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Cucumisin-like protease from the latex of *Euphorbia supina*

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Abstract

A protease has been purified from the latex of *Euphorbia supina* Rafin by two steps of chromatography. The M_r was estimated by SDS-PAGE to be 80 kDa. Its activity was inhibited strongly by diisopropyl fluorophosphate, but not by EDTA, pepstatin, or cysteine protease inhibitors, indicating that the enzyme is a serine protease. The specificity of the protease is broad, but the preferential cleavage sites were C-terminal sites of hydrophobic amino acid residues. The N-terminal sequence of the first fifteen residues was determined and six of the residues match those in cucumisin [EC 3.4.21.25], a protease from the sarcocarp of melon fruit (*Cucumis melo* L. *var*. Prince). The results indicate that the *E. supina* protease is a cucumisin-like serine protease.

Keywords: *Euphorbia supina*; Euphorbiaceae; Cucumisin; Serine protease; Plant protease

1. Introduction

In the course of screening plant tissues for protease activity, we found that milk casein was hydrolyzed by the latex from leaves and stems of several plants. Isolation of proteases from these tissues was interest because the proteolytic activities are not inhibited by various reagents which show pronounced reactivity toward sulfhydryl groups. Serine proteases have been characterized in a number of plants (Vierstra, 1996), although they are not as well studied as are cysteine proteases (Boller, 1986).

Some plants immediately secrete latex when the leaves and stems are injured. Latex itself may act to shield the cambial meristem and the contents of the sieve tubes from predators, or to ward off parasites or pathogens. Therefore, it seems reasonable to assume that the substances and enzymes needed for such purposes are present in latex.

Endopeptidases are presented in the latex of several plants; such enzymes have been found in the latex plants from Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae, and Euphorbiaceae (Boller, 1986). The function of these proteases, which are from different genetic resources, have not been elucidated. One possible function is degradation of proteins during laticifer development or promotion of coagulation.

Most of the proteases isolated from latex are cysteine proteases, which resemble papaya proteases (Rowlings & Barrett, 1994) such as papain [EC 3.4.22.2], chymopapain [EC 3.4.22.6], and caricain [EC 3.4.22.30]. The latex of *Ficus elastica* (Moraceae) and of some of the Euphorbiaceae, however, contain serine endopeptidases (Lynn & Clevatte-Radford, 1986a, 1986b).

Cucumisin [EC 3.4.21.25], a serine protease from *Cucumis melo* var. *Prince*, has been well-characterized (Kaneda & Tominaga, 1975; Uchikoba, Yonezawa &

Abbreviations: DFP, diisopropyl fluorophosphate; Glt, glutaryl; MIA, monoiodoacetic acid; PCMPS, *p*-chloromercuriphenylsulfonic acid; *p*NA, *p*-nitroanilide; PMSF, phenylmethanesulfonylfluoride; TCA, trichloroacetic acid; Tos-Lys-CH_2Cl, *N*-Tosyl-L-lysine chloromethylketone; STI, soybean trypsin inhibitor.

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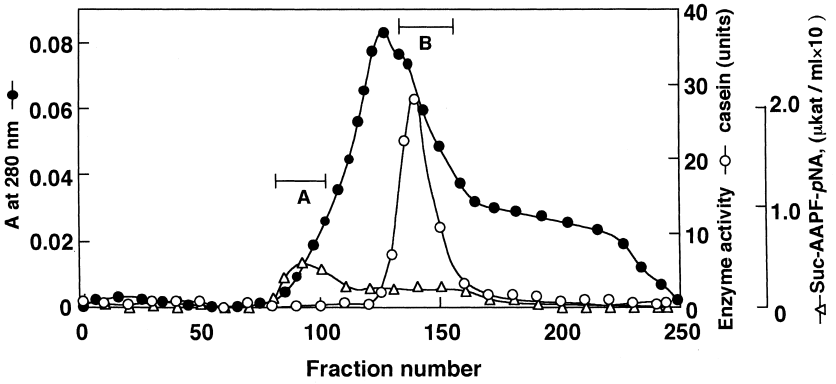

Fig. 1. Elution profiles of protein as monitered by absorbance at 280 nm (●), caseinolytic activity (○), hydrolytic activity of Suc-Ala-Ala-Pro-Phe-*p*NA (Δ) from DEAE-Sepharose column chromatography. The flow rate of the column was 72 ml/h and 9.5 ml fractions were collected. The caseinolytic activity of 0.2 ml aliquots of each fraction was measured at 30°C for 60 min.

