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Note

Isolation and Characterization of a Cysteine Protease of Freesia Corms

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A protease, freesia protease (FP)-A, was purified to electrophoretic homogeneity from regular freesia (*Freesia reflacta*) corms in harvest time. The M_r of FP-A was estimated to be 24 k by SDS-PAGE. The optimum pH of the enzyme was 8.0 using a casein substrate. These enzymes were strongly inhibited by *p*-chloromercuribenzoic acid but not by phenylmethane-sulfonylfluoride and EDTA. These results indicate that FP-A belongs to the cysteine proteases. The amino terminal sequence of FP-A was similar to that of papain, and the sequences was regarded to the conservative residues of cysteine protease. From the hydrolysis of peptidyl-*p*-NAs, the specificity of FP-A was found to be broad. It was thought that FP-A was a new protease from freesia corms.

Key words: plant corm; cysteine protease; plant endopeptidase; freesia; *Freesia reflacta*

During germination and the early growth stage of plants, storage proteins are degraded by proteolysis. Cysteine proteases of higher plants appear to have many proteolytic functions in intracellular and extracellular processes such as degradation of storage proteins in germinating seeds.[1–6] Aleurain from the aleurone layer of barley (*Hordeum vulgare*) is a well-characterized seed cysteine protease.[7,8] In the investigation of plant endopeptidase, many enzymes have been found in latex, fruits, and seeds,[9] however, the endopeptidases from the underground parts of the plants have been poorly understood. Some enzymes were reported as follows: an endopeptidase from maize roots,[10] a protease from *Arabidopsis* roots,[11,12] proteases of sprouting potato (*Solanum tuberosum*) tubers,[13,14] and a cysteine protease of ginger (*Zingiber officinale* Roscoe) rhizomes.[15] We have been interested in measuring the protease activity of plant parts in order to understand the function of proteases toward reserved proteins in corms. In our previous paper, two cysteine proteases (termed FP-A and FP-B) had been found from freesia corms, and then the latter had been isolated.[16] The M_r of FP-B was estimated to be 26 k by SDS-PAGE. The optimum pH of FP-B was 6.0–7.0 at 30°C using a casein substrate. It was thought that the enzyme was a typical papain-like cysteine protease.

In this paper, we have isolated FP-A from freesia corms in harvest time and described the properties and substrate specificity of this protease.

All procedures for purification of the enzyme were done at 7°C. Freesia corms, *Freesia reflacta* ssp. 'White Mary' (200 g, Takii Co., Kyoto, Japan), were homogenized with a domestic mixer in 400 ml of 20 mM Na, K-Pi buffer at pH 7.0. The homogenate was filtered through a cotton cloth and was centrifuged (4,000 × *g*, for 30 min). Solid ammonium sulfate was added to the supernatant to 50% saturation and kept for 24 h. After centrifugation (4,000 × *g*, for 30 min), the pellet was dissolved in 10 mM Na, K-Pi buffer at pH 7.0. The solution was put directly on a column (5.0 × 50 cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with buffer A, and the protease was eluted with 0.2 M Na, K-Pi buffer, pH 7.0. Solid ammonium sulfate was added to the eluate from the column to 50% saturation. After 24 h, the mixture was centrifuged (10,000 × *g*, for 15 min), the pellet was suspended in 10 mM Na, K-Pi buffer, pH 7.0, and then the suspension was dialyzed against the same buffer overnight. The dialysate was centrifuged (10,000 × *g*, for 15 min), and the supernatant was put on a DEAE-Sepharose column (1.8 × 26 cm) equilibrated with 10 mM Na, K-Pi buffer, pH 7.0, and then the proteins adsorbed to the column were eluted with a linear gradient from 10 mM Na, K-Pi buffer, pH 7.0 (1 liter) to 0.2 M Na, K-Pi buffer, pH 7.0 (1 liter).

