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Mode of Antiviral Activity of Water Soluble Components Isolated from *Elfvingia applanata* on Vesicular Stomatitis Virus

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A preparation of water soluble components (EA) was made from carpophores of *Elfvingia applanata* (Pers.) Karst and its *in vitro* antiviral activity on vesicular stomatitis virus [(Indiana serotype, VSV(IND)] was investigated by plaque reduction assay. EA exhibited potent antiviral activity on VSV(IND) growth and negligible cytotoxicity on Vero cells, 50% effective concentration (EC_{50}) of 104 μg/ml and 50% cytotoxic concentration (CC_{50}) of 3,793 μg/ml, respectively. Selectivity index (SI, CC_{50}/EC_{50}) of EA on Vero cell and VSV(IND) was about 36.5. EA did not display either a direct virucidal effect on VSV(IND) or induction of antiviral substance by Vero cells upon its treatment. Thus, the mode of antiviral activity of EA was studied at steps of viral adsorption onto cell. When both EA and virus were added to cell monolayers, titer of cell-free virus in culture supernatant increased in ca. 30-40% compared with that of control group and titer of cell-associated virus was 60-100% higher than that of control group. These results suggested that antiviral activity of EA on VSV(IND) might be due to the hindrance of viral entry to cells at either endocytosis or loss of envelope.

Key words: *Elfvingia applanata* (EA), Antiviral activity, Vesicular stomatitis virus [Indiana serotype, VSV(IND)], Endocytosis

INTRODUCTION

As of oriental folk medicine, the carpophores of *Elfvingia applanata* (Pers.) Karst (Polyporaceace) of *Basidiomycetes* have been used in treatment of various ailments including cancers as like the carpophores of *Ganoderma lucidum* (Kim and Kim, 1990) were widely supplemented to cancer patients.

E. applanata seems to be very valuable biomaterial because of the occurrence of biologically active substances such as bitter triterpenoids (Nishitoba *et al.*, 1989), alnusenone and friedelin (Protiva et al., 1980), α-D-glucan and β-D-glucan (Mizuno *et al.*, 1981; Usui *et al.* 1983).

Recently, components that modulate humoral immune response were detected in a fraction passed by DEAE cellulose ion exchange column (FDP) obtained from *E. applanata* (Kim *et al.*, 1994a). In addition to this, antibacterial and antiviral activities of the aqueous extract of *E. applanata* had been reported (Kim *et al.*, 1994b; Rym *et al.*, 1999). And it is worthwhile to mention that a significant toxicity of these components was not detected in the acute toxicity test (Kim *et al.*, 1994c).

Vesicular stomatitis virus (VSV), which causes only slight vesicular lesions in the mouth and rarely causes fatal diseases, has been extensively studied, especially in terms of its replicative mechanism as a model system of the rhabdovirus replication in culture (Nichol, 1994).

Sulfated polysaccharides such as carrageenan, xylofuranan sulfate, ribofuranan sulfate and dextran sulfate have been attractive candidates for possible antiviral drugs because of their potent *in vitro* activities against human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), herpes simplex virus (HSV) and other enveloped viruses (Baba *et al.*, 1988). The activities of these sulfated polysaccharide are linked to the anionic features of the molecules (Marchetti *et al.*, 1995). The mode of antiviral action of these polysaccharides was suggested to be attributed to an inhibition of the entry process of infection including virus binding to the cells, inhibition of virus-cell fusion, or inhibition of both virus-cell binding and fusion (Hosoya *et al.*, 1991).

In this paper, we studied the antiviral activity of the water soluble components (EA) of carpophores of *E. applanata* against VSV(IND) and the possible mode of its antiviral activity.

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MATERIALS AND METHODS

Materials

The carpophores of *Elfivingia applanata* (Pers.) Karst (Polyporaceae) were purchased from a commercial supplier of Cheongju and authenticated by Dr. W. H. Park, Seoul National Industrial University. A voucher specimen (No. CPM 319) has been deposited at the Medicinal Plants Herbarium at Chungbuk Nat'l University. Eagle's minimum essential medium (MEM), trypsin, penicillin, streptomycin and amphotericin B were purchased from Gibco BRL (Gaithersburg, MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and crystal violet were purchased from Sigma Co. (St. Louis, MO, USA). Vero cell (African green monkey kidney cell, ATCC CCL 81) and vesicular stomatitis virus [Indiana serotype, VSV (IND)] ATCC VR-158 were obtained from American Type Culture Collection (Rocksville, MD, USA).

Extraction of water soluble components

The carpophores of *E. applanata* (500 g) were extracted with hot water for 8 h. Steps of concentration of the extract followed by freeze lyophilization yielded ca. 20 g of dark brownish powder, EA.

