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Purification and Characterization of an Antiviral Ribosome-inactivating Protein from *Chenopodium album* L.

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An antiviral protein (CAP30) with ribosome-inactivating activity was purified from the leaves of *Chenopodium album* L. through ammonium sulfate precipitation and column chromatography using S-Sepharose, Blue-Sepharose, FPLC Suprose12 HR, and FPLC Mono-S. The molecular weight of CAP30 was estimated to be 30 kD. CAP30 was thermostable, maintaing its activity even after incubation at 70°C for 30 min, and was stable in the pH range of 6 to 9. In a cell-free *in vitro* translation system using rabbit reticulocyte lysate, protein synthesis was inhibited by the addition of CAP30 with an IC_{50} of 2.26 pM. The comparison of N-terminal amino acid sequences of this protein with the known ribosome-inactivating proteins (RIPs) revealed that it had some sequence homology with PAP-S and PAP-R from pokeweed (*Phytolacca americana*) and dodecandrin from *P. dodecandra*, but had no sequence homology with RIPs from other plants belonging to different orders. The mosaic symptoms on tobacco leaves caused by cucumber mosaic virus infection was completely inhibited by 100 ng/ml of the pure CAP30 protein.

Key words : Chenopodium album *L, antiviral protein, ribosome-inactivating protein, protein synthesis.*

It is well known that some higher plants produce antiviral proteins. Several potent antiviral proteins with ribosome-inactivating activity have been isolated from these plants and characterized.[1-3] Among these, ribosome-inactivating proteins (RIPs) show N-glycosidase activity depurinating a specific adenine residue from ribosomal RNAs of eukaryotes and prokaryotes.[4-5] Contributing to this cytotoxicity, RIPs are believed to be involved in the pathogen-resistant mechanism in plants. It was reported that RIPs isolated from barley and corn inhibit the growth of several plant pathogens and that plants transformed with these RIP genes were resistant to pathogen.[6-8] RIPs isolated from *Phytolacca americana, Mirabilis jalapa,* and *Amaranthus mangostanus* inhibited viral infection to host plants, and the transgenic tobacco- and potato-containing RIP transgenes were resistant to various viral pathogens.[1,2,9,10]

Many different plant species produce RIPs in various organs such as seeds, leaves, and roots. RIP contents also vary from a few μg to several hundred mg per 100 g tissue.[2,11-13] During the course of screening the RIPs, we found that leaves of *Chenopodium album* L. contained considerable amount of RIPs with antiviral activity. In the present paper, an antiviral protein having ribosome-inactivating activity was purified from the leaves of *C. album* L. and characterized for the development of antiviral transgenic crops.

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Abbreviations: AAP29, *Amaranthus* antiviral protein; BCA, bicinchoninic acid; CAP30, *Chenopodium* antiviral protein; CMV, cucumber mosaic virus; EDTA, ethylendiaminetetraacetic acid; FPLC, fast protein liquid chromatography; PAP-R, pokeweed antiviral protein from root; PAP-S, pokeweed antiviral protein from seed; PMSF, phenylmethylsulfonyl fluoride; RIPs, ribosome-inactivating proteins; TCA, trichloroacetic acid.

Materials and Methods

Materials for purification of antiviral protein. Leaves of *C. album* L. were collected in summer, 1996 in Hwa-Seong Gun, Kyeong-Gi Do, Korea. Leaves were kept at -80 °C in a deep freezer before extracting the antiviral protein.