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Abbreviations: DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); freesia protease, FP; MIA, monoiodoacetic acid; E-64, (L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl-agmatine; PCMB, *p*-chloromercuribenzoic acid; PCMPS, *p*-chloromercuriphenylsulfonic acid; Pi, phosphate; PMSF, phenylmethanesulfonylfluoride; *p*NA, *p*-nitroanilide; STI, soybean trypsin inhibitor; Tos-Lys-CH_2Cl, *N*-Tosyl-L-lysine chloromethylketone; Tos-Phe-CH_2Cl, *N*-Tosyl-L-phenylalanine chloromethyl ketone

Protease (FP-A)-containing fractions were collected, and solid ammonium sulfate was added to the eluate from the column to 50% saturation. The ammonium sulfate precipitate of the eluate from the DEAE-Sepharose column was centrifuged ($10,000 \times g$, for 15 min), then the pellet was dissolved in distilled water and the solution being put on a Bio-gel P-60 gel filtration column (2.4×95 cm) equilibrated with 10 mM Na, K-Pi buffer, pH 7.0. The eluate from the gel filtration was diluted with distilled water, and was put on a Q-Sepharose column (2.0×6.0 cm) equilibrated with the above buffer. The proteins adsorbed to the column were eluted with a linear gradient from 10 mM Na, K-Pi buffer, pH 7.0 (500 ml) to 0.08 M Na, K-Pi buffer, pH 7.0 (500 ml). The pooled active fraction was dialyzed against 10 mM Na, K-Pi buffer, pH 7.0, overnight.

An enzyme, FP-A, was isolated from the freesia corms in harvest time. The peak size of the caseinolytic activity of FP-B was smaller than that of the previous study.[16] The freesia corms contained a relatively large amount of protein. The greater part of an undesired protein was efficiently removed at the initial treatment of a DEAE-Sepharose column (Fig. 1). The yields of each purification step are summarized in Table 1. FP-A was finally purified from the Q-Sepharose column chromatography with 6% recovery. From 200 g of the freesia corms, 13 mg of the purified enzyme was obtained.

The purified FP-A showed as a single band on SDS-PAGE by the method of Laemmli,[17] having a M_r of 24 k as shown in Fig. 1 (box).

Isoelectric focusing of FP-A was done with an acrylamide slab gel containing Ampholine (pH 3.5–10.0) by the procedure of Westermeier[18] using 7% gel. The pI of FP-A (6.9) was estimated by isoelectric focusing on an acrylamide slab gel. The data was consistent with the elution point of FP-A, fraction number 50 to 60 on a DEAE-Sepharose chromatography (Fig. 1).

FP-A from corms was characterized by measuring these protease activities with casein as a substrate at various pHs (Fig. 2(A)). Proteolytic activity was measured with casein as a substrate by the method described previously.[16] The optimum pH of FP-A was observed to be about 8. This profile was similar to that of melain, a cysteine protease from bead tree fruit under the same conditions.[19] The pH stability of FP-A was examined by incubating at various pHs at 37°C for 10 min, before an assay at pH 7.0. At least 80% of the activities of FP-A remained after incubation between pH 7–10.5, (Fig. 2(B)).

The effects of temperature on the proteolytic activities of FP-A are shown in Fig. 2(C). FP-A had an optimal activity in the range of 50°C. The thermal stability of the enzyme was examined by incubating it at various temperatures for 30 min, before an assay at pH 7.0. At least 80% of the proteolytic activities of FP-A remained after incubation at 45°C, as shown in Fig. 2(D).

The effects of various compounds on the enzymatic activity are represented in Table 2. The enzyme (0.5 ml) was added to 0.5 ml of the inhibitor solution in 67 mM K-Pi buffer at pH 7.2 and incubated at 35°C for 60 min. The remaining activity of protease was assayed using casein as a substrate. The activity of FP-A was completely inactivated by 0.1 mM PCMPS. The protease activity of FP-A was strongly inactivated by 1.0 mM DTNB, 0.01 mM E-64, and 1.0 mM PCMB. The effects of antipain (1 mM) and

