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Amino Acid Sequence and Some Properties of Phytolacain R, a Cysteine Protease from Full-Growth Fruits of Pokeweed, *Phytolacca americana*

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A cysteine protease, phytolacain R from full-growth greenish fruits of pokeweed, *Phytolacca americana* L, was purified to electrophoretic homogeneity by a simple purification procedure employing CM-Sepharose ion-exchange chromatography. The enzyme was present in low content in the young fruits about 50 d after flowering but gradually accumulated in growing fruits. Its molecular mass was estimated to be *ca.* 23 kDa by SDS-PAGE, and its sugar content was zero. Its amino acid sequence was established by automated sequence analysis of the peptides obtained by cleavage with *Achromobacter* protease I, chymotrypsin, trypsin, and cyanogen bromide. The enzyme is composed of 218 amino acid residues, of which it shares 110 residues (50%) with papain, 104 (47%) with actinidain, and 87 (40%) with stem bromelain. The amino acid residues forming the substrate-binding the S_2 pocket of papain, Tyr61, Tyr67, Pro68, Trp69, Val133, and Phe207, were predicted to be replaced by Gly, Trp, Met, His, Ala, and Met in phytolacain R, respectively. As a consequence of these substitutions, the S_2 pocket is expected to be less hydrophobic in phytolacain R than in papain.

Key words: amino acid sequence, cysteine protease, endopeptidase, *Phytolacca americana*, pokeweed.

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The fruits of pokeweed, *Phytolacca americana*, contain two cysteine proteases. Phytolacain G was isolated from medium-sized greenish fruits (*1*), whereas phytolacain R, which is contained in low amount in young fruit, was isolated from fully grown greenish fruits (*2, 3*). Analyses of the amino terminal sequences of the two enzymes (*4*) suggest that phytolacain R is not derived from phytolacain G by protein processing during the course of fruit ripening. These enzymes are present in the fruit in higher amounts than appear to be required for the digestion of endogenous substrates. They may act rather as a defense against foreign substrates, *e.g.*, proteins secreted by invading pathogens (*5*). The caseinolytic activity of phytolacain G was completely inhibited by a synthetic peptide containing an *S*-(3-nitro-2-pyridinesulfenyl) group (Npys). The inhibitory activity of this compound against phytolacain G resembled that of papain (*1*). Phytolacain R and papain differed in their specificity for synthetic substrates (*3*). Bz-L-Arg-*p*NA, a good substrate of papain, was not hydrolyzed by phytolacain R. Likewise, a substrate of phytolacain R, Bz-L-Tyr-*p*NA, was not hydrolyzed by papain (*2*).

In this study, we prepared phytolacain R and elucidated some of its properties. We also determined its amino acid sequence in order to obtain structural information on the substrate-recognition site.

MATERIALS AND METHODS

Materials—Greenish full-sized fruits (50-70 d after flowering) of pokeweed were obtained from Kagoshima city. CM-cellulose was purchased from Whatman (Kent, England). CM-Sepharose was a product of Pharmacia (Uppsala, Sweden). Papain and casein were obtained from Merck (Darmstadt, Germany). Thrombin was a product of Sigma (St. Louis, MO, USA). TPCK-trypsin was from Worthington Biochemical (Freehold, NJ, USA). *Achromobacter* protease I, PCMB, and other materials were purchased from Wako Pure Chemical (Osaka).

Assay of Protease—Proteolytic activity was measured by the modified method of Kunitz (*6*) with casein as a substrate. Enzyme solution (1 ml) was added to 1 ml of 2% (w/v) casein in 67 mM sodium-potassium phosphate buffer, pH 7.5. After incubation at 35°C for 20 min, the reaction was stopped by addition of 3 ml of 5% trichloroacetic acid. The mixture was left to stand for 30 min at room temperature, then the precipitate was removed by filtration through Advantec Toyo filter paper No. 5C (Tokyo), and the absorbance of the filtrate was determined at 280 nm. One unit of activity was defined as the activity giving 0.001 A_{280} units of change per min under the above conditions.

Effect of Inhibitor—The Cys(Npys) peptide Phe-Leu-Ser-Cys(Npys)-NH_2 was previously prepared as an inhibi-

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Abbreviations: DTT, dithiothreitol; Npys, *S*-(3-nitro-2-pyridinesulfenyl); PCMB, *p*-chloromercuribenzoic acid; PE-, pyridylethyl; *p*NA, *p*-nitroanilide; TFA, trifluoroacetic acid.

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tor of phytolacain G (*1*). Its inhibitory activity against phytolacain R and papain, a plant cysteine protease chosen as a reference standard, was measured as follows. Phytolacain R and papain were incubated in 67 mM sodium-potassium phosphate buffer, pH 7.2, containing 2 mM DTT, then the enzyme solutions were ultrafiltered to remove DTT by use of a Centriprep (Amicon). The enzyme (0.5 ml) was incubated with Cys(Npys) peptide (0.5 ml) in 67 mM sodium-potassium phosphate buffer, pH 7.2, at 30°C for 60 min, then the remaining activity of the reaction mixture was assayed at pH 7.2 using casein as a substrate as described above.

Enzyme Purification—All purification procedures were performed at 7°C. Frozen pokeweed fruits (300 g) in 20 mM sodium-potassium phosphate buffer, pH 7.0 (1,000 ml) were slowly homogenized, and the seeds were removed from the sarcocarps. The sarcocarps were homogenized and filtered through a cotton cloth. The filtrate was kept at 7°C for 1 h, then centrifuged ($3,500\times g$, 15 min). Solid ammonium sulfate was added to the supernatant to 60% saturation, and the mixture was kept at 7°C for 15 h. The precipitated protein was collected by centrifugation ($3,500\times g$, 15 min) and suspended in distilled water, and the suspension was dialyzed against distilled water for 15 h. The dialyzed enzyme solution was adjusted to pH 5.0 with 0.1 M acetic acid and centrifuged ($10,000\times g$, 15 min) to remove insoluble materials. The supernatant was put on a CM-cellulose column (5.0×40 cm) equilibrated with buffer A (25 mM sodium acetate buffer, pH 5.0), the column was washed with the same buffer, then the enzyme was eluted with buffer B (0.17 M sodium acetate buffer, pH 6.2 containing 0.3 M NaCl). Solid ammonium sulfate was added to 60% saturation to the enzyme-active fractions from the column, and the mixture was kept at 7°C for 15 h, then centrifuged ($10,000\times g$, 15 min). The pellet obtained was dialyzed against water for 6 h and against 25 mM acetate buffer, pH 5.0, containing 4 mM cysteine for 8 h. After centrifugation ($10,000\times g$, 15 min), the supernatant was applied to a column of CM-Sepharose (1.5×18 cm) equilibrated with buffer A. Elution was carried out with a linear gradient from buffer A to buffer C (0.17 M sodium acetate buffer, pH 6.2) at a flow rate of 1.0 ml/min. The pooled active fraction was concentrated by use of a Centriprep (Amicon) and kept at −20°C.

