Antiviral Research, 17 (1992) 323–333

AVR 00535

Yucca leaf protein (YLP) stops the protein synthesis in HSV-infected cells and inhibits virus replication

Kyoko Hayashi[1], Hiroko Nishino[1], Seihachiro Niwayama[1], Kimiyasu Shiraki[1] and Akira Hiramatsu[2]

[1]*Department of Virology, Toyama Medical and Pharmaceutical University, Sugitani, Toyama,* and [2]*Department of Resource Biology, Faculty of Agriculture, Ibaraki University, Ami-machi, Inashiki-gun, Ibaraki, Japan*

(Received 12 March 1991; accepted 23 September 1991)

Summary

Yucca leaf protein (YLP), an inhibitor of tobacco mosaic virus isolated from the leaves of *Yucca recurvifolia* Salisb., exhibited potent activity against herpes simplex virus type 1 (HSV-1) with no cytotoxicity below 300 μg/ml. The inhibitory dose was varied with the time of addition; 50% effective concentrations (ED_{50}) of YLP were 3, 19 and 95 μg/ml when YLP exposure was begun 3 h before virus infection, 0 h and 3 h after infection, respectively. This protein also inhibited the multiplication of herpes simplex virus type 2 and human cytomegalovirus. YLP has been shown to have a weak virucidal activity at higher concentrations. Analysis of early events following infection showed that YLP affected viral penetration in HeLa cells but did not interfere with adsorption to the cells. YLP was found to exert strong inhibition of protein synthesis in virus-infected cells but not in uninfected cells. This selective effect can be considered to attribute mainly to the antiviral activity of YLP.

Yucca leaf protein; Anti-HSV activity; Viral penetration; Protein synthesis inhibition

Introduction

Almost all antiherpes agents such as acyclovir (Elion et al., 1977) and bromovinyl deoxyuridine (De Clercq et al., 1979, 1980) are analogs of normal

Correspondence to: Kyoko Hayashi, Dept. of Virology, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama, Japan.

nucleosides and act via one or both of two enzyme targets, thymidine kinase and DNA polymerase. The development of acquired resistance to these chemical inhibitors has become a troublesome problem associated with herpes simplex virus (Wade et al., 1983; McLaren et al., 1985; Ellis et al., 1987).

A protein, named 'yucca leaf protein' (YLP) is purified from leaves of *Yucca recurvifolia* Salisb. and shown to be a basic simple protein with 208 amino-acid residues and molecular weight of 23000 (Osawa and Hiramatsu, 1987). This substance has been reported to have a preventive effect on tobacco mosaic virus (TMV) infection (Hiramatsu et al., 1987). In the present report, we describe the activity of YLP against HSV-1.

Materials and Methods

Preparation of YLP

YLP was purified from leaves of *Yucca recurvifolia* Salisb., as described elsewhere (Osawa and Hiramatsu, 1987).

Cells and viruses

HeLa and Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 6% fetal bovine serum (FBS) for use in growth of virus or plaque assay. Human embryonic lung (HEL) cells were cultured in MEM containing 10% FBS. HSV-1 strain HF was propagated at a low multiplicity and plaque-assayed on HeLa or Vero cell monolayers, HSV-2 strain UW-268 on Vero cells and human cytomegalovirus (HCMV) strain Towne on HEL cells.

Cytotoxicity assay

For growth inhibition studies, 2×10^4 cells in 0.5 ml MEM plus 6% FBS were seeded into each well of 24-well plates, cultured for 24 h at 37°C, and allowed to grow for additional 72 h in the presence of increasing amounts of the protein. After the medium was removed, cells were trypsinized and the cell number was determined by a conventional haemocytometer using the trypan blue-exclusion method (McLimans et al., 1957). The inhibition data were plotted as dose-effect curves (not shown), from which the 50% inhibitory doses (ID_{50}) were obtained.