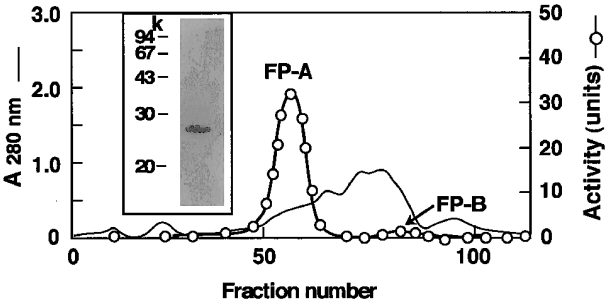

Fig. 1. Elution Profile of Protease Activity of Freesia Corms on DEAE-Sepharose Ion-exchange Chromatography and SDS-PAGE of Purified FP-A from a Q-Sepharose Column.

The flow rate of the column was 0.9 ml/min. Each fraction was 15 ml. Solid bars, absorbance at 280 nm; (○), caseinolytic activity. Box: SDS-PAGE of purified FP-A. The samples were electrophoresed in a 15% polyacrylamide gel. The gel was stained in Coomassie Brilliant Blue R-250 for 15 min and then destained.

Table 1. Purification of Freesia Protease A from Corms

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
Extract	16,000	100,000	6.4	100	1
Ammonium sulfate precipitation	12,000	210,000	18	210	3
DEAE-cellulose	1,800	94,000	53	92	8
DEAE-Sepharose	380	9,800	26	10	4
Bio-gel P-60	42	8,100	190	8	30
Q-Sepharose	13	6,000	480	6	75

One unit of activity was defined as the activity giving 0.001 A_{280} units of change per min under these conditions.

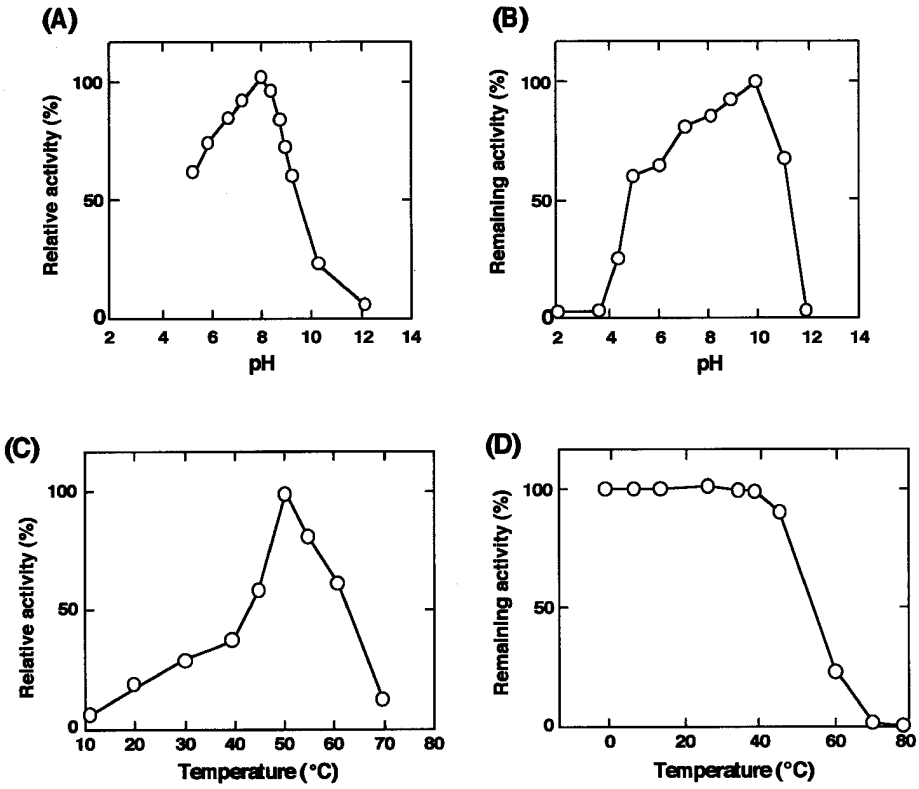

Fig. 2. The Effects of pH and Temperature on the Proteolytic Activity and the Stability.