Molecular Mass Determination—The molecular mass of the enzyme was estimated by HPLC gel filtration on a TSK-gel G2000SW column (600×7.5 mm, Tosoh) equilibrated with 50 mM sodium-potassium phosphate buffer, pH 5.5, containing 0.3 M NaCl by elution with the same buffer at a flow rate of 0.5 ml/min. The effluent was monitored by measuring its absorbance at 280 nm. The column was calibrated using BSA (67 kDa), chymotrypsinogen (26 kDa), myoglobin (18 kDa), and cytochrome *c* (12 kDa).

The subunit molecular mass was estimated by SDS-PAGE under reducing conditions according to the procedure of Laemmli (*7*) using 15% gel. Molecular mass markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonate dehydratase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). The proteins were stained with Coomassie Brilliant Blue R-250.

Determination of Carbohydrate—The carbohydrate of the enzyme preparation was determined by the phenol-sulfuric acid method (*8*). D-Mannose was used as a standard.

Preparation of S-Pyridylethylated Phytolacain R—Reduction and *S*-pyridylethylation of phytolacain R were performed by the method of Ruegg and Rudinger (*9*). The phytolacain R (10 mg, 380 nmol) was dissolved in 2.4 ml of 0.5 M Tris-HCl buffer, pH 8.5, containing 6 M guanidine-HCl and 10 mM EDTA. The solution was kept under N_2 gas at 25°C for 30 min, then DTT (23 mg, 150 μmol) was added. The mixture was incubated at 25°C for 2 h, then 4-vinylpyridine (52 μl, 450 μmol) was added, and the incubation was continued for 2 h. The mixture was dialyzed against 1 mM HCl containing 4 M urea at 4°C for 48 h. PE-phytolacain R was purified on an Aquapore RP-300 column (4.6×30 mm, Brownlee) equilibrated with solvent A (TFA/H_2O, 1:1,000) by elution with a linear gradient from 30% B to 100% B [(CH_3CN/2-propanol, 3:7)/H_2O/TFA, 700:300:0.7] for 30 min at a flow rate of 0.5 ml/min. The eluate was monitored by measuring the absorbance at 220 nm with a Gilson HPLC system. The PE-phytolacain R fraction was collected and lyophilized.

Enzymatic Digestions—*Achromobacter* protease I: PE-Phytolacain R (2 mg, 76 nmol) was digested with 40 μg (1.4 nmol) of *Achromobacter* protease I in 280 μl of 0.2 M Tris-HCl buffer, pH 8.3, containing 1 mM EDTA, and 2 M urea at 37°C for 24 h. The reaction was stopped by addition of formic acid (40 μl).

Chymotrypsin: A solution of PE-phytolacain R (2 mg, 76 nmol) in 30 μl of 1% ammonium bicarbonate, pH 8.0, containing urea (24 mg) was preincubated at 37°C for 1 h, then mixed with 170 μl of 1% ammonium bicarbonate and bovine α-chymotrypsin (100 μg, 4 nmol) in 20 μl of the ammonium bicarbonate/urea solution. The reaction mixture was incubated at 37°C for 5 h. The reaction was stopped by addition of formic acid (100 μl).

Trypsin: Trypsin digestion of PE-phytolacain R (2 mg, 76 nmol) was performed at 37°C for 12 h by using TPCK-treated trypsin in 1% ammonium bicarbonate containing 1 mM $CaCl_2$ by the method described above for chymotrypsin digestion.

Cyanogen bromide cleavage: PE-phytolacain R (1 mg, 38 nmol) was treated at 25°C for 24 h in 100 μl of 70% formic acid containing 1 mg of cyanogen bromide, using a ratio of the modified protein to cyanogen bromide of 1:250 (mol/mol). The reaction mixture was diluted with distilled water, then lyophilized.

Separation of Peptides—The digests obtained with *Achromobacter* protease I, chymotrypsin and cyanogen bromide were chromatographed on an RP-HPLC C_4 column (Aquapore butyl column, 4.6×100 mm, Brownlee) equilibrated with solvent A by elution with a linear gradient from 100% solvent A to 60% solvent B for 90 min at a flow rate of 0.5 ml/min. The eluate was monitored by measuring the absorbance at 220 nm with the HPLC system as described above.

The digests obtained with trypsin were chromatographed on an RP-HPLC C_8 column (Aquapore RP-300 column, 4.6×100 mm, Brownlee) under the conditions described above.

Amino Acid Analysis—Samples were hydrolyzed with 100 μl of 6 M HCl containing 0.1% phenol *in vacuo* at 110°C for 24 h in tubes into a sealed vial with a Pico·Tag work

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station (Waters). The amino acids were derivatized with phenylisothiocyanate in the work station, then the phenylthiocarbamyl derivatives of amino acid were analyzed on a Pico•Tag HPLC amino acid analysis system (Waters).

Amino acid analysis of whole PE-phytolacain R protein and the cyanogen bromide-cleaved peptide was carried out with a Hitachi L-8500 amino acid analyzer (Hitachi) by the standard ninhydrin method.

Protein Sequencing Analysis—Automated Edman degradation was performed with an Applied Biosystems 477A protein sequencer (Applied Biosystems). The phenylthiohydantoin derivatives were identified by use of an Applied Biosystems 120A analyzer.

