Antiviral Activity of a Type 1 Ribosome-inactivating Protein from *Chenopodium album* L.

Si-Myung Lee*, Kang-Jin Cho, Yeong-Tae Kim, Hee-young Park, Su-il Kim[1], Young-Soo Hwang and Donghern Kim

Division of Biochemistry, National Institute of Agricultural Science and Technology, Suwon 441-707
[1]Department of Agricultural Chemistry, Seoul National University, Suwon 441-707, Korea

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The antiviral activity of CAP30 from *Chenopodium album*, a type1 ribosome-inactivating protein (RIP), was examined against 5 different plant viral pathogens, and its activity against Tobacco mosaic virus was compared to those of well known antiviral proteins such as Pokeweed Antiviral protein from leaves and seeds. When the inoculating concentration of Tobacco mosaic virus was varied from 0.4 to 400 μg/ml, it was observed that CAP30 at the concentration of 1 μg/ml suppressed the viral infection of *C. amaranthicolor* and *C. quinoa* almost completely up to 40 μg/ml Tobacco mosaic virus. Results from the assays for the inhibitions of *in vitro* translation of rabbit reticulocyte lysate and the suppression of Tobacco mosaic virus infection (10 μg/ml) to *C. quinoa* indicated that CAP30 is a strong inhibitor of protein synthesis and virus infection. The infection of several viruses other than Tobacco mosaic virus to host plants were also inhibited by 5 μg/ml CAP30, suggesting that a gene encoding CAP30 can be used to develop transgenic virus-resistant plants.

Key words: *Antiviral protein, CAP30,* Chenopodium album, *ribosome inactivating protein.*

Ribosome-inactivating protein (RIP), inhibitors of protein synthesis, are widely distributed throughout the plant kingdom.[1,2] The inactivation of ribosome is due to a highly specific RNA N-glycosidase activity of the enzyme. It hydrolyzes a single N-glycosidic bond between adenine and ribose within a highly conserved sequence (5'-AGUACG AGAGG A-3') on the large rRNA of eukaryotes and *E. coli.*[3] This causes the interruption of GTP-dependent binding of the elongation factors to ribosome.[4,5]

Based on their subunit composition, RIPs are divided into two groups: type1 RIPs, consisting of a single polypeptide chain, and type2 RIPs, consisting of A (active) chain and B (binding) chains with lectin properties.[6-8] Even though these two types of RIPs are different from each other in terms of protein structure and functional properties such as cytotoxicity and substrate specificity, both types are believed to protect plants against pathogen infection. In particular, type1 RIPs, represented by pokeweed antiviral protein (PAP) purified from *Phytolacca americana*, have been shown to inhibit viral infection to plants when applied on the surface of plant leaves with viral pathogens.[9,10] PAP, localized in the cell wall matrix, has been hypothesized to be released into the cytosol, causing a localized death of tissue at the site of viral infection when plants are damaged by mechanical forces and vectors (e.g. aphids) transmitting viruses to plants.[10,11] Besides PAP, several RIPs also have been shown to have the antiviral activity presumably by the same hypothetical mechanism.[10,12] However, this hypothesis has yet to be tested, since RIP activity depurinating conspecific rRNA is being questioned. Cereal seed ribosomes were reported to be resistant to the action of endogeneous RIPs.[13-15] On the other hand, type1 RIPs from a variety of dicot plant species have been reported to inactivate conspecific ribosomes, providing evidences supportive to the hypothesis. Regardless of the mechanism by which RIPs inhibit viral infection, such antiviral property of PAP has been utilized to produce virus-resistant transgenic plants by genetic engineering. Lodge *et al.* have shown that transgenic tobacco and potato plants harboring PAP cDNA are resistant against broad spectrum of viruses such as cucumber mosaic virus (CMV) and potato tuber viruses X and Y (PVX and PVY, respectively).[16]

Recently, we purified and characterized an RIP (CAP30) from *Chenopodium album* (Manuscript in preparation). Based on its properties such as its basic pI value and amino acid sequence similarity to other known RIPs, CAP30 was revealed to belong to type1 group. This result may indicate that CAP30 could be used for the biotechnological protection of crop plants against viral infection, but it needs to be confirmed that CAP30 actually inhibits viral infection.