(A) The effects of pH on the caseinolytic activity. The incubation mixture consisted of 1.0 ml of an enzyme solution containing 10 mM cysteine (60 mg/ml) and 1.0 ml of a 2% (w/v) casein in various pH buffers. The assay solution was incubated at 37°C for 60 min. Buffers: 0.1 M citric acid-HCl (pH 2.0), 67 mM K, Na-Pi (pH 5.0–8.0), 0.1 M glycine-NaOH buffer (pH 8.0–12.3). The activity assay could not be done at pH 2–5, because casein was insoluble in that range of pH. (B) The pH stability of the enzyme was assayed with the incubation in various pHs for 30 min. Buffers: 0.1 M citric acid-HCl (pH 2.0), 0.1 M Na-acetate buffer (pH 3–6), 33 mM Na, K-Pi (pH 5.0–8.0), 50 mM glycine-NaOH buffer (pH 8.0–12.9). (C) The effects of temperature on the caseinolytic activity. The incubation mixture consisted in 1.0 ml of an enzyme solution containing 10 mM cysteine and 1.0 ml of a 2% (w/v) casein in 67 mM Na, K-Pi buffer, pH 7.0. The assay solution was incubated at various temperatures for 30 min. (D) Thermal stability of the enzyme. The protease solution was incubated at various temperatures for 30 min and the residual activities were assayed.

chymostatin (1 mM) were weak. PMSF and EDTA had no effect on the enzymatic activity. The inactivation of Tos-L-Lys-CH_2Cl towards FP-A was more effective than those of Tos-L-Phe-CH_2Cl. The activity of FP-B was strongly inhibited by Tos-L-Lys-CH_2Cl. All protease activities were weakly inactivated by 2.0 mM $ZnCl_2$. The enzyme activity were gradually decreased by an addition of DTNB. In the course of inhibition, an addition of 2-mercaptoethanol to this reaction mixture reversed this effect and restored the initial activity (data not shown). Therefore, these results indicated that FP-A belong to the cysteine proteases. It was found that the effect of compounds using in Table 2 against the activity of FP-A was similar to those of FP-B.

The substrate specificity of FP-A was investigated with synthetic substrates of Ala-Ala-Pro-X-*p*NAs (X = Val, Leu, Lys, Ala, Phe, Gly, and Glu). The rate of enzymatic hydrolysis for peptidyl-*p*NA substrates was measured by the method described previously.[19] As shown in Table 3, all substrates were hydrolyzed by the protease. The preferential cleavage sites for FP-A were large hydrophobic residues at the P_1 position, like Val or Leu. The substrates having Gly or Glu at the P_1 position were barely cleaved by FP-A. From the digestion of seven peptidyl substrates, the specificity of FP-A was found to be approximately broad. The specificity of FP-A toward peptidyl-*p*NA may be broader than for FP-B.

The N-terminal sequence of the FP-A was identified. The sequence of FP-A is aligned with those of other cysteine proteases from plant tissues for maximum similarity (Fig. 3). Some consensus sequences were found in the sequences of these proteases. The N-terminal sequence of FP-B was not identical with that of FP-A. FP-A and FP-B are different proteins, judging from the N-terminal sequences. It can therefore be presumed that these proteases arise independently from translation of diffeent mRNAs.

From this study, it was thought that the enzyme properties of FP-A were similar to those of FP-B, but it was perhaps an other enzyme than FP-B, and no mentioned similarity was detected between FP-A and the known cysteine proteases from underground

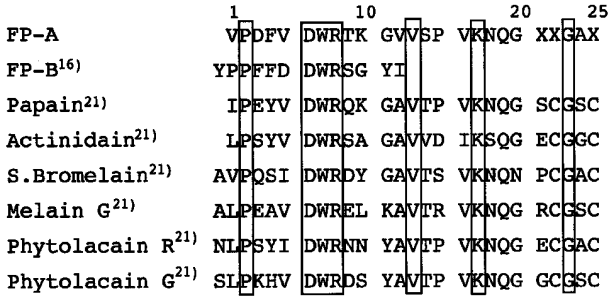

Fig. 3. The Comparison of the N-Terminal Amino Acid Sequence of FP-A and Other Plant Cysteine Proteases.