Antiviral activity

HeLa or Vero cell monolayers in 24-well culture plates were washed and infected with HSV-1 or HSV-2 at a multiplicity of infection (MOI) of 0.2, adsorbed for 1.5 h at room temperature, and refed with maintenance medium

(MEM plus 2% FBS) containing various concentrations of YLP. Cultures were incubated for 24 h at 34°C in 5% CO_2. Similarly, HEL cells infected with HCMV at an MOI of 0.2 were treated with the protein for 48 h. The harvested cultures were disrupted by three cycles of freezing and thawing. Viral yields were determined by plaque assay. Each concentration was assayed at least twice. The antiviral activity was expressed as 50% (ED_{50}) or 90% (ED_{90}) effective dose for viral replication which was the lowest drug concentration of reducing plaque numbers by 50% or 90% in the treated cultures as compared to untreated ones.

Inhibition of virus adsorption to cells

HeLa cells were grown to confluency in 24-well plates. YLP was added to the dishes for 3 h at 37°C. The cells were washed with phosphate-buffered saline (PBS), pH 7.2 and inoculated with HSV-1 at an MOI of 1 for 1.5 h at room temperature in the absence or presence of YLP. The inocula were recovered, dishes were washed twice with PBS, and each wash was harvested. The unadsorbed viruses in these samples were assayed by plaque titration in 10-mm^2 dishes.

Assay for rate of virus penetration

Virus penetration was measured by inactivation of unpenetrated viruses with a low-pH buffer as described by Huang and Wagner (1964) and modified by Highlander et al. (1987). HeLa cell monolayers in 35-mm dishes were pretreated with the protein for 3 h at 37°C. After washing with PBS, the cells were inoculated with HSV-1 (200 PFU/0.1 ml per dish) and incubated for 1 h at 4°C. The dishes were washed twice with PBS, added 1 ml of MEM plus 2% FBS and shifted to 37°C. Every 30 min, each dish was treated with 1 ml of citrate buffer, pH 3.0, for 1 min. After washing twice with PBS, the monolayers were overlaid with 0.5% methylcellulose. After incubation for 2 days, the monolayers were stained with 0.06% crystal violet in 20% EtOH to count the number of plaques.

Preparation of antiserum

Rabbit antiserum was prepared by the procedure according to the method described by Showalter et al. (1981) with a slight modification, i.e. HeLa cells were infected with HSV-1 at an MOI of 1 for 1 h at room temperature, incubated for 18 h at 34°C, harvested, submitted to three cycles of freezing-thawing and centrifuged at 3000 × *g* for 10 min. 0.5 ml of the supernatant (10^8 PFU/ml) per animal was used as an immunogen. The immunization schedule for rabbits used a primary injection of the sample (a 1:1 emulsion of antigen in Freund's complete adjuvant) in five subcutaneous sites along the back. Booster immunizations were at 7, 14 and 21 days in Freund's incomplete adjuvant. Rabbits were bred at day 45.

Tran ^{35}S-labeling of cells and analysis of labeled protein

Vero cells with or without pretreatment with YLP for 1 h or 3 h were mock-infected or infected with HSV-1 at an MOI of 0.2 or 10 at 4°C and radiolabeled immediately after infection. In one experiment, cells were infected with UV-inactivated HSV-1 at the amount corresponding to an MOI of 10. After washing and replenishing the monolayer with methionine-free medium, 10 μCi of Tran ^{35}S-label (1000 Ci/mmol; ICN Biomedicals, Inc.) was added per 60-mm dish and incubated for 4 h at 37°C. Infected cells were harvested and extracted with RIPA buffer (0.05 M Tris-HCl, pH 7.0, 0.15 M NaCl, 1% SDS, 1% Triton X-100) followed by centrifugation at 25000 rpm for 1 h at 4°C. An aliquot of the cell lysates was treated with rabbit antiserum and protein A Sepharose CL-4B overnight at 4°C with rocking. The immunoprecipitates and the cell lysates were analyzed by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis the gels were soaked in 1 M sodium salicylate for 30 min, dried and exposed to X-ray films (Sambrook et al., 1989).