Kaneda, 1995). The amino acid sequences around the reactive serine and histidine of cucumisin have been identified (Kaneda, Omine, Yonezawa & Tominaga, 1984; Yonezawa, Uchikoba & Kaneda, 1995). In addition, the primary structure of cucumisin has been predicted from the cDNA (Yamagata, Matsuzawa, Nagaoka, Onishi & Iwasaki, 1994). Its structure was homologous to those of proprotein-processing proteases such as Kex2 from yeast (Mizuno, Nakamura, Oshima, Tanaka & Matsuo, 1988) and human furin (Van den Ouweland, Van Duijinhoven, Keizer, Dorssers & Van de Ven, 1990).

In this study, we describe the isolation, general properties, and substrate specificity of a new serine protease from the latex of *Euphorbia supina*, and compare its enzymatic characteristics and structural properties to those of other cucumisin-like proteases.

2. Results

Little caseinolytic activity was detected in the homogenate from the stems and leaves of *E. supina*. However, the latex of *E. supina* had strong caseinolytic activity. Therefore, the stems and leaves were cut to a small size (3–4 mm), and the latex secreted from the terminal positions was extracted with 17 mM K, Na–Pi buffer, pH 7.0. The extract soln. from the latex was maintained at −20°C until purification of protease.

The elution profile from DEAE-Sepharose column chromatography showed a main activity peak, termed *E. supina* protease B, and a minor activity peak, termed *E. supina* protease A (see Fig. 1). The fractions containing *E. supina* protease B were collected and the enzyme was further purified by gel filtration on Sephacryl S-300. *E. supina* protease B was purified 270-fold by a three-step procedure (see Table 1), with 28% recovery. Approximately 1.6 mg of the purified enzyme was obtained from 3 kg of *E. supina* stems and leaves.

As shown in Fig. 2, SDS-PAGE of the Sephacryl S-300 purified *E. supina* protease B revealed a single protein band. The M_r was estimated to be 80 kDa, which is greater than that of cucumisin [EC 3.4.21.25] (67 kDa) isolated from prince melon (*Cucumis melo* L. *var.* Prince) (Uchikoba et al., 1995).

The caseinolytic activity of *E. supina* protease B was measured under various pH and temperature conditions. The optimum pH for enzyme activity was ca. 8. At pH 7.0, proteolytic activity remained at 80% of the initial value after incubation for 20 min at 60°C.

The effects of various compounds on the enzymatic activity for casein are shown in Table 2. Almost all enzyme activity was lost on incubation with 2.0 mM diisopropylfluorophosphate (DFP) and phenylmethane-sulfonylfluoride (PMSF) for 60 min at 30°C. Low sensitivity of *E. supina* protease B to inhibition by DFP was similar to results for cucumisin (Uchikoba et al., 1995) and a cucumisin-like protease from *Benincasa hispida* (Uchikoba, Yonezawa & Kaneda, 1998). Leupeptin and soybean trypsin inhibitor (STI)

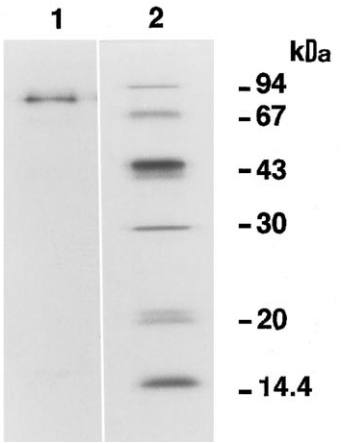

Fig. 2. SDS-PAGE of purified *E. supina* protease B. Lane 1, 10 μg of pooled fractions from from a gel filtration on Sephacryl S-300. Lane 2, M_r standards.