Cells and virus

Vero cells were cultured with MEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured twice a week. VSV(IND) stock was prepared in Vero cell cultures and stored at -70°C until use. Viral titer of VSV(IND) stock solution was determined by plaque assay.

Cytotoxicity assay

For cytotoxicity assay of EA, Vero cells were seeded in 96 well culture plates (Falcon, NJ, USA) at an initial density of 3.5×10^4 cells per well. The 16-18 h cultured cells in 5% CO_2 at 37°C were received various concentrations of EA and further incubated for 48 h. Viable cell yield was deter-mined by MTT reduction assay (Scubiero *et al.*, 1988). The cytotoxicity was expressed as 50% cytotoxic concen-tration (CC_{50}) that is the concentration of EA needed to inhibit the growth of cells up to 50% by regression analysis.

Plaque reduction assay

The antiviral activity was essentially evaluated by a plaque reduction assay (Shigeta *et al.*, 1992). Host cell monolayers grown in 24 well culture plates (Falcon, NJ, USA) were infected with about 150 pfu of virus per well in presence of various concentrations of EA. After 1 h adsorption period eclipsed, agar overlay medium containing EA at various concentrations was overlaid to cell monolayer. After further incubation of cell culture at 37°C was followed for the formation of viral plaques and enumeration of viral plaques was made. The activity of antiviral agents was expressed as the 50% effective concentration (EC_{50}), the concentration of substance required for reduction of viral plaque up to 50% by regression analysis. Selectivity index of EA was evaluated by dividing the CC_{50} with the EC_{50}.

Virucidal effect of EA

Host cell independent virucidal effect of EA was examined as follows. Virus stock solution ($>10^9$ pfu/ml) was mixed with various concentration of EA, 0.2, 1.0 and 2.0 mg/ml, and the virus-EA mixture was incubated at 5% CO_2, 37°C for 1, 2 and 4 h, and titers of survived virus were determined by the plaque reduction assay in Vero cells (Barnard *et al.*, 1993).

Antiviral activity in preincubation

Antiviral activity of Vero cells pretreated with EA was examined by the method (Katz *et al.*, 1991) with a slight modification as followed. Prior to VSV infection, Vero cells in the medium containing EA at 0.1, 0.5 and 1.0 mg/ml at 37°C were incubated for 1, 2 and 24 h. Then, the cells were rinsed with phosphate buffered saline (PBS, pH 7.4) and infected with virus. After incubation of the cell culture at 5% CO_2, 37°C, enumeration of viral plaques was made. Prolong exposured (PE) cell group to EA was made as follows. Confluent monolayer of cell was cultured in a medium containing EA and washed with PBS (pH 7.4). And the cells were infected with VSV and agar overlay medium containing EA at various concentrations was overlaid on the cells. After adsorption of virus by the cells for 1 h, plaque reduction assay was performed.

One-step growth curve

The growth characteristics of VSV(IND) were examined by one-step growth experiment (Burleson *et al.*, 1992; Fenner *et al.*, 1987). Vero cell monolayers grown in 35-mm culture dishes were infected with VSV(IND) at a multiplicity of infection (MOI) of 5~10 pfu/cell at 37°C. After viral adsorption for 30 min, the unadsorbed viruses in culture supernatant were removed by washing with PBS (pH 7.4), and then fresh medium containing either EA of 0.2 mg/ml or Interferon alpha (IFN-α, Boehringer Mannheim, Mannheim, Germany) of 200 IU/ml was added to the cell monolayer. At various time interval of post-infection, an aliquot of culture supernatant was subjected to virus titration by plaque forming assay and this was

scored as cell-free virus. The culture medium was removed and cells were disrupted by freezing at -70°C and thawing three times. Cell debris was removed by low-speed centrifugation (1000 × g) and an aliquot of disrupted cell solution was subjected to virus titration. And this was considered as cell-associated virus. The total virus at any given time point is the sum of the cell-free and the cell-associated virus. One-step growth curve was constructed with virus titer at various time-intervals.

Assay of viral adsorption onto cell

The effect of EA on adsorption of VSV(IND) to Vero cells was examined by the methods (Burleson *et al.*, 1992; Fenner *et al.*, 1987) with slight modificaton. Vero cell monolayers in 35-mm culture dishes were infected with virus (MOI=5~10) and EA (0.0, 0.2, 1.0 mg/ml). At various times at post-infection, an aliquot of culture supernatant and of disrupted cells are subjected to titration of cell-free virus and cell-associated virus, respectively. Titers of cell-free virus and cell-associated virus were determined by plaque assay.