In this article, we present our results on the antiviral

*Corresponding Author
Phone: 82-331-290-0364, Fax: 82-331-290-0391
E-mail: smlee@niast.go.kr

Abbreviations: AMV, alfalfa mosaic virus; BBWV, broad bean wilt virus; CAP30, *Chenopodium* antiviral protein; CMV, cucumber mosaic virus; PAP, pokeweed antiviral protein; PAP-S, pokeweed antiviral protein from seed; PRIP2, pumpukin ribosome-inactivating proteins 2; PVX, potato virus X; RIP, ribosome-inactivating proteins; TMV, tobacco mosaic virus; TuMV, turnip mosaic potyvirus.

activity of CAP30 against six different viruses. For comparison, we examined the ribosome-inactivating and antiviral activities of PAP, PAP-S (RIP from *Phytolacca americana* seeds), and PRIP2 (RIP purified from pumpkin leaves). Results indicate that CAP30 is more potent in the inhibition of *in vitro* translation as well as viral infection.

Materials and Methods

Materials. CAP30 was purified from leaves of *Chenopodium album* by a series of chromatography such as S-sepharose, Blue-sepharose, FPLC mono-S, and FPLC superose 12HR as described in a companion paper (manuscript in preparation). PAP and PAP-S were purchased from Calbiochem Co. (La Jolla, California, USA). Protein concentrations of RIPs were determined by Bradford method with BSA as a standard.

Nicotinia tabaccum cv. Samsun, *Chenopodium amaranthicolor,* and *Chenopodium quinoa* were used as test plants for examining antiviral activities of RIPs. Plants were grown in a greenhouse until either fourth leaf, in the case of tobacco plants, or sixth to eighth leaf, in the case of *C. amaranthicolor* and *C. quinoa*, emerged.

Assay for inhibitory activity on cell-free protein sysnthesis. Ribosome-inactivating activities of CAP30, PAP, and PAP-S were assayed by using Brome mosaic virus RNA and cell-free lysate from rabbit reticulocytes, which was purchased from Promega Co. according to manufacturer's protocol with minor modification. Briefly, RIP was added to the assay solution to make a final volume of 25 μl to the final concentration as designated in Table 3. After 1 h of reaction at 30, the radioactivity incorporated into newly synthesized protein was measured by the use of a liquid scintillation counter.

Virus Preparation. Tobacco mosaic virus (TMV) strain U1 was kindly donated by Dr. Jeong-Uk Chun (Division of Plant Pathology, the National Institute of Agricultural Science and Technology). Plant leaves infected with viruses obtain cucumber mosaic virus (CMV), potato virus X (PVX), alfalfa mosaic virus (AMV), turnip mosaic potyvirus (TuMV) and broad bean wilt virus (BBWV) were donated by Mr. Heung-Soo Choi (Division of Plant Pathology, the National Institute of Agricultural Science and Technology). Virus particles were extracted from infected leaves with 10 vol. of 10 mM phosphate buffer (pH 7.2).

Assay for the antiviral activity. In order to facilitate the virus infection for the assay of antiviral activity of RIPs, carborundum powders were dispersed over the leaf surface of host plants. Then the surface was rubbed gently with a cotton swab wetted with a 1 to 1 (vol/vol) mixture of virus solution and RIP solution. The antiviral activities of RIPs were estimated either by observing the formation of local lesion on the leaf surface co-treated with virus and RIPs for one week or the systemic viral infection to test plants treated for one month.

Prior to comparing the antiviral activity of CAP30 with those of other type1 RIPs, the inhibition of TMV infection at different concentrations by CAP30 was examined. For this purpose, aliquots of 0.4 - 400 /ml TMV were mixed with the same volume of CAP30 (2 μg/ml). The mixture was then applied on one half of the leaves to be tested (*C. quinoa and C. amaranthicolor*). TMV solution without RIPs was also applied on the other half of the leaves as a control. The number and size of local lesions between control and treated sides were compared one week after the inoculation. Twelve leaves of three test plants were infected for each treatment.

Comparison of the antiviral activity of CAP30 to those of PAP and PAP-S was performed as follows. RIPs were diluted with distilled water to adjust the concentrations to 0.125, 0.25, 0.5, 1, and 2 μg/ml and mixed with an equal volume of TMV (10 μg/ml). The mixtures were then applied on leaves (12 leaves for each treatment) of *C. quinoa,* and the number of local lesions were counted after one week.