Purification of antiviral protein. Leaves of *C. album,* 1 kg, were mixed with Buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol) containing 50 mM NaCl and 0.2 mM PMSF and were homogenized in a Waring blender. After overnight stirring at 4°C, the extract was filterated with cheese cloth and was centrifuged at 15,000 × g at 4°C. The supernatent was precipitated with 30~80% ammonium sulfate saturation. After centrifugation as described above, the precipitated protein was resuspended in Buffer A and then dialyzed in 10 mM Tris-HCl Buffer (pH 7.5). The crude protein was chromatographed on an S-Sepharose colum (5 × 20 cm), equilibrated, and eluted with a linear gradient of NaCl (0~0.5 M) Buffer A. Fractions having protein synthesis inhibitory activity were pooled and concentrated with 80% ammonium sulfate saturation. The S-Sepharose fraction was applied to a Blue Sepharose column (2.5 × 20 cm) equilibrated with Buffer A and eluted with 20 mM NaCl Buffer A. The active fractions from Blue Sepharose was applied to a Superose 12 HR (25 m*l*) column with FPLC system (Phamacia) and then eluted with Buffer

A. The fractions obtained from Superose 12 HR were dialyzed in Buffer A and then applied to a Mono-S column (1 m*l*) of FPLC system equilibrated with Buffer A. After rinsing the column with the same buffer, proteins were eluted with the same buffer containing linear gradient of NaCl (0~0.5 M). The Superose 12 and Mono-S chromatographies were repeated several times to obtain sufficient proteins for further purification. The active fractions from Mono-S were pooled and lyophillized.

Inhibition of protein synthesis in cell free system. Ribosome-inactivating activity of protein was assayed in a cell-free system with a rabbit reticulocyte lysates purchased from Promega Co. The following supplements were added to the reticulocyte lysate to produce a maximal rate of protein synthesis as described by Walsh *et al.*[14] Different concentrations of protein were added to 17.5 μ*l* recticulocyte, 3.5 μ*l* ribonuclease-free H_2O, 0.5 μ*l* RNase ribonuclease inhibitor (40 u/μ*l*), 0.5 μ*l* amino acid mixture (minus leucine, 1 mM), 2.5 μ*l* [^{14}C]leucine (50 μCi/m*l*), and 1.0 μ*l* of Brome mosaic virus mRNA (0.5 μg/μ*l*) in triplicate. Each sample was incubated at 30°C for 60 min. The labeled amino acid incorporation into protein was determined with the Liquid Scintillation Counter (Beckman LS5801). The protein was precipitated with TCA, and processing was done according to the Promega manual.[15] The concentration required for 50% inhibition (IC_{50}) of incorporation was calculated from the concentration curve.

Polyacrylamide gel electrophoresis (PAGE). Electrophoretic analysis of proteins was carried out using 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels according to the method of Laemmli.[16] Proteins were visualized using Coomassie brillant blue R-250 or silver staining method. For molecular weight determination of proteins, phosphorylase B (M.W. 97,400), bovine serum albumin (M.W. 66,200), ovalbumin (M.W. 45,000), carbonic anhydrase (M.W. 31,000), trypsin inhibitor (M.W. 21,500), and lysozyme (M.W. 14,400) were used as standards.

Protein Determination. Protein concentration was determined using the BCA method,[17] and bovine serum albumin was used as a standard.

Thermal inactivation point and optimum pH determination. The pure antiviral protein diluted in Tris/HCl buffer, pH 7.5, was kept at various temperatures for 30 min and cooled, and ribosome-inactivating activity of the heat-treated samples was assayed using the method described above. The antiviral protein was incubated at 25°C for 15 min at various pH for determination of the optimum pH using 10 mM sodium-acetate (pH 3-5), 10 mM Tris-HCl (pH 6-8), and 10 mM sodium carbonate buffer (pH 9-12).

Protein sequencing. Purified antiviral protein, 4 μg, was electrophoresised, and electroblotted onto the ProBlott®-type membrane (Applied Biosystems) with 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) in 10% MeOH buffer system. Proteins on the ProBlott membrane were stained with Coomassie brilliant blue and destained by soaking in 50% methanol in distilled H_2O. The protein band was excised with a clean razor blade, and the membrane was fully dried. The protein band on the membrane was inserted in the cartridge of a protein sequencer (Applied Biosystem protein sequencer 476A) and analyzed through the liquid phase system.