These sequences were aligned for maximum similarily. Numbering is according to that of papain. Abbreviations of amino acids follow the alphabetical system.

Table 2. Effects of Various Compounds on the Activity of Freesia Proteases

Compounds	Concentration (mM)	Relative activity (%)	
		Protease A	Protease B
None	—	100	100
MIA	2.0	22	56
PCMB	1.0	16	13
PCMPS	0.1	0	—[b]
Antipain	0.001	78	—[b]
E-64	0.01	25	10
DTNB	1.0	0	15
DFP	2.0	70	100
PMSF	2.0	123	93
Pefabloc SC[a]	1.0	82	—[b]
Tos-Lys-CH_2Cl	2.0	12	4
Tos-Phe-CH_2Cl	2.0	30	71
Chymostatin	0.001	89	—[b]
Ovomucoid	0.01	116	—[b]
STI	0.01	104	—[b]
EDTA	2.0	143	100
$MgCl_2$	2.0	97	99
$CaCl_2$	2.0	92	95
$MnCl_2$	2.0	91	89
$CoCl_2$	2.0	81	94
$ZnCl_2$	2.0	72	49

[a] 4-(2-Aminoethyl)-benzenesulfonyl fluoride.
[b] No data were presented.

parts such as potato[13] and ginger.[15] On the other hand, the M_r and optimum pH of FP-A activity were similar to those of asclepain A3 from the latex of *Asclepias syriaca* L.[20]

In our previous paper, FP-B was isolated from freesia corms.[16] This is because in the previous study, the corms were stored at 4–7°C for several months, and then the corms were used for the purification of FP-B. In this study, the corms using for purification of proteases were obtained at harvest time. The amount of FP-B eluted from the DEAE-Sepharose column was observed to increase as a preservation period of freesia corms at 7°C (data not shown). We thought that the chilling tolerance in the corms was regulated, and some proteases (specially FP-B) appeared in response to chilling impossible for germination of the corms.

We plan to study, for future characterization, the roles of proteases in corm dormancy and in normal growth.

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References

1) Akasofu, H., Yamauchi, D., Mitsuhashi, W., and Minamikawa, T., Nucleotide sequence of cDNA for sulfhydryl-endopeptidase (SH-EP) from cotyledons of germinating *Vigna mungo* seeds. *Nucleic Acids Res.*, **17**, 6733 (1989).
2) Kembhavi, A. A., Buttle, D. J., Knight, C. G., and Barrett, A. J., The two cysteine endopeptidases of legume seeds: purification and characterization by use of specific fluorometric assays. *Arch Biochem. Biophys.*, **303**, 208–213 (1994).
3) Takeda, O., Miura, Y., Mitta, M., Matsushita, H., Kato, I., Abe, Y., Yokosawa, H., and Ishii, S., Isolation and analysis of cDNA encoding a precursor of *Canavalia ensiformis* asparaginyl endopeptidase (legumain). *J. Biochem.*, **116**, 541–546 (1994).
4) Okamoto, T. and Minamikawa, T., Purification of a

Table 3. Hydrolysis of Peptidyl-*p*-nitroanilides by Freesia Proteases

Substrates	Hydrolysis rate with protease A (nmol/min/ml)	Relative activity (%)	
		Protease A	Protease B
Ala-Ala-Pro-Val-*p*NA	0.25	100[a]	2
Ala-Ala-Pro-Leu-*p*NA	0.14	56	9
Ala-Ala-Pro-Lys-*p*NA	0.11	44	100[b]
Ala-Ala-Pro-Ala-*p*NA	0.10	40	7
Ala-Ala-Pro-Phe-*p*NA	0.092	37	4
Ala-Ala-Pro-Gly-*p*NA	0.053	21	0
Ala-Ala-Pro-Glu-*p*NA	0.015	6	0