In order to examine the inhibitory activity of CAP30 against viral infections other than TMV, solutions containing PVX, CMV, AMV, BBWV, and TuMV were diluted 10 times with distilled water and mixed with an equal volume of 5 μg/ml CAP30. For the inoculation, the PVX solution mixed with CAP30 was applied on one half of the tobacco leaves and the other half of the leaves were treated PVX without CAP30. In the case of CMV, the mixture was applied on the leaves of *C. quinoa*. For testing the effectiveness of CAP30 to protect plants against systemic viruses such as AMV, BBWV, and TuMV, the mixtures were applied on the uppermost two leaves of *C. quinoa* at 4 to 6 leaf stage. Twenty-four days after the inoculation, symptoms of viral disease such as leaf-wrinkling and lesion formation were monitored and compared with control plants which were treated with virus only.

Results and Discussion

Ribosome-inactivating proteins have been of considerable interest recently not only due to their therapeutic potential as chimeric toxins,[17] which can be targeted to a cancer cell, but also due to its potential as a candidate for developing virus-resistant plants by genetic transformation. CAP30, which is a recently purified type1 RIP from *Chenopodium album,* could be one of such candidate (Manuscript in preparation). However, to use a gene encoding CAP30 for the genetic transformation it is prerequisite to confirm that CAP30 shows the antiviral activity.

In order to determine the concentration range of virus treatment at which the viral infection is effectively suppressed by RIPs, leaves of *C. quinoa* and *C. amaranticolor* were treated with various concentrations of TMV in the presence and absence of 1 μg/ml CAP30. As shown in Fig. 1, half of the *C. amaranthicolor* leaves treated with 0.4, 4, 40, and 400 μg/ml TMV only formed small necrotic lesions, indicating that the virus successfully

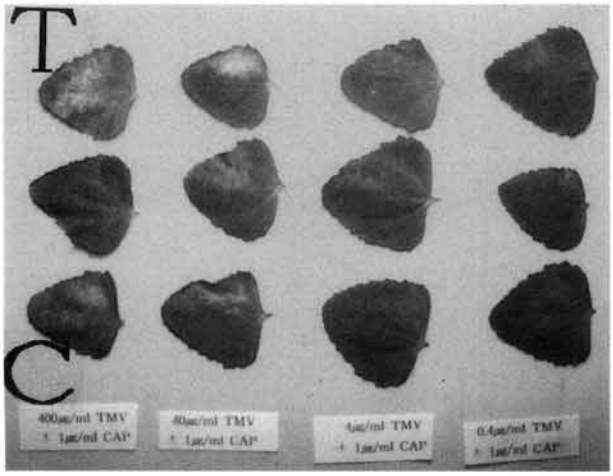

Fig. 1. Photographic illustration of the antiviral activity of CAP30 against TMV infection of *C. amaranthicolor*. Upper half of each leaf was trated with TMV only(designated T) and the opposite half was treated with CAP30(1 μg/ml) pus TMV(designated C). A: TMV concetration 400 μg/ml; B: TMV concetration 40 μg/ml; C: TMV concetration 4 μg/ml; D: TMV concetration 0.4 μg/ml

Table 1. Effects of CAP30 on TMV infection to leaves of two different host plants, *C. amaranthicolor* and *C. quinoa*.

Concentration of TMV (μg/ml)	Number of Local Lesion[1]			
	Chenopodium quinoa		*Chenopodium amaranticolor*	
	TMV only	CAP30 + TMV	TMV only	CAP30 + TMV
400	57±19	54±15	137±23	126±17
40	46±13	2±2	108±21	1±1
4	25±10	0	58±12	0
0.4	15±4	0	17±4	0

Table 2. Effect of CAP30 on *in vitro* translation of rabbit reticulocyte.

Ribosome Inactivating Protein	CAP30	PAP	PAP-S
IC_{50} (pM)	9.2	23	14

1 μg of CAP30, PAP, PAP-S was used for *in vitro* translation assay

infected the host plants. However, the other half of the leaves co-treated with TMV and CAP30 did not show any local lesions, when the concentration of TMV was less than 40 μg/ml. The mean numbers of lesions on the leaves of *C. amaranthicolor* and *C. quinoa* formed by TMV infection are shown in Table 1. According to the table, the number of lesions on the leaves of *C. amaranticolor* and *C. quinoa* formed by the infections of 40 μg/ml TMV were reduced to about 1 and 5% of the control, respectively. Such high suppression of the lesion formation observed in this study indicates that CAP30 appears to be at least as effective as other type1 RIPs such as PAP and Dianthin 32 as an antiviral

Table 3. Effects of CAP30 and other RIPs on TMV infection at different concentrations.