RESULTS

Purification of Phytolacain R—Phytolacain R from pokeweed fruit was purified by a simple procedure, which is summarized in Table I. The elution profile of CM-Sepharose column chromatography showed a sharp (phytolacain G, fractions 87-93) and a broad (phytolacain R) active peak (Fig. 1C). The pooled active fraction (phytolacain R, fractions 120-145) from the preparation of ripe fruits was concentrated by use of a Centriprep (Amicon) and kept at −20°C. The enzyme was effectively isolated by the single CM-Sepharose column chromatography, with 300 g of fruits yielding 45 mg of purified phytolacain R, representing 13-fold purification and 13% recovery. In our previous study (*2*), 30 mg of the purified phytolacain R was obtained from 30 kg of ripe fruits. The procedure involved several steps, and it is thought that the enzyme degraded autolytically in the course of isolation. In the present study, phytolacain R was easily isolated in the pure form by one step of chromatography. In this regard phytolacain G had been obtained from fractions 86 to 95 in the same condition as shown in Fig. 1B described previously.

As shown Fig. 1A, phytolacain G was the major enzyme fraction found in greenish small-sized fruits (2 wk after flowering), and this enzyme increased with the growth of fruits (Fig. 1, A-D). Phytolacain R, another basic proteolytic enzyme, was found in full-sized greenish fruits (50-70 d after flowering, Fig. 1C) and reddish ripe fruits (80-100 d after flowering, Fig. 1D). Full-sized greenish fruits (Fig. 1C) and reddish ripe fruits (Fig. 1D) of pokeweed thus contained phytolacains G and R. The pass-through fraction shown in Fig. 1, C and D, also contained proteolytic activity. These activities were inactivated by PCMB, and no significant difference was detected among the active fractions in the sensitivity to various compounds and pH of maximum activity (data not shown). It is suggested that these enzymes are isozymes of phytolacain. Phytolacain R had no activity toward such synthetic substrates as Leu-*p*-nitroanilide (Leu-*p*NA), Ala-*p*NA for aminopeptidase activity, and benzyloxycarbonyl-Phe-Leu (Z-Phe-Leu) for carboxy-

TABLE I. **Purification of phytolacain R from pokeweed fruits.**

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Extract	4,570	47,300	10.4	100
Ammonium sulfate	1,280	36,000	28.1	76
CM-Sepharose	45	6,330	141	13

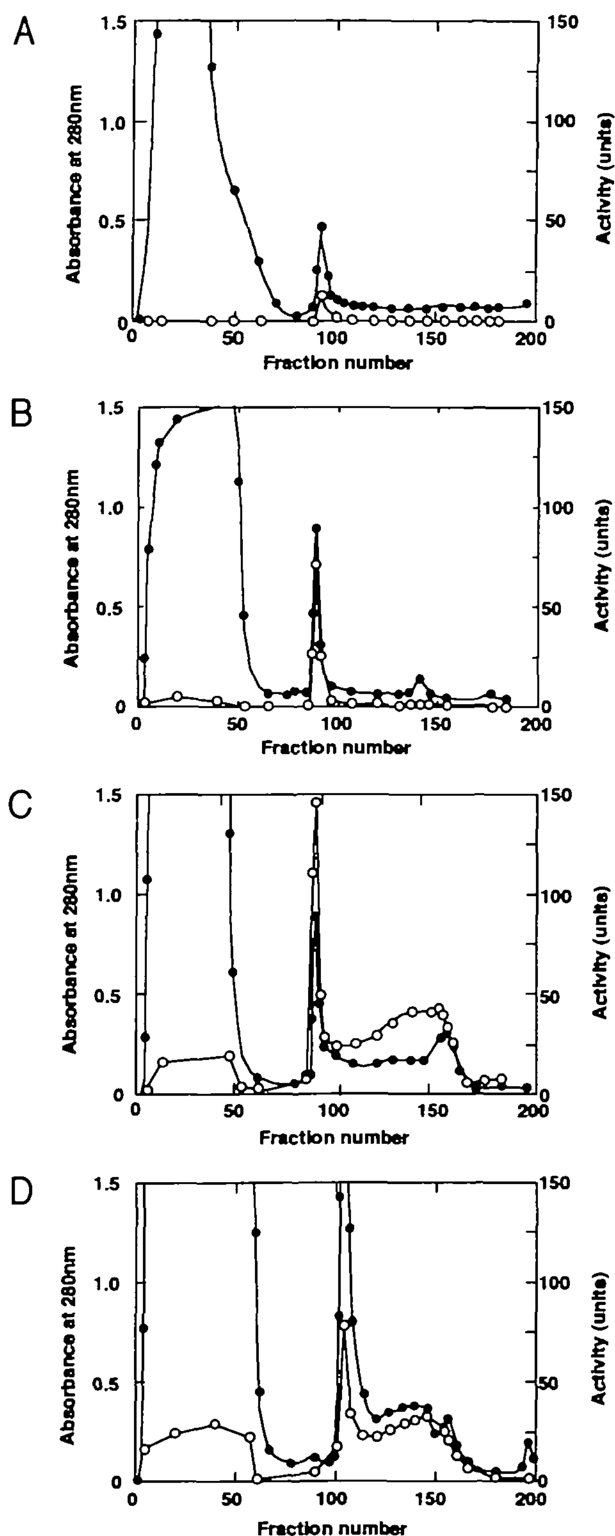

Fig. 1. **Elution profiles on CM-Sepharose cation ion-exchange chromatography of cysteine protease activity from pokeweed fruits at various stages of growth.** The flow rate of the column was 72 ml/h. Each fraction was 15 ml. ●, absorbance at 280 nm; ○, caseinolytic activity. A, small greenish fruits (2 wk after flowering, diameter less than 3 mm); B, medium-sized greenish fruits (20-45 d after flowering, 3-5 mm); C, full-sized greenish fruits (50-70 d after flowering, 6-8 mm); D, ripe reddish fruits (80-100 d after flowering).

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peptidase activity (data not shown). Therefore, it is presumed that phytolacain R is an endopeptidase.

Molecular Mass of Phytolacain R—Purified protein migrated as a single band of 23 kDa on SDS-PAGE in a 15% polyacrylamide as shown in Fig. 2. A molecular mass of 25 kDa was also obtained by using TSK-gel HPLC gel filtration. Molecular masses of well-known plant cysteine proteases occupy the range 23-30 kDa (*10*). Thus, phytolacain R is typical of plant proteases in its molecular mass.