Results

Inhibition of cell growth and viral replication by YLP

After 72 h of treatment at 37°C, concentrations of YLP below 300 μg/ml showed no inhibition of HeLa cell growth. At the highest concentration tested (1000 μg/ml), 87% inhibition of cell growth was seen, the ID_{50} calculated being 595 μg/ml, as shown in Table 1. The ID_{50}s for Vero and HEL cells were 492 and 958 μg/ml, respectively.

To examine the effect of time of addition on the reduction of virus yield, YLP was added to the medium 3 h pre-infection, immediately after infection (0 h) or 3 h post-infection. When added 3 h before and then throughout incubation, YLP was found to be effective in suppressing viral replication and the ED_{50} was 3 μg/ml (Table 1). YLP was less effective when added at 0 and 3 h post-infection and showed the ED_{50}s of 19 and 95 μg/ml, respectively. The in vitro therapeutic indices determined by dividing the ID_{50} by the ED_{50} were 198, 31 and 6 when added 3 h before, 0 h and 3 h after virus infection. These results suggest that YLP might interfere with the early events of virus replication such as adsorption and/or penetration of virus. The replication of HSV-1 in Vero cells was also inhibited by YLP at almost same degree as that in HeLa cells, the ED_{50} being 16 μg/ml. Furthermore, YLP exerted anti-HSV-2 and anti-HCMV activities, where the therapeutic indices were somewhat lower compared with the value for HSV-1. ED_{90} values obtained from each experiment was usually 4 to 13 times higher than ED_{50} values.

TABLE 1

Effect of YLP on the cell growth and replication of virus

Virus	Host cell	Time of addition (h)	Cytotoxicity ID_{50} (μg/ml)	Antiviral activity		Therapeutic index (ID_{50}/ED_{50})
				ED_{50} (μg/ml)	ED_{90} (μg/ml)	
HSV-1	HeLa	−3	595 ± 21	3 ± 0.6	22 ± 2.2	198 ± 47
		0	595 ± 21	19 ± 2.0	168 ± 24	31 ± 2.1
		+3	595 ± 21	95 ± 2.2	419 ± 13	6 ± 0.1
HSV-1	Vero	0	492 ± 18	16 ± 2.6	204 ± 23	31 ± 3.8
HSV-2	Vero	0	492 ± 17	21 ± 1.5	213 ± 5	23 ± 2.6
HCMV	HEL	0	958 ± 59	71 ± 7.8	430 ± 21	13 ± 1.5

For cytotoxicity assay, cells were treated with YLP for 72 h at 37°C. In the assay for anti-HSV-1, -HSV-2 and -HCMV activities, infected cells were harvested after incubation at 34°C for 24, 24 and 48 h, respectively. YLP was added 3 h before (−3 h), immediately after (0 h) or 3 h after infection (+3 h). Each value represents the mean ± SD of two independent experiments.

Effect of YLP on virus adsorption and penetration

To determine whether YLP has any effect on adsorption, or inhibition of virus binding to cells was measured after pretreatment of HeLa cells with 5, 50 or 200 μg of YLP per ml for 3 h at 37°C. Inocula harvested after 1.5 h adsorption at room temperature and washes were subjected to plaque assay. The numbers of viruses which were not bound to monolayers were then compared with those recovered from untreated cells. Table 2 shows the remaining infectivity in each sample. YLP did not interfere with an attachment to cell membranes in both absence and presence of YLP during virus infection.

Inhibition of virus penetration is one of possible steps which might be affected by this protein. In the penetration assay employed in our experiments, the kinetics of penetration could be determined by inactivating the extracellular viruses with a low-pH citric acid buffer at various times after temperature shift

TABLE 2

Effect of YLP on HSV-1 adsorption

Material	Residual infectivity (PFU/ml) × 10^{-4}						
	Untreated	Pretreated					
		5 μg/ml		50 μg/ml		200 μg/ml	
		−	+	−	+	−	+
Inoculum	20 ± 2.8	22 ± 1.0	23 ± 1.0	19 ± 1.0	23 ± 5.6	20 ± 1.0	20 ± 2.2
First wash	0.7±0.13	0.7±0.06	0.7±0.09	0.7±0.09	0.8±0.04	0.7±0.13	0.6±0.04
Second wash	0.2±0.06	0.2±0.06	0.2±0.05	0.2±0.01	0.2±0.06	0.2±0.01	0.2±0.08

HeLa cells were pretreated with YLP for 3 h at 37°C, then washed and infected with HSV-1 at an MOI of 1 in the absence (−) or presence (+) of YLP. After 1.5 h adsorption at room temperature, inocula were harvested and the monolayers were washed twice with PBS. Each wash was also harvested and non-adsorbed viruses were titered by plaque assay. Values are means ± SD of two independent experiments.