Table 1
Purification of *E. supina* protease B

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Crude extract	1600	3600	2.3	100	1
DEAE-Sepharose	18	2400	130	67	57
Sephacryl S-300	1.6	1000	625	28	270

were only weak inhibitors. *N*-Tosyl-L-lysine chloromethylketone (Tos-Lys-CH_2Cl), EDTA, and pepstatin had no effect on the activity. These properties are consistent with those of cucumisin (Uchikoba et al., 1995). Based on the results, it was suggested that the *E. supina* protease B is a serine protease.

The results of hydrolysis of several peptidyl-*p*NA substrates by *E. supina* protease are shown in Table 3. The most favorable substrate for *E. supina* protease B was glt-Ala-Ala-Pro-Leu-*p*NA. Suc-Ala-Ala-Pro-Phe-*p*NA and Suc-Ala-Ala-Ala-*p*NA were hydrolyzed by *E. supina* protease B. Boc-Leu-Gly-Arg-*p*NA and Tos-Gly-Pro-Lys-*p*NA were also hydrolyzed by the enzyme, which exhibits broad specificity.

The oxidized B-chain of bovine insulin was hydrolyzed by *E. supina* protease B and the amino acid compositions of the resulting peptides were determined, from which the it was possible to locate each peptide in the primary structure of the B-chain of insulin (see Fig. 3). Fourteen sites on the oxidized insulin B-chain were cleaved by *E. supina* protease B during 24 h of hydrolysis. Some cleavage sites resembled those on cucumisin (Kaneda & Tominaga, 1975; Uchikoba et al., 1995), however, the *C*-terminal bond of the Val residue was not split by *E. supina* protease B.

The N-terminal sequence of the first 15 residues of *E. supina* protease B was determined. The sequence was aligned with that of cucumisin family proteases for maximum homology (see Fig. 4). *E. supina* protease B is comparable with those of seven plant serine proteases, namely, that expressed in melon fruit (Yamagata et al., 1994); white gourd protease (Uchikoba et al., 1998); PR-P69 (also known as P69A) (Tornero, Conejero & Vera, 1997); P69B pathogen-induced in tomato (Tornero, Conejero & Vera, 1996); ag12, expressed in the early stages of actinorhizal nodule formation in *Alnus glutinosa* (Ribeiro, Akkermans, Kammen, Bisseling & Pawlowski, 1995); lily protease LIM9, induced in meiotic prophase (Kobayashi et al., 1993; Taylor et al., 1997); *Arabidopsis thaliana* protease (EMBL accession number X85974). Six out of the fifteen N-terminal residues of *E. supina* protease B were identical to those of cucumisin.

3. Discussion

A previous study has identified proteases in the latex of several *Euphorbia* species as serine proteases (Lynn & Clevatte-Radford, 1988), but did not examine the structure of these proteases. The relation between the proteases of *Euphorbia* species and other plants is not known. In the present study, a new protease purified from the latex of *E. supina* was identified as a serine protease. A large portion of the caseinolytic activity of *E. supina* latex is due to *E. supina* protease B at neutral pH. This is the optimum pH for the caseinolytic activity of this protease, similar to that of proteases from

Table 2
Effect of various compounds on the caseinolytic activity of *E. supina* protease B

Compounds	Concentration (mM)	Class	Relativity activity (%)
None	–		100
DEP	2.0	Serine	15
	0.5		20
	0.01		80
PMSF	2.0	Serine	1
Tos-Lys-CH_2Cl	2.0	Serine trysin-like/cysteine	122
Leupeptin	0.2	Serine/cysteine	88
Aprotinin	0.01	Serine	92
STI	0.01	Serine trypsin-like	73
PCMPS	2.0	Cysteine	81
MIA	2.0	Cysteine	79
Antipain	0.01	Cysteine	92
EDTA	2.0	Metallo	111
Pepstatin	0.1	Aspartic	107