Statistical analysis

The data were expressed as mean ± S.D. The statistical significance of the difference between mean values was determined by student's *t*-test.

RESULTS AND DISCUSSION

To investigate the antiviral activity of water soluble components of *E. applanata*, EA was prepared from the carpophores of *E. applanata* with hot water. As shown in Table I, EA exhibited potent antiviral activity against VSV(IND) grown in Vero cell, but negligible cytotoxicity on Vero cell. An EC_{50} of EA on plaque formation of VSV (IND) was 104 μg/ml whereas its CC_{50} on Vero cell was 3,792 μg/ml. Therefore, EA seemed to be a good antiviral substance against VSV(IND) with SI of 36.5. Prior to one-step growth experiment and investigation of mode of antiviral activity of EA, virucidal effect of EA and capacity of EA-pretreated Vero cell supporting virus propagation were examined. After a few hour incubation of virus particles with various concentration of EA, plaque assay of each preparations revealed insignificant difference in the survival viral titers in a dose-dependent manner between EA-treated and non-treated groups as shown in Table II. These results suggested that EA seemed not to display direct killing on VSV(IND).

Since there is a report that *Ganoderma tsugae* mycelium enhances splenic natural killer cell activity and serum IFN production in mice (Won *et al.*, 1992), we examined whether the antiviral effect of EA is due to the induction of inadequate state of VSV(IND) propagation in Vero cells. However, capacity of the EA-pretreated Vero cells supporting VSV multiplication was not significantly varied from the non-treated group while the prolong exposured group (PE) to EA showed reduction of propagation of VSV(IND) in a dose-dependent manner as seen in Table III. These results suggested that EA seemed not to influence Vero cell capacity supporting viral propagation, not like interferon treated-cells became an antiviral state. This was also confirmed by one-step growth curve of VSV

Table I. Antiviral activity of EA on vesicular stomatitis virus (Indiana serotype) in Vero cells by plaque reduction assay

Substance	CC_{50}[b] (μg/ml)	EC[c] (μg/ml)			SI[d]
		EC_{50}	EC_{70}	EC_{90}	
EA[a]	3,793	104	300	869	36.5

The values represent the means of quadraplicate experiments.
[a]Water soluble components of *Elfvingia applanata*. carpophores.
[b]50% cytotoxic concentration(CC_{50}) is the concentration of EA required to show 50% cytotoxic effect.
[c]Effective concentration(EC) is the concentration of antiviral substance required to reduce plaque formation of the virus by 50 %, 70% and 90%.
[d]Selectivity index (SI) = CC_{50}/EC_{50}

Table II. Virucidal effect of EA on vesicular stomatitis virus (Indiana serotype)

EA[a] (mg/ml)	Plaque forming unit (PFU/ml)		
	1 h	2 h	4 h
0.0	114 ± 10	54 ± 6	38 ± 6
0.1[b]	83 ± 8	40 ± 6	29 ± 4
0.5	85 ± 10	44 ± 3	25 ± 5
1.0	112 ± 4	55 ± 5	23 ± 1

The results were expressed as mean ± S.D. of quadraplicate experiments.
[a]Water soluble components of *Elfvingia applanata*. carpophores
[b]Viral suspension was mixed with various concentrations of EA (0.1, 0.5 and 1.0 mg/ml) and the mixture was incubated for hours. The viral titer in the mixture was measured as plaque assay in Vero cells.

Table III. Antiviral activity of in preincubation of Vero cells with EA prior to infection of vesicular stomatitis virus

EA[a] (mg/ml)	Plaque forming unit (PFU/ml)			
	PE[b]	1 h[c]	2 h	24 h
0.0	161 ± 15	161 ± 9	161 ± 10	161 ± 10
0.1	89 ± 3*	137 ± 4	131 ± 11	141 ± 5
0.5	31 ± 9**	128 ± 5	160 ± 9	147 ± 6
1.0	6 ± 1***	149 ± 11	144 ± 13	129 ± 11

The results were expressed as mean ± S.D. of quadraplicate experiments.
[a]Water soluble components of *Elfvingia applanata* carpophores
[b]PE represents prolonged exposure of cells to EA, pre-and post-virus infection.
[c]Preincubation time of cells with EA prior to virus infection.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ (Student *t*-test)