RIP concentration (μg/ml)	Number of Local Lesion[1]		
	CAP30	PAP	PAP-S
2.0	0	11	11
1.0	11	157	42
0.5	42	208	64
0.25	3312	277	196
0.125	4714	5617	5813
TMV only		649	

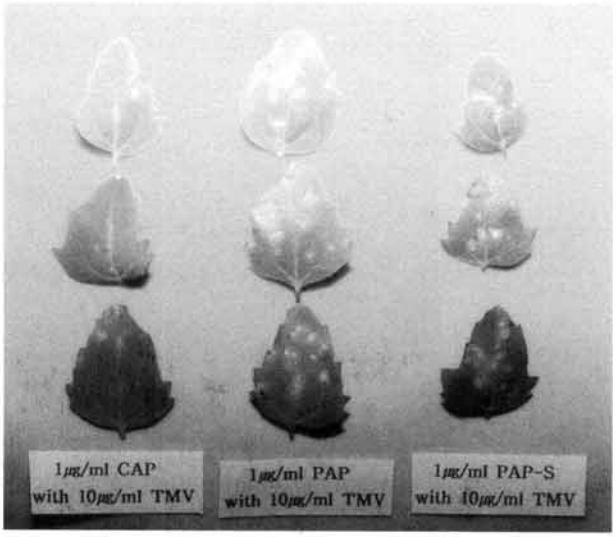

Fig. 2. Comparison of the antiviral activities of CAP30, PAP, and PAP-S against TMV infection of *C. quinoa* leaves. Mixtures of 10 μg/ml TMV and 1 μg/ml RIPs were applied on the leaves.

protein.[18] In order to confirm this possibility, biochemical and antiviral activities of CAP30 were compared to those of other RIPS in terms of the inhibitory activities of *in vitro* translation and TMV infection to leaves of *C. quinoa*. The concentrations of three different RIPs which caused 50% inhibition of *in vitro* protein synthesis (IC_{50}) are shown in Table 2 IC_{50} values of CAP30, PAP, and PAP-S were 9.2, 23, and 14 pM, respectively, indicating that CAP30 inhibits the *in vitro* translation of the rabbit reticulocyte lysate most effectively. In addition, CAP30 is the most effective antiviral agent among them as shown in Table 3 and Fig. 2. CAP30 at 1 μg/ml almost completely inhibited the lesion formation while considerable numbers of lesions were still observed on the leaves co-treated with 10 μg/ml TMV and 1 μg/ml PAP or PAP-S. From these results, it was concluded that CAP30 is a ribosome-inactivating protein with a stronger antiviral activity than PAP and PAP-S.

To develop transgenic plants using an antiviral protein gene, it is important to estimate the spectrum of plant virus of which infection to host plants is inhibited by the protein. In order to achieve this, the effect of CAP30 on the infection of PVX, CMV, AMV, BBWV, and TuMV to test plants were