Table 3
Hydrolysis of peptidyl-*p*NA with *E. supina* protease B

Substrates	Hydrolysis rate (μkat/μmol protein)	Relative activity (%)
Glt-Ala-Ala-Pro-Leu-*p*NA[a]	1.6	100[b]
Suc-Ala-Ala-Pro-Leu-*p*NA	1.2	75
Suc-Ala-Ala-Pro-Phe-*p*NA	1.0	63
Suc-Ala-Ala-Ala-*p*NA	0.72	45
Boc-Ala-Ala-Pro-Ala-*p*NA	0.58	36
Pyr-Phe-Leu-*p*NA	0.58	36
Boc-Leu-Gly-Arg-*p*NA	0.52	33
Boc-Ser(Bzl)-Gyl-Arg-*p*NA	0.52	33
Tos-Gly-Pro-Lys-*p*NA	0.52	33
Z-Ala-Ala-Leu-*p*NA	0.43	27
Bz-Arg-*p*NA	0.43	27
Z-Gly-Gly-Leu-*p*NA	0.28	18
Suc-Ala-Pro-Ala-*p*NA	0.28	18
Suc-Ala-Ala-*p*NA	0.08	5
Bz-Tyr-*p*NA	0	0

[a] Glt-, glutaryl-.
[b] The enzyme activity toward Glt-Ala-Ala-Pro-Leu-*p*NA was assigned the value of 100%.

other *Euphorbia* species which exhibit pH optima of 6 to 8 (Lynn & Clevatte-Radford, 1988), but is in contrast to the cucumisin-like protease family from Cucurbitaceae, which has an optimum pH in the range 8–10 (Uchikoba et al., 1995, 1998). However, with the exception of optimal pH and certain structural differences, *E. supina* protease B was similar to cucumisin from the sarcocarp of *Cucumis melo*.

The M_r of *E. supina* protease B is similar to that of a serine protease, hevain l (80 kDa) isolated from latex of *Hevea brasiliensis* (Lynn & Clevatte-Radford, 1986a, 1986b); the majority of latex proteases from *Euphorbia* species have a M_r of between 60 and 80 kDa (Lynn & Clevatte-Radford, 1988). The M_r values of well-known plant serine proteases of the cucumisin family are in the range of 60–70 kDa (Uchikoba et al., 1995; Tornero et al., 1996, Tornero, Conejero & Vera, 1997; Ribeiro et al., 1995; Curotto, González, O'Reilly & Tapia, 1989; Yonezawa, Uchikoba & Kaneda, 1997). The N-terminal sequence of *E. supina* protease B also resembled those of cucumisin-like proteases.

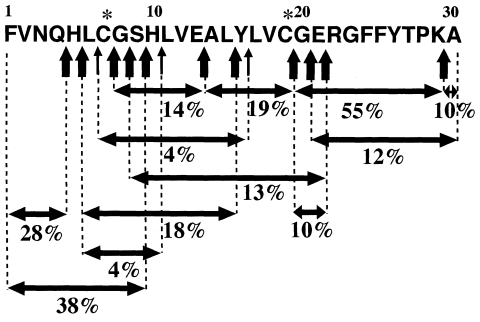

Fig. 3. Patterns of cleavage of oxidized insulin B-chain by *E. supina* protease B. The oxidized insulin B-chain (100 nmol) was digested with 0.1 nmol of protease at 37°C for 24 h. Comparison of the amino acid composition of the recovered peptides with the published insulin B-chain sequence permitted unequivocal identification of the cleavage sites by the enzymes. Peptides produced by the digestion are indicated by horizontal double-headed arrows. Number s indicate recovery percentage as determined by amino acid analysis of each peptide produced by the digestion. The amount of the initial substrate showed as 100%. Thin vertical arrows mark the cleavage sites yielding less than 10% recovery and the thick vertical arrows denote more than 10% recovery. C* is cysteic acid.

Five of the six cleavage sites occupied by charged amino acid residues at the P_1 position in the oxidized insulin B-chain were cleaved by *E. supina* protease. The substrates that contained Arg at the P_1 position were cleaved (see Table 3), while acidic residues such as Glu or Asp were little hydrolyzed by *E. supina* protease B (data not shown). *E. supina* protease B, was like cucumisin-like proteases (Uchikoba et al., 1995, 1998; Yonezawa et al., 1997), exhibits broad specificity at the P_1 position.