Assay for antiviral activity. Virus-inoculum containing cucumber mosaic virus (CMV) was extracted from infected tobacco leaves with 10 volume of 50 mM Na-phosphate buffer (pH 7.0). The treated purified antiviral protein in 50 mM Na-phosphate buffer (pH 7.0) mixed with virus inoculum was rubbed on to the leaves of tobacco (*Nicotiana tabacum* cv. Samsun NN) using carborandum (600 mesh) as an abrasive. Only virus inoculum for positive control and buffer for negative control were treated as above. Each treatment was replicated five times and randomized on the leaves of the test plants. Mosaic symtoms were observed at 8, 11, and 15 days after the inoculation.

Results and Dscussion

Protein purification. A ribosome-inactivating protein (RIP) was purified from the crude extracts of *C. album* L. leaves using several different chromatographic techniques such as DE-52 cellulose anion exchange, S-sepharose cation exchange, Blue sepharose, FPLC Superose-12, and FPLC Mono-S column chromatography. After obtaining protein fractions, the presence of RIP was monitored by measuring the inhibitory activity of each fraction against *in vitro* translation of rabbit reticulocyte ribosome. A fraction with RIP activity from the FPLC Superose-12 contained single protein as revealed by SDS-PAGE experiment (Fig. 1). In order to determine the molecular weight, the standard proteins, Ig G (158,000), chick ovalbumin (44,000) and equine myoglobin (17,000) were also applied to FPLC

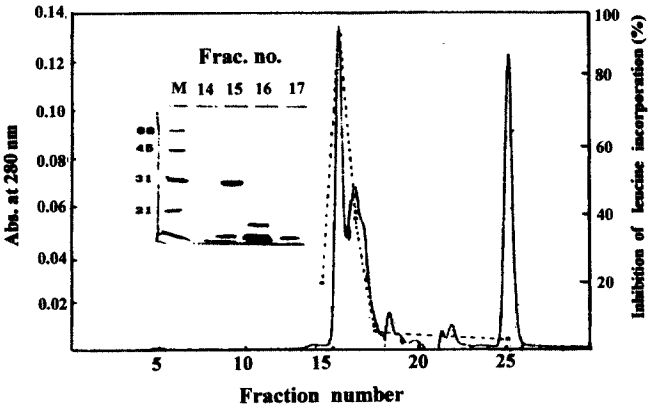

Fig. 1. FPLC Superose 12 column chromatography of the active fraction separated through ammonium sulfate precipitation and column chromatography using DE-52 cellulose, S-Sepharose, and Blue Sepharose from crude extract of *Chenopodium album* L. (—), absorbance at 280 nm; (•···•), inhibition of leucine incorporation in *in vitro* translation system. The insert shows SDS-PAGE profiles of the fractions.

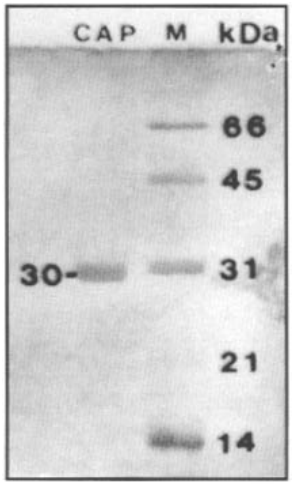

Fig. 2. SDS-PAGE analysis of the purified CAP30. Proteins were visualized by silver staining method. The mobility of CAP30 in SDS-PAGE (12.5%) was determined and compared to the standard proteins. Lane M, mixture of low molecular weight markers; Lane CAP, CAP30 (*Mr* 30,000).

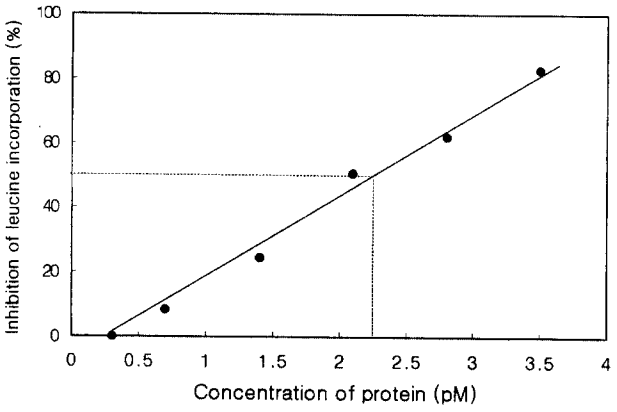

Fig. 3. Inhibition of protein synthesis by CAP30 in a cell-free system. IC_{50} was determined through three replications.