Carbohydrate Determination—No carbohydrate was found in phytolacain R by the phenol-sulfuric acid method. No sugar was found in phytolacain G by the same method (*4*).

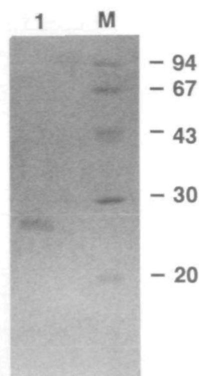

Fig. 2. **SDS-PAGE of purified phytolacain R.** The samples were electrophoresed in 15% polyacrylamide gel. The gel was stained in Coomassie Brilliant Blue for 15 min and then destained. Lane 1, purified phytolacain R; lane M, molecular mass standards (Da $\times 10^{-3}$).

Amino Acid Composition—The amino acid composition of purified phytolacain R was determined (data not shown). The values are expressed as the number of residues per molecule, assuming a molecular mass of 24 kDa. Taking the nearest integral numbers of residues, we calculated that phytolacain R consisted of 218 amino acid residues per molecule. Phytolacain R contained a methionine residue, unlike papain.

Enzymatic Properties—Matsueda *et al.* employed an affinity labeling method to study the inhibition of cysteine proteases including a cytoplasmic Ca^{2+}-dependent pro-

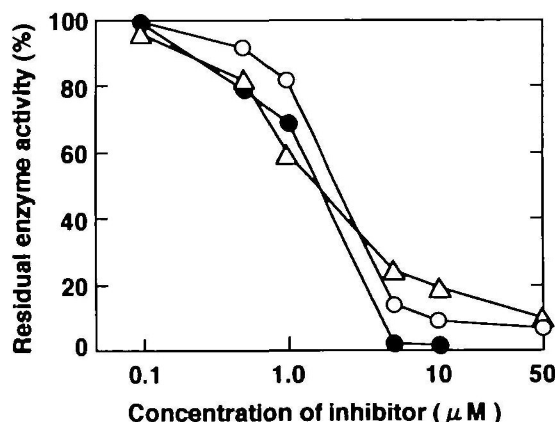

Fig. 3. **Inhibitory effect of the Cys(Npys) peptide on phytolacains R and G and papain.** Phytolacains R (○) and G (●) and papain (△) protease activities were measured using casein as a substrate. Enzyme concentrations were 2.3 μM.

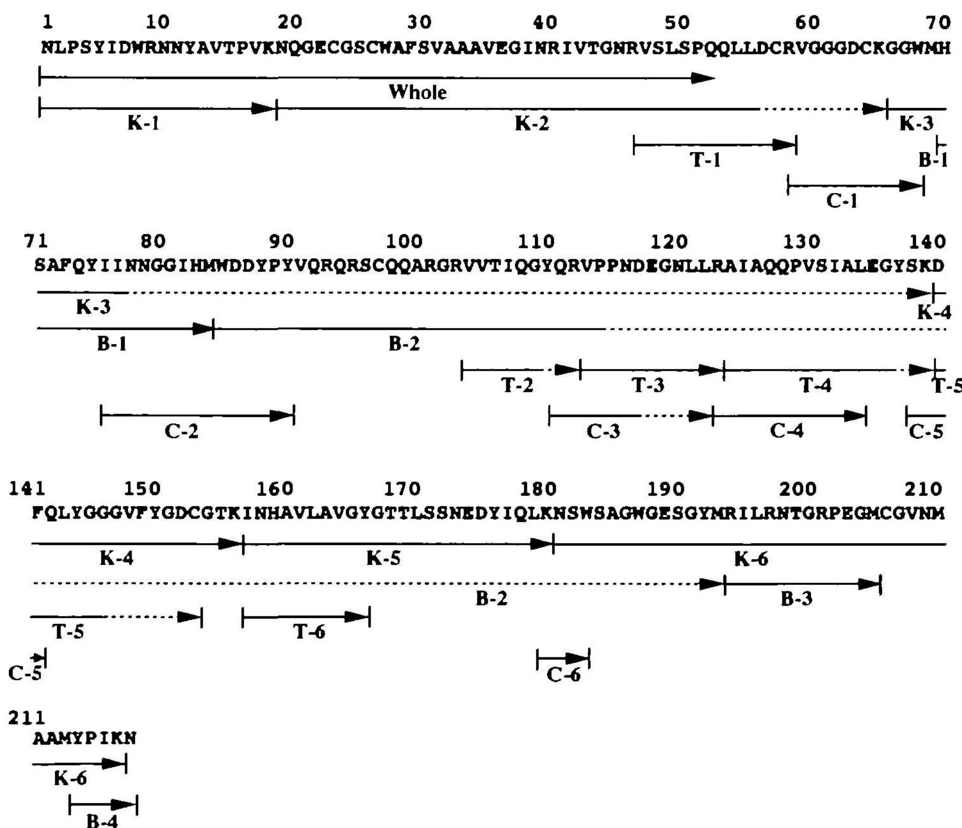

Fig. 4. **Sequence of phytolacain R.** Sequences derived by Edman degradation of specific peptides are given below the overall sequence (bold type). The peptides derived from PE-phytolacain R by treatment with *Achromobacter* protease I, chymotrypsin, trypsin, and cyanogen bromide are designated by the letters K, C, T, and B, respectively. The peptides are numbered from the N-terminus.