[a] Activity with Ala-Ala-Pro-Val-*p*NA was taken as 100%.
[b] Activity with Ala-Ala-Pro-Lys-*p*NA was taken as 100%.

processing enzyme (VmPE-1) that is involved in post-translational processing of a plant cysteine endopeptidase (SH-EP). *Eur. J. Biochem.*, **231**, 300–305 (1995).
5) Cercos, M., Santamaria, S., and Carbonell, J., Cloning and characterization of TPE4A, a thiol-protease gene induced during ovary senescence and seed germination in pea. *Plant Physiol.*, **119**, 1341–1348 (1999).
6) Ho, S. L., Tong, W. F., and Yu, S. M., Multiple mode regulation of a cysteine proteinase gene expression in rice. *Plant Physiol.*, **122**, 57–66 (2000).
7) Rogers, J. C., Dean, D., and Heck, G. R., Aleurain: a barley thiol protease closely related to mammalian cathepsin H. *Proc. Natl. Acad. Sci. USA*, **82**, 6512–6516 (1985).
8) Li, X. Y., Rogers, S. W., and Rogers, J. C., A copy of exon 3-intron 3 from the barley aleurain gene is present on chromosome 2. *Plant Mol. Biol.*, **17**, 509–512 (1991).
9) Boller, T., Roles of proteolytic enzymes in interactions of plants with other organisms. In: ''Plant Proteolytic Enzymes'', ed. Dalling, M. J., vol. 1, CRC Press, Boca Raton, FL, pp. 67–96 (1986).
10) Batt, R. and Wallace, W., Characteristics of the active site and substrate specificity of a maize root endopeptidase. *Biochem. Biophys. Acta*, **990**, 109–112 (1989).
11) Reddy, A. S., Safadi, F., Beyette, J. R., and Mykles, D. L., Calcium-dependent proteinase activity in root cultures of *Arabidopsis*. *Biochem. Biophys. Res. Comm.*, **199**, 1089–1095 (1994).
12) Safadi, F., Mykles, D. L., and Reddy, A. S. N., Partial purification and characterization of a Ca^{2+}-dependent proteinase from *Arabidopsis* roots. *Arch. Biochem. Biophys.*, **348**, 143–151 (1997).
13) Kitamura, N. and Maruyama, Y., Purification and properties of cysteine protease in sprouting potato tubers. *Agric Biol. Chem.*, **50**, 381–390 (1986).
14) Kitamura, N., Okitani, A., and Maruyama, Y., Substrate specificity of cycteine proteinase from sprouting potato tubers. *Agric Biol. Chem.*, **53**, 1159–1160 (1989).
15) Ohtsuki, K., Taguchi, K., Sato, K., and Kawabata, M., Purification of ginger proteases by DEAE-Sepharose and isoelectric focusing. *Biochim. Biophys. Acta*, **1243**, 181–184 (1995).
16) Kaneda, M., Yonezawa, H., and Uchikoba, T., Purification and characterization of a cysteine protease from corms of freesia, *Freesia reflacta*. *Biosci. Biotechnol. Biochem.*, **61**, 1554–1559 (1997).
17) Laemmli, U. K., Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* (London), **227**, 680–685 (1970).
18) Westermeier, R., Isoelectric forcusing. In ''Electrophoresis in Practice'', Ed 2, VCH, Weinheim, pp. 45–56 (1997).
19) Arima, K., Uchikoba, T., Yonezawa, H., Shimada, M., and Kaneda, M., Cucumisin-like protease from the latex of *Euphorbia supina*. *Phytochemistry*, **53**, 639–644 (2000).
20) Brockbank, W. J. and Lynn, K. R., Purification and preliminary characterization of two asclepains from the latex of *Asclepias syriaca* L. (milkweed). *Biochim. Biophys. Acta*, **578**, 13–22 (1979).
21) Uchikoba, T., Arima, K., Yonezawa, H., Shimada, M., and Kaneda, M., Amino acid sequence and some properties of phytolacain G, a cysteine protease from growing fruit of pokeweed, *Phytolacca americana*. *Biochim. Biophys. Acta*, **1523**, 254–260 (2000).