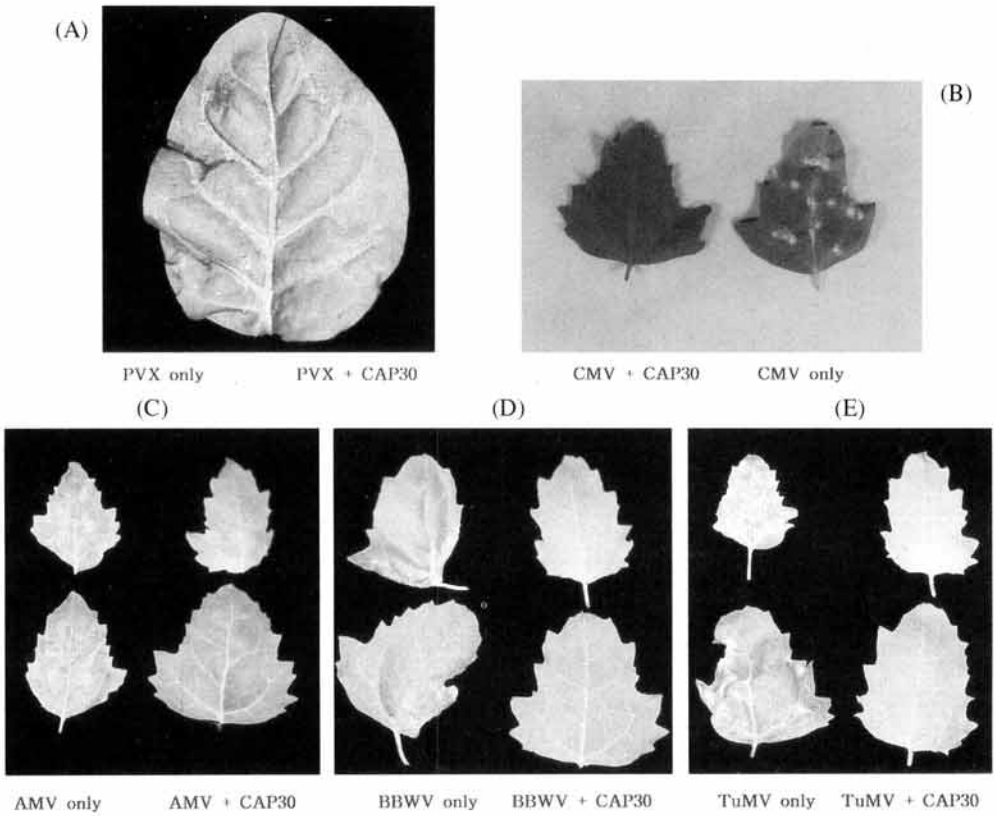

Fig. 3. The inhibitory activity of CAP30 against infections of host plants by PVX(A), CMV(B), AMV(C), BBWV(D), and TuMV(E). The concentration of CAP30 treated was 5 μg/ml. For the systemic infection of C. qunioa, the uppermost leaf of the plants at 4 to 6 leaf stages were rubbed either with the mixture of virus and CAP30 or with virus alone. Twelve leaves were used for each treatment

Table 4. Effects of CAP30 on the development of symptoms of local and systemic viral infections.

Days after Treatment	Virus	CMV		PVX		Days after Treatment	AMV		BBWV		TuMV	
	CAP30	-	+	-	+		-	+	-	+	-	+
3		24	0	0	0	6	-#	-	-	-	-	-
6		56	0	0	0	12	++++	-	+	-	-	-
9		96	8	48	9	18	++++	-	+++	-	+++	+
12		112	16	126	26	24	++++	-	++++	+	++++	++

CMV : cucumber mosaic virus PVX : potato virus X AMV : alfafa mosaic virus
BBWV : broad bean wilt virus TuMV : trunip mosaic potyvirus
#-, +, ++, +++, ++++ : Intensity of symptom

examined in this study. As shown in Fig. 3A, numbers of small necrotic lesions were noticed on one half of tobacco leaves infected by PVX, while no noticeable symptoms on the other half of the leaves co-treated with PVX and 5 μg/ml of CAP30 were observed. Fig. 3B demonstrates that CAP30 at 5 μg/ml protects leaves of *C. quinoa* from the local CMV infection almost completely, while the control leaves treated with CMV only developed several local lesions caused by virus infection. AMV, BBWV, and TuMV infected *C. quinoa* systematically. When they were inoculated on the uppermost leaf of the host plants at 4 to 6 leaf stages, symptoms of systemic viral infections were noticed on newly emerged leaves located at higher position than the virus-infected leaves (Fig. 3C-D). However, such symptoms were not observable when CAP30 was co-treated to leaves with these viruses (Fig. 3C-D). These results indicate that the antiviral activity of CAP30 is effective against broad spectrum of plant pathogenic virues.

The effect of CAP30 treatment against viral infection is primarily due to its activity delaying the development of symptoms of viral infection. As shown in Table 4, the local lesions caused by CMV infection and CMV plus CAP30 co-inoculation were observed at 3 and 9 days after the inoculation, respectively and the number of lesions increased with the time duration. In the case of PVX, the lesions were observable on the leaves treated with the virus plus CAP30 as well as the virus alone. And the number of the lesion caused by the virus and CAP30, which is less than 20% of

that of the lesion by PVX only, also increased at 12 days after the inoculation. Similaly symptoms of systemic viral infection became observable for BBWV and TuMV at the later period of monitoring, even though the intensity of such symptoms were much milder than the controls. From these observations, it was concluded that CAP30 acts against viral pathogen by suppressing the progress of viral infection.

In this study, we attempted to demonstrate that CAP30, a ribosomal inactivation protein from *Chenopodium album* L., has an antiviral activity. This is necessary if one intends to use a gene encoding the protein for the plant transformation toward virus-resistant crops. Based on results obtained through this study, we concluded that the antiviral activity of CAP30 is stronger than the other well-known RIPs and acts against viral pathogens of broad spectrum. In order to develop virus-resistant crops, we have isolated a cDNA clone encoding CAP30, and genetic tranformation using the gene is currently under study.

