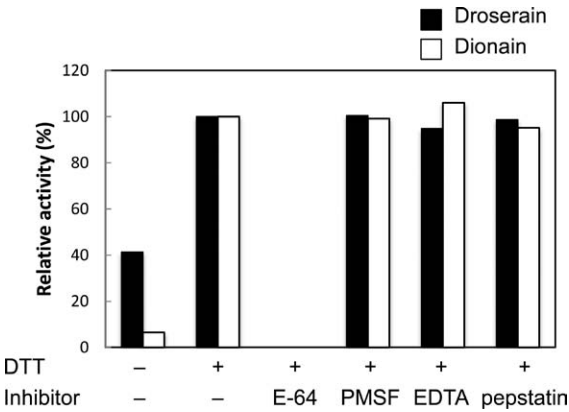

Figure 2: Effects of inhibitors on the activities toward Z-Phe-Arg-MCA of the digestive fluids of *Drosera indica* (droserain) and *Dionaea muscipula* (dionain) in the presence of 10 mM DTT at pH 6.0. The final concentrations of the inhibitors were E-64: 10 μM, PMSF: 1mM, EDTA: 5 mM, and pepstatin A: 1 μM.

added, and the mixture was preincubated at 37°C for 5 min before addition of the substrate. For comparison, the activity of dionain in the digestive fluid of *Dionaea muscipula* was also measured under the same conditions using 2 μl of the crude digestive fluid in a total volume of 100 μl made up with distilled water.

Results and Discussion

Figure 1 shows the pH dependence of endopeptidase activity toward Z-Phe-Arg-MCA in the presence of 10 mM DTT. The maximal activity was observed at pH 6.0, and about 20% and 40% of the maximal activity was observed even at pH 3.0 and 9.0, respectively. This indicates that the enzyme is capable of acting in a wide range of pH extending from acidic to weakly alkaline region. In the absence of DTT, about 40% of the activity in the presence of 10 mM DTT was observed (Fig. 2). A similar pH dependence of activity was observed with dionain in the presence of 10 mM DTT except that the present enzyme showed notable activity at pH 8-9 unlike dionain. The shoulder of activity at pH 8-9 may indicate the presence of the second cysteine endopeptidase.

Furthermore, the enzyme was completely inhibited by 10 μM E-64 (a cysteine peptidase inhibitor) whereas it was not inhibited by phenylmethanesulfonyl fluoride (a serine peptidase inhibitor), EDTA (a metallopeptidase inhibitor), and pepstatin A (aspartic peptidase inhibitor) as shown in Figure 2. The inhibitory profiles of these inhibitors were essentially the same with dionain (Fig. 2). These results show the occurrence of a cysteine endo-

peptidase similar to dionain in the digestive fluid of *Drosera indica*. We propose the name "droserain" for this enzyme. In this connection, we also propose the names of aspartic endopeptidases from the digestive fluids of *Dionaea muscipula* (Venus Flytrap), *Drosera* (sundew) sp., and *Cephalotus follicularis* as "dionaeasin", "droserasin", and "cephalotusin", respectively, as previously suggested (Takahashi 2003).

In the present study, 20 µl of the diluted digestive fluid was used for the assay of droserain, whereas 2 µl of the crude digestive fluid was used for the assay of dionain, and the activity observed for the latter was 4.2 times higher than that for the former. Therefore, one leaf of *D. indica* was calculated to contain approximately 8 µl equivalent of the digestive fluid of *D. muscipula*. This value appears to be fairly reasonable. The present enzyme is thought to work in concert with the aspartic endopeptidase "droserasin" in the digestive fluid of *Drosera* sp. like dionain and dionaeasin in *Dionaea muscipula*. This resemblance is reasonable since *Dionaea* and *Drosera* sp. are closely located in the phylogenetic tree. It remains to be clarified what kind of reducing agent is present in the digestive fluids of these carnivorous plants to activate the cysteine endopeptidases.

References

Athauda, S.B.P., Inoue, H., Iwamatsu, A., and Takahashi, K. 1998. Acid proteinase from *Nepenthes distillatoria* (Badura). Adv. Exp. Med. Biol. 436: 453-458.

Athauda, S.B.P., Inoue, H., Iwamatsu, A., and Takahashi, K. 2002. Purification and enzymatic characterization of an aspartic proteinase (Nepenthesin) from the insectivorous plant *Nepenthes distillatoria*. Proc. 4th Interntl. Carnivor. Plant Conf. Tokyo, 117-124.

Athauda, S.B.P., Matsumoto, K., Rajapakshe, S., Kuribayashi, M., Kojima, M., Kubomura-Yoshida, N., Iwamatsu, A., Shibata, C., Inoue, H., and Takahashi, K. 2004. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. Biochem. J. 380: 295-306.

Takahashi, K., Athauda, S.B.P., Matsumoto, K., Rajapakshe, S., Kuribayashi, M., Kaga, T., Muramatsu, M., Niwa, H., Shimaya, K., Kubota, K., Nishii, W., Kojima, M., Inoue, H., Kubomura, N., Iwamatsu, A., and Shibata, C. 2003. Unique characteristics of carnivorous plant aspartic proteinases with special reference to nepenthesin. Program and Abstracts, Interntl. Conf. Asp. Proteases and Inhibitors, Kyoto, 20.

Takahashi, K., Athauda, S.B.P., Matsumoto, K., Rajapakshe, S., Kuribayashi, M., Kojima, M., Kubomura-Yoshida, N., Iwamatsu, A., Shibata, C., and Inoue, H. 2005. Nepenthesin, a unique member of a novel subfamily of aspartic proteinases: enzymatic and structural characteristics. Curr. Protein Pept. Sci. 6: 513-525.

Takahashi, K., Matsumoto, K., Nishii, W., Murakami, M., Kubota, K., Shibata, C., and Athauda, S.B.P. 2009. Comparative studies on the acid proteinase activities in the digestive fluids of *Nepenthes*, *Cephalotus*, *Dionaea*, and *Drosera*. Carniv. Pl. Newslett. 38: 75-82.

Takahashi, K., Suzuki, T., Nishii, W., Kubota, K., Shibata, C., Isobe, T., and Dohmae, N. 2011. A cysteine endopeptidase ("dionain") is involved in the digestive fluid of *Dionaea muscipula* (Venus's fly-trap). Biosci. Biotechnol. Biochem. 75: 346-348.

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