Superose-12 gel filtration chromatography. The fraction with the eluted ribosome-inactivating activity fraction was found between ovalbumin and myoglobin (data not shown). The active fraction was pooled and finally separated through the FPLC Mono-S. From the FPLC Superose 12 chromatography and SDS-PAGE of the purified protein (Fig. 2), RIP from *C. album* was revealed to be a single polypeptide chain with an approximate molecular weight of 30,000. Based on these observations, the purified RIP was named as CAP30. The yield of CAP30 at each separation step is shown in Table 1. The final yield was 10.1% in terms of total RIP activity.

RIPs are, in general, classified into two groups, types 1 and 2, by their structural characteristics. Type 2 RIPs, which are represented by ricin from castor bean, consist of two different peptides and are highly cytotoxic.[18,19] On the other hand, type 1 RIPs are 25-32 kD basic proteins with a single peptide.[13,20-22] Due to their less cytotoxicity and higher inhibitory activity against protein synthesis, they have been utilized for the development of pathogen-resistant plants and immunotoxin drugs.[23] Based on the results obtained from chromatography and SDS-PAGE experiments, we could conclude that CAP30 is included in type 1 RIP group. Type 1 RIPs have been purified from various plant sources. Sairam and Marcil[24] isolated three different type 1 RIPs from *Gelonium multiflorum,* with molecular weights ranging from 29,200 to 31,500, and Irvin *et al.*[25] and Irvin[26] isolated type I RIPs of 27 to 32 kD from *P. americana.*

Inhibition of protein synthesis in cell free system. RIP activity of CAP30 was determined by measuring the inhibition of *in vitro* protein synthesis using rabbit reticulocyte lysate and brome mosaic virus mRNA in the presence of different amounts of CAP30. The concentration of CAP30 causing 50% inhibition of protein synthesis (IC_{50}) was 2.26 pM (Fig. 3). Since IC_{50} of Gelonin and Bryodin were reported to be 0.4[27] and 0.12 nM,[28] respectively, RIP activity of CAP30 is considerably higher than the other type 1 RIPs tested.

Thermostability and optimun pH. Thermal stability and optimum pH of the purified CAP30 were examined. CAP30 was stable up to 70°C heat treatment and pH 7, but was completely inactivated after keeping at 80°C for 30 min (Figs. 4 and 5), suggesting that CAP30 was very thermostable and adapted to a broad pH range. Similar results had been previously reported that RIPs were relatively stable to heat treatment.[13,28]

Table 1. Purification of the antiviral protein, CAP30, from the leaves of *Chenopodium album* L.

Purification steps	Total protein (mg)	IC_{50} (ng/ml)	Specific Activity (10^{-3}units/mg protein)	Total activity (units*)	Yield (%)
Crude extract	14356	105.0	9.52	136.7	100
Ammonium sulfate saturation	6380	51.1	17.86	113.9	83.3
S-Sepharose	294	4.22	237.0	69.7	51.0
Blue Sepharose	23.5	1.05	952.4	22.4	16.4
FPLC Superose12	4.2	0.25	4000	16.8	12.3
FPLC Mono-S	1.1	0.08	12500	13.8	10.1

*One unit is defined as the amount of protein necessary to inhibit protein synthesis by 50% in 1 m*l* rabbit reticulocyte lysate reaction mixture. (IC_{50} = ng of protein/m*l*).