References

1. Jimenez, A. and Vazquez, D. (1985) Plant and fungal protein and glycoprotein toxins inhibiting eukaryote protein synthesis. *Ann. Rev. Microbiol.* **39**, 649-672.
2. Stripe, F., Barbieri, L., M. Battelli, G., Soria, M. and Lappi, D. A. (1992) Ribosome-inactivating proteins from plants: Present status and future prospects. *Bio/Technology* **10**, 405-412.
3. Endo, Y. and Wool, I. G. (1982) The site of action for -sarcin on eukaryotic ribosomes. *J. Biol. Chem.* **257**, 9054-9060.
4. Endo, Y. and Tsurugi, K. (1988) The RNA N-glycosidase activity of ricin A-chain: The characteristic for the enzymatic activity of ricin A-chain with ribosomes and with rRNA. *J. Biol. Chem.* **263**, 8735-8739.
5. Endo, Y., Tsurugi, K. and Lambert, J. M. (1988) The site of action of six different ribosome-inactivating proteins from plants on eukaryotic ribosomes: the RNA N-glycosidase activity of proteins. *Biochem. Biophys. Res. Commun.* **150**, 1032-1036.
6. Barbieri, L., Aron, G. M., Irivin, J. D. and Stripe, F.(1982) Purification and partial characterization of another form of antiviral protein from the seeds of *Phytolacca americana* L. *Biochem. J.* **203**, 55-59.
7. Lewis, M. S. and Youle, R. J. (1986) Ricin subunit association. Thermodynamics and the role of the disulfide bond in toxicity. *J. Biol. Chem.* **261**, 11571
8. Load, J. J. (1985) Precursors of ricin and *Ricinus Communis* agglutinin glycosylation and processing during synthesis and intracellular transport. *Eur. J. Biochem.* **146**, 411-416.
9. Irivin, J. D. (1975) Purification and partial characterization of the antiviral protein from *Phytolacca americana* which inhibits eukaryotic protein ribosomes. *Arch. Biochem. Biophys.* **200**, 418-425.
10. Chen, Z. C., White, R. F,. Antoniw, J. F. and Lin, Q. (1991) Effect of pokeweed antiviral protein(PAP) on the infection of plant viruses. *Plant Physiol.* **40**, 612-620.
11. Ready, M. P., Brown, D. T. and Reinbothe, S. (1986) Extracellular localization of pokeweed antiviral protein. *Proc. Natl. Acsd. Sci.* USA **83**, 5053-5056.
12. Yoshinari, S., Koresawa, S., Yokota, S., Sawamoto, H., Tamura, M. and Endo, Y. (1997) Gypsophilin, a new type-1 ribosome-inactivating protein from *Gypsophila elegans*: purification, enzymatic characterization and subcellular localization. *Biosci. Biotech. Biochem.* **61**, 324-331.
13. Bonness, M. S., Ready, M. P., Irivin, J. D. and Mabry, T. J. (1994) Pokeweed antiviral protein in activates pokeweed ribosome; implication for the antiviral mechanism. *The Plant J.* **5**, 173-183.
14. Stripe, F. and Hughes, R. C. (1989) Specificity of ribosome-inactivating proteins with RNA N-glycosidase activity. *Biochem J.* **262**, 1001-1002.
15. Prestle, J., Schonfelder, M., Adams, G. and Mundy, K.-W., 1992, Type-1 ribosome-inactivating proteins depurinate plant 25S rRNA without species specificity. *Nuc. Acid. Res.* **20**, 3179-3182.
16. Lodge, J. K., Kaniewski, W. K. and Tumer, N. E. (1993) Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proc. Natl. Acsd.* Sci. USA **90**, 7089-7093.
17. Lin, J. Y., Tsering, K. Y., Chen, Z. C., Lin, L. T. and Tung, T. C. (1970) Abrin and ricin: New anti-tumour substances. *Nature* **227**, 292-293.
18. Stripe, F., Williams, D. G., Onyon, J., Legg, R. F. and Stevens, W. A. (1981) Dianthins, ribosome-damaging proteins with anti-viral properties from *Dianthus caryophyllus* L.(carnation). *Biochem J.* **195**, 399-405.