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TABLE II. **Amino acid composition of peptides obtained from phytolacain R by *Achromobacter* protease I digestion.**

Amino acid	K-1	K-2	K-3	K-4	K-5	K-6
Asx	4.3(4)	4.7(5)	6.2(7)	2.0(2)	2.1(3)	3.2(3)
Glx		6.3(5)	11.0(11)	2.2(1)	1.8(2)	3.3(2)
Ser	1.0(1)	3.2(4)	4.1(4)		1.8(2)	2.1(3)
Gly		6.5(7)	10.9(8)	4.9(5)	3.6(2)	6.0(6)
His			2.0(2)		0.8(1)	
Arg	1.2(1)	1.9(3)	5.9(6)			2.4(3)
Thr	1.1(1)	2.4(1)	2.2(1)	0.9(1)	1.8(2)	1.3(1)
Ala	1.4(1)	4.0(4)	7.0(5)		2.7(2)	3.3(3)
Pro	2.4(2)	0.8(1)	5.2(4)			2.3(2)
Tyr	2.2(2)		5.5(5)	1.2(2)	2.4(2)	2.6(2)
Val	2.3(2)	3.9(5)	6.8(5)	1.5(1)	2.4(2)	2.2(1)
Met			1.7(2)			4.0(4)
PE-Cys		4.0(4)	1.0(1)	1.0(1)		1.0(1)
Ile	1.5(1)	2.4(2)	7.0(6)		2.5(2)	3.2(2)
Leu	1.5(1)	3.3(3)	5.4(3)	1.4(1)	3.8(3)	2.1(1)
Trp	– (1)	– (1)	– (2)			– (2)
Phe		1.0(1)	1.7(1)	1.7(2)		
Lys	1.1(1)	1.0(1)	1.6(1)	1.1(1)	1.0(1)	1.0(1)
Total	18	47	74	17	24	37
Position	1-18	19-65	66-139	140-156	157-180	181-217

TABLE III. **Amino acid composition of peptides obtained from phytolacain R by trypsin digestion.**

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6
Asx	1.4(1)		2.3(3)		2.0(2)	0.9(1)
Glx	2.4(2)	1.0(1)	1.4(1)	2.7(3)	0.7(1)	
Ser	2.1(2)			1.8(2)		
Gly		1.3(1)	1.2(1)	1.5(1)	3.8(4)	1.2(1)
His						0.8(1)
Arg	1.1(1)		1.3(1)			
Thr		1.1(1)				
Ala				2.6(3)		1.9(2)
Pro	0.8(1)		1.5(2)	1.3(1)		
Tyr		1.4(1)		1.1(1)	1.7(2)	1.3(1)
Val	0.8(1)	1.5(2)	1.4(1)	1.3(1)	0.7(1)	1.8(2)
Met						
PE-Cys	0.5(1)				0.5(1)	
Ile		1.2(1)		1.6(2)		1.0(1)
Leu	2.1(3)		1.4(2)	1.5(1)	2.2(1)	1.1(1)
Trp						
Phe					1.0(2)	
Lys				1.0(1)		1.0(1)
Total	12	7	11	16	14	10
Position	47-58	104-112	113-123	124-139	140-153	157-166

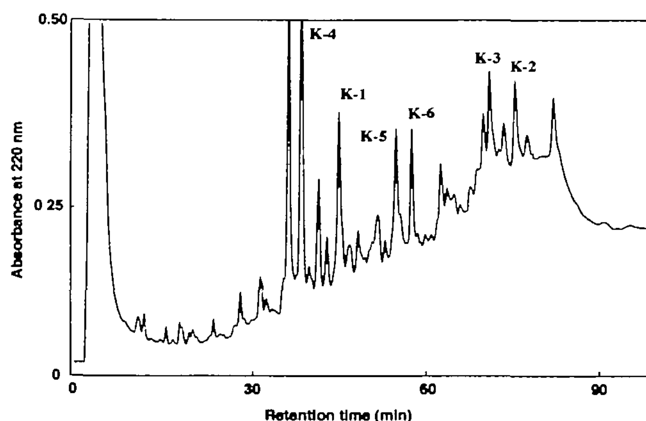

Fig. 5. **Separation of peptides from a *Achromobacter* protease I digest of PE-phytolacain R.** PE-phytolacain R was digested with *Achromobacter* protease I, and peptides were separated by chromatography on an Aquapore butyl column as described in the text.

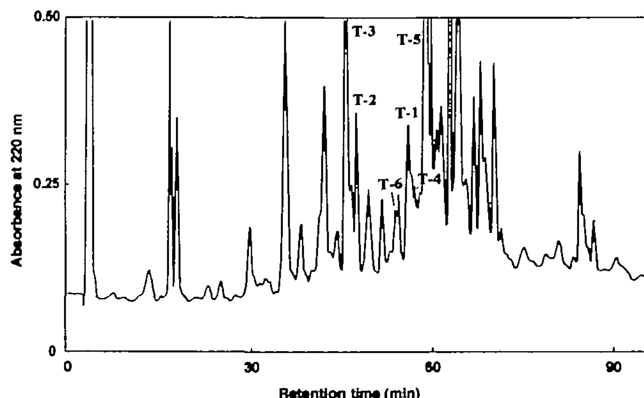

Fig. 6. **Separation of peptides from a trypsin digest of PE-phytolacain R.** PE-phytolacain R was digested with trypsin, and peptides were separated by chromatography on an Aquapore RP-300 column as described in the text.

tease, rat liver lysosome cathepsins B, H, and L (*11*), and calpain (*12*). The inhibitors had a homologous sequence to a good substrate of these enzymes, and the P_1 residue of the substrate was replaced with a Cys(Npys) residue. The inhibitory action of a Cys(Npys) peptide was specific for the cysteine proteases, and the Cys(Npys) residue of the inhibitor was presumed to bind to the SH-group of the active site on the enzymes. The inhibitory activity of the Cys(Npys) peptide toward phytolacains R and G and papain is shown in Fig. 3. On addition of 5.0 μM inhibitor, 15% of the original caseinolytic activity of phytolacain R (2.3 μM) and 24% of the original activity of papain remained, while the caseinolytic activity of phytolacain G was completely inhibited (Fig. 3) (*1*).

Amino Acid Sequence—The sequencing is summarized in Fig. 4. The peptides showing proteolytic digestions and chemical cleavage were further purified by reverse-phase HPLC for sequencing in some cases.

The N-terminal amino acid sequence of the enzyme was determined to position 52. It matched perfectly the amino acid sequence up to position 35 reported previously (*3*). Most of the sequence could be deduced from the peptides obtained with *Achromobacter* protease I.

Six peptides were produced by *Achromobacter* protease I cleavage of the PE-phytolacain R (Table II and Fig. 5) and sequenced at least partly. K-1, K-4, K-5, and K-6 were completely sequenced and had a lysine at the C-terminus. The sequences of peptides K-1 and K-2 were aligned with the help of the N-terminal sequence of PE-phytolacain R.