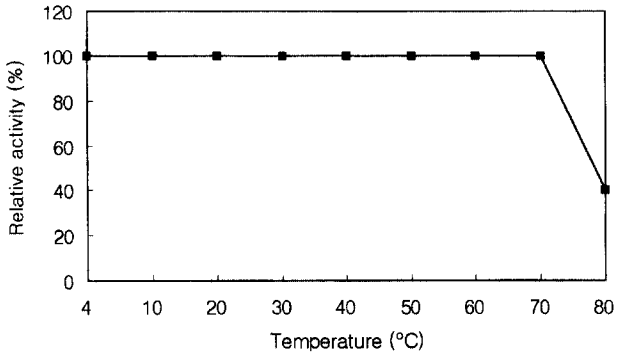

Fig. 4. Effect of temperature on the stability of CAP30. CAP30 (1 ng/m*l*) 20 μl was incubated at various temperature, (4, 10, 20, 30, 40, 50, 60, 70, and 80°C) for 30 min, and the inhibition of protein synthesis was examined.

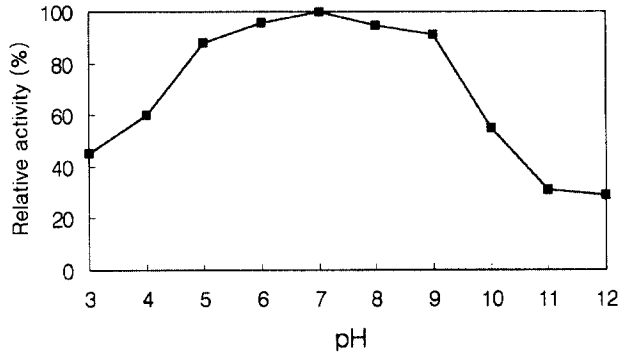

Fig. 5. Effect of pH on the stability of CAP30. CAP30 was incubated at 25°C for 15 min at different pH. The buffers used were 10 mM sodium acetate (pH 3-5), 10 mM Tris-HCl (pH 6-8), and 10 mM sodium carbonate (pH 9-12).

Amino-terminal amino acid sequence of CAP30. Table 2 shows the amino-terminal amino acid sequence of the eight RIPs arranged to maximize their similarities. Identical residues among the proteins are shown in white letter with black background. CAP30 was revealed to have limited homologies with PAP-S,[29] dodecandrin,[29] PAP-R,[30] and AAP29,[1] but was quite different from luffin-a[31] and tricokirin.[32] All the above proteins from *Phytolaccaceae* i.e. PAP-S, PAP-R, PAP and dodecandrin, were more similar to each other than to CAP30 from *Chenopodium*, AAP29 from *Amarnthus*, luffin-a from *Luffa* or tricokirin from *Trichosanthes*, indicating a great phyllogenetic difference between the two orders, *Centrospermales* and *Cucurbitaceae*. It has been pointed out that RIPs from different plant species belonging to the same order are similar as *Phytolacca*, *Chenopodium* and *Amaranthus* belong to *Centrospermales*, and *Luffa* and *Trichosanthes* belong to *Cucurbitaceae*. Ready *et al.*[29] and Bolognesi *et al.*[33] also reported that the similarity of amino-terminal amino acid sequences of RIPs depends on the taxonomically closeness.

Table 3. Antiviral activity of CAP30 against CMV infection on tobacco leaves.

Treatments* (protein ng/ml)		Symptom**		
		8 DAT[1]	11 DAT	15 DAT
1. PAP[2]	1000	-	-	-
2. PAP	500	-	++	+++
3. PAP	250	++	+++	+++
4. CAP30	250	-	-	-
5. CAP30	100	-	-	-
6. CAP30	50	-	-	++
7. CAP30	25	-	+	+++
8. CAP30	12.5	+	++	+++
9. CMV[3] only		+++	+++	+++
10. Buffer solution only		-	-	-

*In each treatment, equal volume of CAP30 solution and CMV extracts were mixed and rubbed on tobacco leaves except for treatments 9 and 10. PAP was used as a reference for the antiviral activity. **Symptom: -, no infection; +, ++, +++, degrees of infection. [1]DAT, days after treatment. [2]PAP, antiviral protein from pokeweed. [3]CMV, cucumber mosaic virus.