Additionally, twelve of fourteen cleavage sites having uncharged amino acids at the P_2 position were cleaved. It seems that this protease also exhibits broad specificity at the P_2 position. It is interesting that the P_2 position did not require an aromatic residue such as Tyr or Phe. This observation concerning the subsite of *E. supina* protease B may be useful in designing a good substrate for this enzyme. Three of four cleavage sites having Leu at the P_1' position were cleaved. However, there was no cleavage of peptide bonds having Val at the P_1' position.

The N-terminal sequence of *E. supina* protease B showed some similarity to that of other plant serine proteases as shown in Fig. 4. The amino acid residues common among cucumisin-like proteases (Uchikoba et al., 1998) were also conserved in the sequence of *E.*

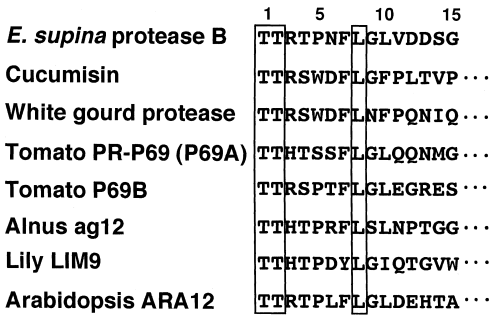

Fig. 4. Comparison of the N-terminal amino acid sequences of eight plant serine proteases: *E. supina* protease B from the present study; cucumisin (Yamagata et al., 1994); white gourd protease (Uchikoba, 1998); lily protease (LIM9) (Kobayashi et al., 1993; Taylor et al., 1997); *Arabidopsis* protease (ARA12, EMBL accession number X85974); *Alnus* ag12 (Ribeiro et al., 1995); tomato PR-P69 (P69A) (Tornero et al., 1997), and tomato P69B (Tornero, Conejero & Vera, 1996). The enclosures show those amino acid residues identical in all the sequences.

supina protease B, suggesting that *E. supina* protease B, cucumisin, and white gourd protease may have homologous protein structures. Interestingly, *E. supina* protease B is a serine protease, while papain is a cysteine protease located in the vacuoles (Vierstra, 1996). Although these proteases differ genetically, they may have achieved a homologous substrate specificity and similar function through convergent evolution.

4. Experimental

4.1. Materials

E. supina was collected in Kagoshima City, Japan. DEAE-cellulose was purchased from Whatman. CM-Sepharose was a product of Pharmacia. Casein was obtained from MERCK. Tos-Lys-CH_2Cl was a product of Sigma. Other materials were purchased from Wako Pure Chemical.

4.2. Assay of protease

Proteolytic activity was measured by the modified method of Uchikoba et al. (1998) with casein as a substrate. Enzyme soln. (0.5 ml) was added to 2 ml of 1% (w/v) casein in 67 mM Na, K–Pi buffer, pH 7.5. After incubation at 35°C for 20 min, the reaction was stopped by the addition of 3 ml of 5% TCA. After standing for 30 min at room temp, the ppt. was removed by filtration through Advantec Toyo filter paper No. 5C (Tokyo, Japan) and the absorbance of the filtrate was determined at 280 nm. A unit of activity was defined as the activity giving 0.001 A_{280} units of change per min under the above conditions.

4.3. Hydrolysis of peptidyl-pNAs

Enzymatic hydrolysis was carried out in 0.2 M Tris–HCl buffer, pH 7.5 at 25°C. The enzyme solution (0.2 ml) was added to 10 μl of 10 mM peptidyl-*p*NA/DMSO soln. and 0.6 ml of 0.2 M Tris–HCl buffer, pH 7.5. The rate of enzymatic hydrolysis for peptidyl-*p*NA substrates was followed spectrophotometrically with a Hitachi U-1100 spectrophotometer. An extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm was used for the yield of the hydrolysis product, *p*-nitroaniline (Erlanger, Kokowsky & Cohen, 1961). The hydrolysis activity was expressed in μkat (μmol *p*-nitroaniline formed per second).