The remaining sequence and the overlaps were obtained by the cleavage of PE-phytolacain R with trypsin (Table III and Fig. 6) and chymotrypsin (Table IV and Fig. 7), and by chemical cleavage with cyanogen bromide (Table V and Fig. 8). The sequence of peptide C-6 was used to link the sequences of peptides K-5 and K-6 obtained with *Achromobacter* protease I. Similarly peptides T-2 and T-3 were linked by peptide C-3, T-3, and T-4 by C-4, T-4, and T-5 by C-5, and B-1 and B-2 by C-2. The carboxy-terminal residue of phytolacain R was Asn, obtained by sequencing of

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TABLE IV. **Amino acid composition of peptides obtained from phytolacain R by chymotrypsin digestion.**

Amino acid	C-1	C-2	C-3	C-4	C-5	C-6
Asx	1.4(1)	3.2(4)	3.1(3)		1.0(1)	0.9(1)
Glx			2.2(2)	1.8(2)		
Ser					1.0(1)	1.0(1)
Gly	5.2(5)	2.0(2)	1.3(1)			
His		0.6(1)				
Arg	1.2(1)		1.1(1)	0.9(1)		
Thr	0.8(1)					
Ala				2.9(3)		
Pro		0.9(1)	1.9(2)	1.1(1)		
Tyr		1.6(2)				
Val			1.2(1)	1.0(1)		
Met		0.6(1)				
PE-Cys	1.0(1)					
Ile		1.6(3)		2.0(2)		
Leu			1.6(2)	1.1(1)		
Trp	− (1)	− (1)				− (1)
Phe					1.1(1)	
Lys	1.0(1)				1.1(1)	1.0(1)
Total	11	15	12	12	4	4
Position	58-68	76-90	111-122	123-134	138-141	180-183

TABLE V. **Amino acid composition of peptides obtained from phytolacain R by BrCN cleavage.**

Amino acid	B-1	B-2	B-3	B-4
Asx	1.8(2)	10.3(11)	1.0(1)	0.9(1)
Glx	1.1(1)	14.8(14)	1.0(1)	
Ser	1.0(1)	7.4(8)		
Gly	2.0(2)	13.8(14)	1.6(2)	
His	2.1(2)	1.4(1)		
Arg		7.8(6)	2.6(3)	
Thr		2.1(4)	0.8(1)	
Ala	1.2(1)	7.1(7)		
Pro		4.7(4)	1.1(1)	1.0(1)
Tyr	0.6(1)	9.1(9)		2.3(1)
Val		7.1(8)		
H·Ser[a]	1.0(1)	0.8(1)	1.3(1)	
PE-Cys		1.9(1)		
Ile	2.1(3)	4.2(5)	1.1(1)	0.8(1)
Leu		7.3(7)	1.4(1)	
Trp				
Phe	0.6(1)			
Lys		2.3(3)		0.8(1)
Total	15	109	12	5
Position	70-84	85-193	194-205	214-218

[a]H·Ser shows homoserine residue.

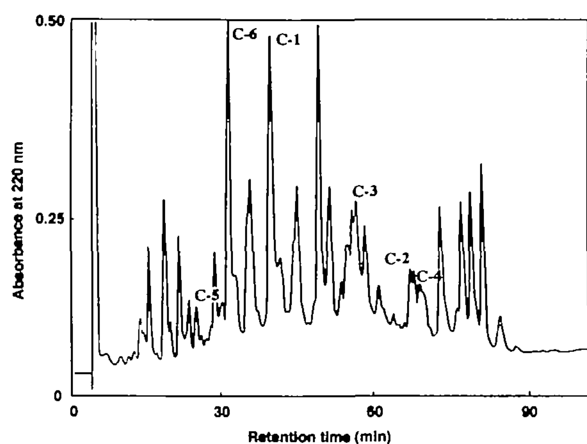

Fig. 7. **Separation of peptides from a chymotrypsin digest of PE-phytolacain R.** PE-phytolacain R was digested with chymotrypsin, and peptides were separated by chromatography on an Aquapore butyl column as described in the text.

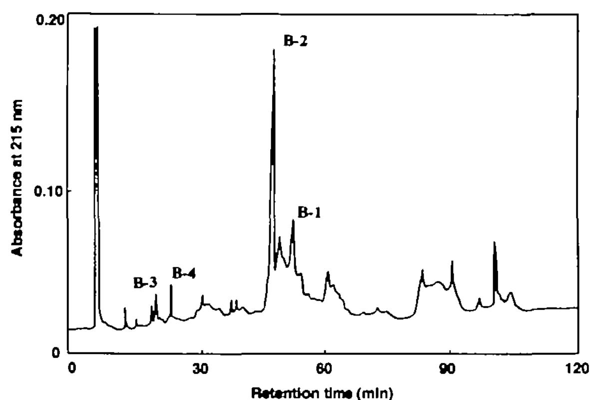

Fig. 8. **Separation of peptides from a cyanogen bromide digest of PE-phytolacain R.** PE-phytolacain R was cleaved with cyanogen bromide, and peptides were separated by chromatography on an Aquapore butyl column as described in the text.

peptide B-4, which was shown not to contain homoserine or homoserine lactone by amino acid analysis. The amino acid composition of phytolacain R was consistent with that of the enzyme described above.

DISCUSSION

The sequence of PE-phytolacain R was aligned with that of papain [EC 3.4.22.2] (*13, 14*), actinidain [EC 3.4.22.14] (*15*), and stem bromelain [EC 3.4.22.32] (*16*) (Fig. 9). Of its 218 amino acid residues, phytolacain R shares 110 residues (50%) with papain, 104 (47%) with actinidain and 87 (40%) with stem bromelain. It was found to have 7 cysteines by sequencing and amino acid analysis, suggesting that it has three disulfide bridges, like other members of the papain family. At the N-terminus, phytolacain R is longer than papain by one residue, asparagine. Stem bromelain is longer by one alanine residue (*16*). A proline residue at position 2 (position 3 in phytolacain R and stem bromelain) is conserved in the papain family. This residue is considered to prevent unwanted N-terminal proteolysis (*17*). In comparison with papain, phytolacain R shows an insert of six amino acid residues between positions 167 and 168. In the same region, chymopapain [EC 3.4.22.6] (*18*), caricain [EC 3.4.22.30] (*19*), and actinidain (*16*) have a four amino acid insert.