Inhibitory activity of CAP30 against virus infection. Effect of CAP30 on the virus infection was tested using bioassay with tobacco and cucumber mosaic virus (CMV). The results showed that concentrations of CAP30 higher than 100 ng/m*l* were strongly inhibitory to CMV infection (Table 3 and Fig. 6), indicating that CAP30 is an antiviral protein with RIP activity.

Table 2. Comparison of N-terminal amino acid sequence of CAP30 with known RIPs.

Name	Amino acid sequence																											
CAP30	1	A	**D**	I	**T**	**F**	K	L	E	P	K	P	**T**	Q	**N**	T	**Y**	N	**T**	**F**	**M**	Q	V	I	**R**	**N**	Q	**A**	27
PAP-S	1	I	N	T	I	T	F	D	A	G	H	A	**T**	I	N	K	**Y**	A	**T**	**F**	**M**	E	S	L	**R**	**N**	E	**A**	27
Dodecandrin	1	V	N	T	I	I	Y	N	V	B	S	T	**T**	I	S	N	**Y**	A	**T**	**F**	**M**	D	N	L	**R**	**N**	E	**A**	27
PAP-R	1	V	N	T	I	I	Y	N	V	G	S	T	**T**	I	S	K	**Y**	A	**T**	**F**	L	N	D	L	**R**	**N**	E	**A**	27
PAP	1	V	N	T	I	I	Y	N	V	G	S	T	**T**	I	S	K	**Y**	A	**T**	K	L	N	D	L	**R**	**N**	E	**A**	27
AAP29	1	A	**D**	L	**T**	**F**	T	V	T	K	D	G	**T**	S	Q	S	**Y**	-	**T**	L	-	N	-	Y	**R**	-	M		26
Luffin-a	1	D	V	R	F	S	L	S	G	S	S	S	T	S	Y	S	K	F	I	G	D	L	R	K	A	L	P		26
Tricokirin	1	D	V	S	F	S	L	S	G	G	G	T	A	S	Y	E	K												16

The protein amino acid sequences were aligned to maximize similarities between proteins. The numbers indicate the number of amino acid residues from N-terminal.

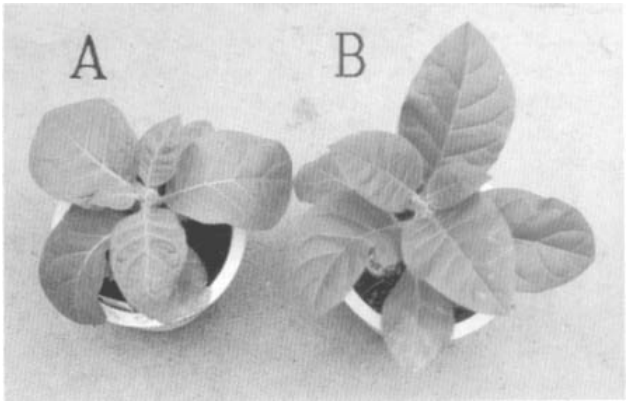

Fig. 6. CAP30 antiviral activity suppressing CMV infection on tobacco leaves. Photograph was taken 15 days after the treatment. A, CMV only; B, CMV+CAP30.

RIP-type antiviral proteins are known to suppress viral amplification by inhibiting protein synthesis of the host cell ribosome after the viral infection.[26,28,34] Therefore, they are effective against broad spectrum of viral pathogens as demonstrated by Chen *et al.*[9] and Kubo *et al.*[35] Since CAP30 is also an RIP-type antiviral protein and shows higher RIP activity as compared to those of other known RIPs tested, it could be a useful agent acting against viral pathogens. Amino acid sequence information obtained in this study would be helpful in isolating a gene encoding CAP30. Studies on the isolation of CAP30 gene and transformation of several crop plants with the gene to develop virus resistant transgenic crops are on going.

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