4.4. Electrophoresis

SDS-PAGE — The method of Laemmli (1970) was followed, using a 15% polyacrylamide gel. The purified enzyme was treated 67 mM DFP for 30 min at 30°C to avoid autolysis. The diisopropyl-phosphorylated enzyme was subjected to SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue. The M_r standards used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactoalbumin (14 kDa) (Pharmacia).

4.5. Enzyme purification

Enzyme purification was performed at 7°C. Leaves and stems of *E. supina* (3 kg) were cut and placed in an appropriate volume of buffer A (17 mM Na, K–Pi buffer, pH 7.0). The soln. containing latex secreted from the tissues was filtered through a filter paper, and the supernatant (3 l) was applied to a column of DEAE-cellulose (5 × 43 cm) equilibrated with buffer A. The column was washed with buffer A (400 ml) and developed with buffer B (0.2 M Na, K–Pi buffer, pH 7.0). The active fractions eluted were combined, dialyzed against 33 mM Na, K–Pi buffer, pH 7.0, and chromatographed on a column of DEAE-Sepharose fast-flow (2.0 × 11 cm) with a linear gradient to buffer B (1 l) from buffer A (1 l). The major peak fractions of proteolytic activity were combined, and then solid ammonium sulfate was added to 40% saturation. The enzyme soln. from DEAE-Sepharose fast-flow was applied to a column of Butyl-Toyopearl (1.0 × 5 cm) equilibrated with 67 mM Na, K–Pi buffer, pH 7.0 containing ammonium sulfate (40% saturation), and was eluted with buffer C (67 mM Na, K–Pi buffer, pH 7.0). The fractions containing protease from the Butyl-Toyopearl column were applied to a Sephacryl S-300 gel-filtration column (2.4 × 132 cm) equilibrated with buffer C and eluted. Proteolytic activity was measured against casein as substrate. The active fractions were collected and kept at −20°C.

4.6. pH effects on proteolytic activity

The activity of protease (0.5 ml, 60 μg/ml) was measured at various pHs with 2 ml of 1% casein as substrate at 30°C for 20 min. To measure the pH stability of the enzyme, 1.0 ml of enzyme soln. (60 μg/ml) was incubated in various pH buffers at 35°C for 20 min. The enzyme solutions were adjusted to pH 7, and activity was assayed according to the method used for protease described above.

4.7. Temperature effects on proteolytic activity

The residual activity of the enzyme was assayed after the enzyme (0.5 ml, 60 μg/ml) had been incubated in buffer C at various temperatures (20–65°C) for 20 min. Activity was then assayed by the method used for protease as described above.

4.8. Inhibitor effects on proteolytic activity

The enzyme soln. (0.5 ml, 60 μg/ml) was added to 0.5 ml of inhibitor soln. (0.01–2 mM final concentration) in buffer C and incubated at 30°C for 60 min. Activity was then measured as described above. A control assay consisted of enzyme soln. without an inhibitor.

4.9. N-terminal sequencing analysis

Automated Edman degradation of *E. supina* protease was performed with an Applied Biosystems 477A protein sequencer. The phenylthiohydantoin derivatives were identified with an Applied Biosystems 120A analyzer.

4.10. Hydrolysis of oxidized insulin B-chain

The oxidized bovine insulin B-chain (100 μg, 29 nmol) was digested with 0.1 nmol of *E. supina* protease B in 100 μl of 0.1 M Tris–HCl buffer, pH 7.2, at 30°C. After incubation for 24 h, 50 μl of 0.1% (v/v) TFA was added to the mixture. The digested peptides were separated on a TSK-gel ODS-120T column (4.6 × 250 mm, Toso) with a linear gradient in acetonitrile containing 0.1% TFA, 0 to 85%, by using a Gilson HPLC system. The eluate was monitored by measuring absorbance at 220 nm. For analysis of the amino acid composition, the purified peptides were hydrolyzed in 6 M HCl containing 0.1% phenol at 110°C for 24 h. The samples were derivatized with phenylisothiocyanate using a Pico·Tag work station (Waters). Phenylthiocarbamyl derivatives of amino acids were analyzed with a Pico·Tag HPLC system.

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