The relationship of structure and function in cysteine proteases has been investigated, especially for papain and actinidain, whose tertiary structures are known (*20*). Cys25 and His159 (based on the residue numbering of papain) of phytolacain R were predicted to be the catalytic residues by homology to papain. Asn175 of papain, oriented in the vicinity of the imidazole of His159 (*17*), was also conserved in phytolacain R. Of the residues surrounding the putative oxianion hole in papain, Asn64, Gly65, and Asp158 (*21*) were replaced by Lys, Gly, and Asn, respectively, in phytolacain R, while Gln19, Gly66, and Trp177

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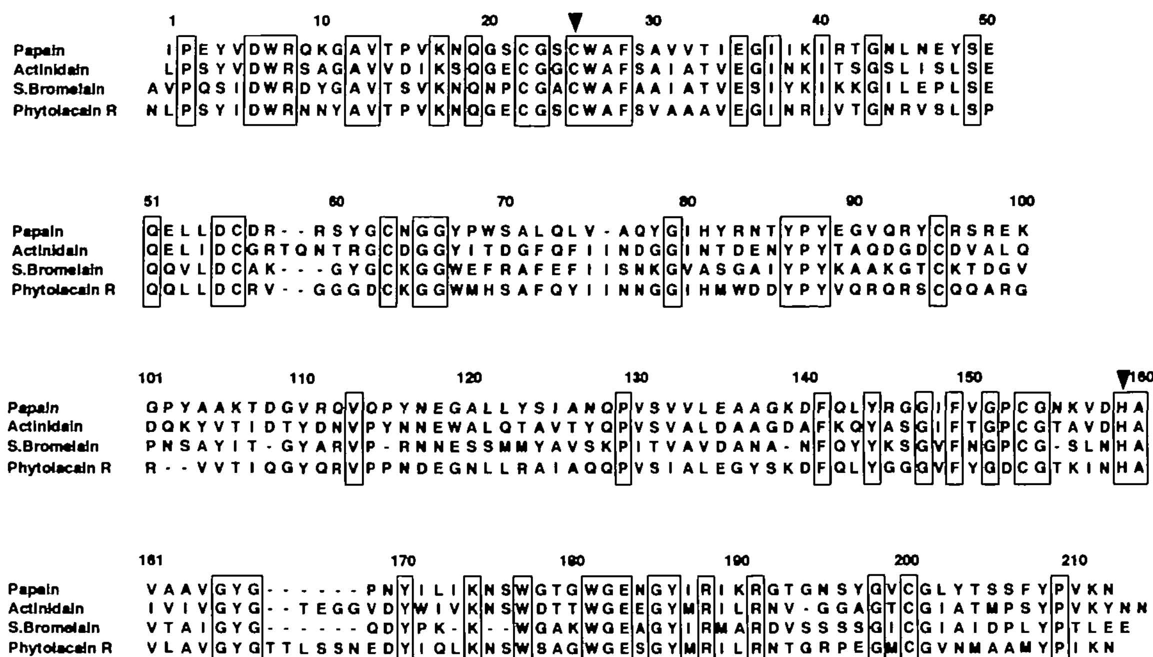

Fig. 9. **Comparison of the amino acid sequences of phytolacain R and other plant cysteine proteases.** The four sequences were aligned for maximum homology. Numbering is according to that of papain.

were conserved. These substitutions probably account for the difference in substrate specificity observed between phytolacain R and papain: Bz-L-Arg-*p*NA, a good substrate of papain, was not hydrolyzed by phytolacain R; and Bz-L-Tyr-*p*NA, a substrate of phytolacain R, was not hydrolyzed by papain (*2*). In particular, the replacement of Asn64 by Lys may lead to positive charge repulsion between Lys64 of phytolacain R and the side chain of Arg of the substrate Bz-L-Arg-*p*NA. We previously classified the substrate specificities of phytolacain R and papain as broad type, based on the P_1 position of the cleavage sites (*3*). On the other hand, the P_2 position of the sites of cleavage by phytolacain R was occupied mainly by a hydrophobic amino acid residue such as Val, Tyr, or Phe (four of nine cleavage sites of insulin B-chain) or by a small amino acid residue, glycine or serine. In the case of papain, however, the P_2 position was mainly occupied by a hydrophobic amino acid residue such as Val, Leu, Tyr, or Phe (six of eight cleavage sites). Papain was reported to prefer hydrophobic amino acid residues at P_2 of the cleavage sites (*22*), while phytolacain R also preferred small amino acid residues such as Ser (P_2) or Gly (P_2) substrates. The papain substrate pyroglutamyl-Phe-Leu-*p*NA (*23*) was not hydrolyzed by phytolacain R (data not shown). In some positions, the amino acid residues forming the pocket of the subsite S_2 of phytolacain R differed from those of papain. The amino acid residues forming the substrate binding S_2 pocket of papain (*20, 24*), Tyr61, Tyr67, Pro68, Trp69, Val133, and Phe207 were replaced by Gly, Trp, Met, His, Ala, and Met in phytolacain R, respectively (Table VI). These substitutions, especially Tyr61→Gly and Val133→Ala, presumably make the S_2 pocket is less hydrophobic in phytolacain R than in papain.

Phytolacain R may react with the Cys(Npys) residue of Phe-Leu-Ser-Cys(Npys)-NH_2 as a P_1' site or a P_1 site,

TABLE VI. **Amino acid residues forming the pocket of subsite S_2 in phytolacain R and papain.**

Enzymes	Position No[a]							
	61	67	68	69	133	157	205	207
Phytolacain R	Gly	Trp	Met	His	Ala	Ile	Ala	Met
Papain	Tyr	Tyr	Pro	Trp	Val	Val	Ser	Phe

[a]Based on the residue numbering of papain.

because it is thought to prefer a hydrophobic residue such as Leu or a small hydrophilic residue such as Ser at position P_2. In brief, it was found that the inhibition by an affinity labeling approach using Npys peptide was effective against the protease activity of phytolacain R or G. The activity of phytolacain G was completely inhibited by the Npys peptide, whereas the caseinolytic activity of phytolacain R remained. Phytolacains R and G thus differ somewhat in substrate specificity.