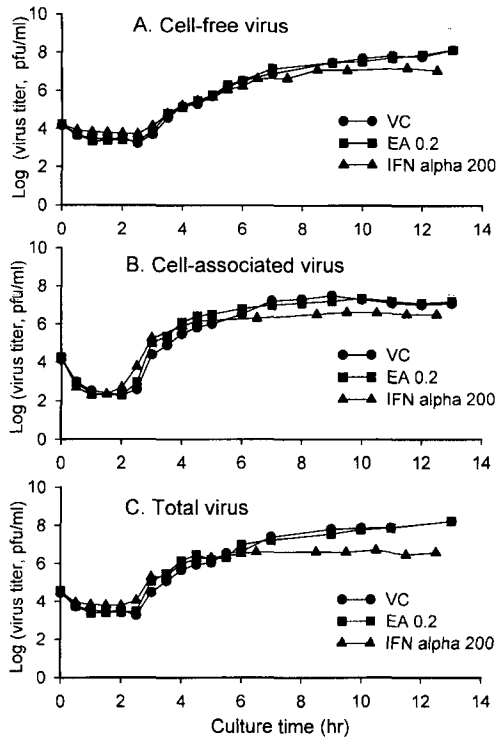

Fig. 1. Effect of EA and IFN alpha on the one-step growth of vesicular stomatitis virus (Indiana serotype). The cells in EA-group were cultured with 0.2 mg/ml EA and the cells in IFN alpha group were treated with 200 IU/ml for 1 h prior to virus infection. Virus titers were expressed as logarithmic scale of plaque forming unit (pfu).

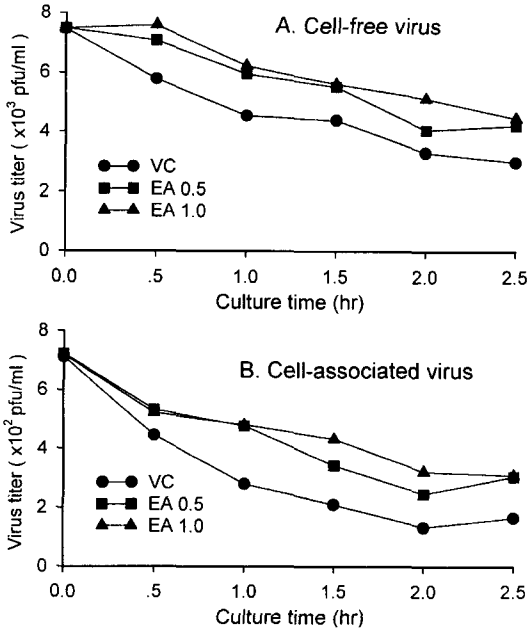

Fig. 2. Effect of EA on the adsorption of vesicular stomatitis virus (Indiana serotype) to Vero cells. Cells were infected with virus in the presence of EA (0.5 and 1.0 mg/ml) and cultured for given times. Cell-free virus and cell-associated virus titer were expressed as plaque forming unit (pfu).

(IND) replication in the presence of either EA or interferon as shown in Fig. 1. The VSV(IND) titer increased sharply between 2.5 and 7 h of post-infection and viral titers were level-off with approximately 2×10^8 pfu/ml. The adsorption and eclipse periods of VSV(IND) seemed to be 30~60 min and 120~150 min, respectively. One-step growth curve of VSV(IND) in the presence of EA was dissimilar to that in the presence of interferon. This result suggests that antiviral activity of EA would be distinct from that of substances induced upon viral infection, such as IFN or IFN-like materials.

The chemical nature of EA was mainly of polysaccharides and proteins. It was reported that the antiviral or antitumor constituents of the hot water extract of *E. applanata* are polysaccharides of β-(1→3), (1→4) linked-D-glucose (Sasaki *et al.*, 1971). The antiviral activity of polysaccharides seems to be associated with the anionic feature of the molecules, interfering the viral attachment and penetration to host cells during early stages of viral replication cycle (Marchetti *et al.*, 1994).

Vesicular stomatitis virus, a member of Rhabdoviruses, is an enveloped virus with extensive and complex lipid envelope surrounding the nucleocapsid. The infection of an enveloped virus to animal cells is proceeded to uptake of an enveloped virion by endocytosis, loss of viral envelope, and uncoating nucleocapsid prior to processes of virus multiplication. To examine which steps of VSV(IND) replication are affected by EA, viral titers during propagation in the cell monolayers in the presence of EA were measured as depicted in Fig. 2. Titers of cell-free virus in the culture supernatant with EA were about 30~40% higher than that of control group. And titers of cell-associated virus were also higher about 60~100% than that of control group. These results suggest that EA causes to reduce virus particles being endocytosised and casted-off the envelope by host Vero cells.

In conclusion, the antiviral activity of EA seemed to be due to the inhibition of the entry process including endocytosis, loss of viral envelope and nucleocapsid uncoating of VSV(IND) onto Vero cells. Main disturbance by EA on VSV(IND) replication may be due to inhibition of glycoprotein G binding of VSV(IND) to host cell surface (White and Fenner, 1994).

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