A cysteine protease has been implicated in the digestive process during the later stages of fruit development (*25*). However, to our knowledge, a comparison of the proteases of ripe and unripe fruits of the same plant has not hitherto been conducted. Phytolacains G and R both originated from the fruits of pokeweed, but differences were observed between then. Nine sites of oxidized insulin B-chain were cleaved by phytolacain G during 20 h of hydrolysis. Six of the sites cleaved by phytolacain G were also cleaved by phytolacain R. The substrate specificity of phytolacain G was broad but resembled that of papain in the preference for a hydrophobic residue at the P_2 position. The N-terminal sequences of phytolacains G and R also differed, but the amino acid residues conserved in the papain family were also conserved in these enzymes (*4*).

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REFERENCES

1. Uchikoba, T., Yonezawa, H., Hamada, T., Matsueda, R., and Kaneda, M. (1995) Isolation of phytolacain G and its inhibition measured by affinity labeling. *Biosci. Biotech. Biochem.* **59**, 1784-1785
2. Kaneda, M., Izumi, S., Fukuda, T., Uchikoba, T., and Tominaga, N. (1988) Isolation and characterization of a protease from *Phytolacca americana*. *Phytochemistry* **27**, 3661-3662
3. Kaneda, M., Nagatome, S., and Uchikoba, T. (1995) Comparison of phytolacain R, a cysteine protease from *Phytolacca americana*, with papain. *Phytochemistry* **39**, 997-999
4. Uchikoba, T., Yonezawa, H., Shimada, M., and Kaneda, M. (1998) Comparison of phytolacain G, a cysteine protease from *Phytolacca americana*, with phytolacain R. *Biosci. Biotech. Biochem.* **62**, 2058-2061
5. Boller, T. (1986) Roles of proteolytic enzymes in interactions of plants with other organism in *Plant Proteolytic Enzymes* (Dalling, M.J., ed.) Vol. 1, pp. 67-96, CRC Press, Florida
6. Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. *J. Gen. Physiol.* **30**, 291-310
7. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
8. Dubois, M., Gilles, K.A., Hamilton, J.K., Robert, P.A., and Smith, F. (1956) Colorimatric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350-356
9. Ruegg, U.T. and Rudinger, J. (1977) Reductive cleavage of cysteine disulfides with tributylphosphine in *Methods in Enzymology* (Hirs, C.H.W. and Timacheff, S.N., eds.) Vol. 47, pp. 111-116, Academic Press, New York
10. Boller, T. (1986) Roles of proteolytic enzymes in interactions of plants with other organisms in *Plant Proteolytic Enzymes* (Dalling, M.J., ed.) Vol. 1, pp. 67-96, CRC Press, Florida
11. Matsueda, R., Umeyama, H., Kominami, E., and Katunuma, N. (1988) Activation of conventional *S*-protecting groups of cysteine by conversion into the 3-nitro-2-pyridinesulfenyl (Npys) group. *Chem. Lett.* 1857-1860
12. Matsueda, R., Umeyama, H., Puri, R.N., Bradford, H.N., and Colman, R.W. (1990) Potent affinity labeling peptide inhibitor of calpain. *Chem. Lett.* 191-194
13. Dayhoff, M.O., Hunt, L.T., McLaughlin, P.J., and Barker, W.C. (1972) Other proteases in *Atlas of Protein Sequence and Structure* Vol. 5, D-121, National Biomedical Foundation, Washington, D.C.
14. Cohen, L.W., Coghlan, V.M., and Dihel, L.C. (1986) Cloning and sequencing of papain encoding cDNA. *Gene* **48**, 219-227
15. Carne, A. and Moore, C.H. (1978) The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*. *Biochem. J.* **173**, 73-83
16. Ritonja, A., Rowan, A.D., Buttle, D.J., Rawlings, N.D., Turk, V., and Barrett, A.J. (1989) Stem bromelain: amino acid sequence and implications for weak binding of cystatin. *FEBS Lett.* **247**, 419-424
17. Rawlings, N.D. and Barrett, A.J. (1994) Families of cysteine peptidases in *Methods in Enzymology* (Barrett, A.J., ed.) Vol. 244, pp. 461-486, Academic Press, New York
18. Jacquet, A., Kleinschmidt, T., Schnek, A.G., Looze, Y., and Braunitzer, G. (1989) The thiol proteinases from the latex of *Carica papaya* L. III. The primary structure of chymopapain. *Biol. Chem. Hoppe-Seyler* **370**, 425-434
19. Dubois, T., Kleinschmidt, T., Schnek, A.G., Looze, Y., and Braunitzer, G. (1988) The thiol proteinases from the latex of *Carica papaya* L. II. The primary structure of Proteinase Ω. *Biol. Chem. Hoppe-Seyler* **369**, 741-754
20. Kowlessur, D., O'Driscoll, M., Topham, C.M., Templeton, W., Thomas, E.W., and Brocklehurst, K. (1989) The interplay of electrostatic fields and binding interactions determining catalytic-site reactivity in actinidin. A possible origin of differences in the behaviour of actinidin and papain. *Biochem. J.* **259**, 443-452
21. Drenth, J., Kalk, K.H., and Swen, H.M. (1976) Binding of chloromethyl ketone substrate analogues to crystalline papain. *Biochemistry* **15**, 3731-3738
22. Keil, B. (1992) *Specificity of Proteolysis. Essential Substrate Residues for Action of Endopeptidases*, pp. 43-228, Springer-Verlag, Berlin, Heidelberg
23. Filippova, I.Y., Lysogorskaya, E.N., Oksenoit, E.S., Rudenskaya, G.N., and Stepanov, V.M. (1984) L-Pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide—A chromogenic substrate for thiol protease assay. *Anal. Biochem.* **143**, 293-297
24. Jaziri, M., Kleinschmidt, T., Walraevens, V., Schnek, A.G., and Looze, Y. (1994) Primary structure of CC-III. The glycosylated cysteine proteinase from the latex of *Carica candamarcensis* Hook. *Biol. Chem. Hoppe-Seyler* **375**, 379-385
25. Lin, E., Burns, D.J.W., and Gardner, R.C. (1993) Fruit developmental regulation of the kiwifruit actinidin promoter is conserved in transgenic petunia plants. *Plant Mol. Biol.* **23**, 489-499

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