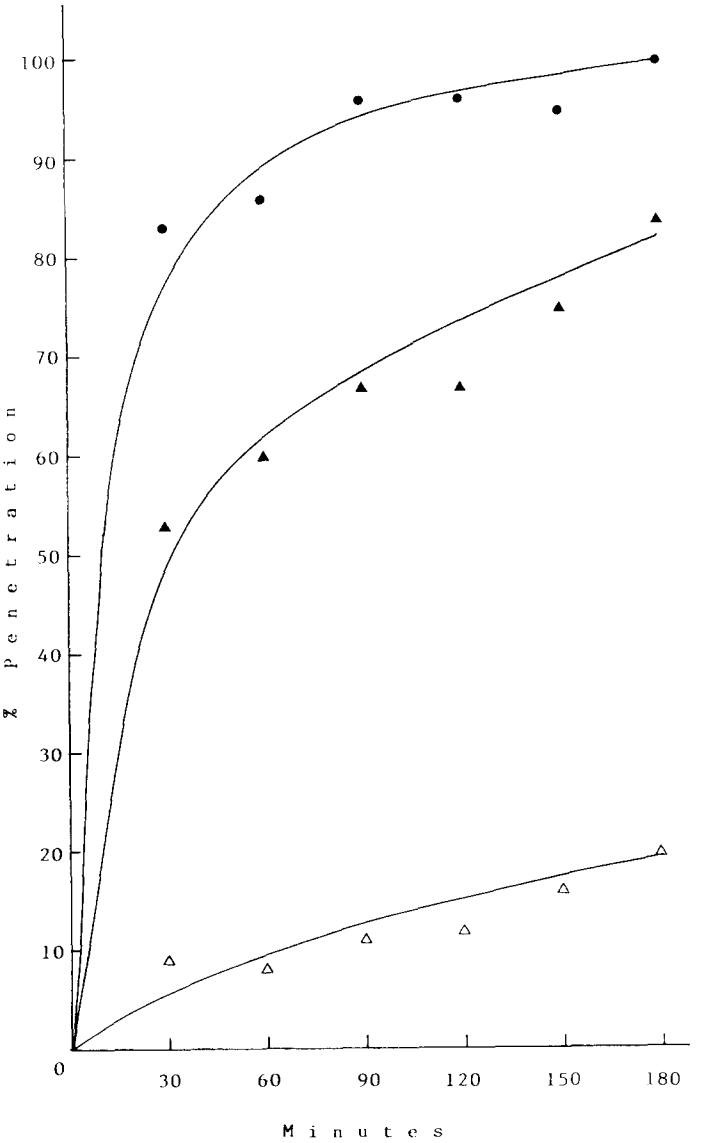

Fig. 1. Effect of YLP on HSV-1 penetration. Appropriate dilutions of virus were adsorbed for 1 h at 4°C on HeLa cells pretreated for 3 h with YLP (●, 0 μg/ml; ▲, 50 μg/ml; △, 200 μg/ml). Then cultures were washed with PBS, shifted to 37°C and treated with citric acid buffer for 1 min at 30-min intervals. Survived virus plaques were scored after 2 days. The results were expressed as % penetration of virus on untreated cells as 100%. Each value is mean of two independent experiments.

from 4°C to 37°C. The results are shown in Fig. 1, where each value represents the average of two experiments. The cells pretreated with YLP inhibited virus penetration by about 20% or 80% at the concentration of 50 or 200 μg/ml, respectively. These data suggest that YLP exerts its effect partially by preventing virus penetration of the host cell surface.

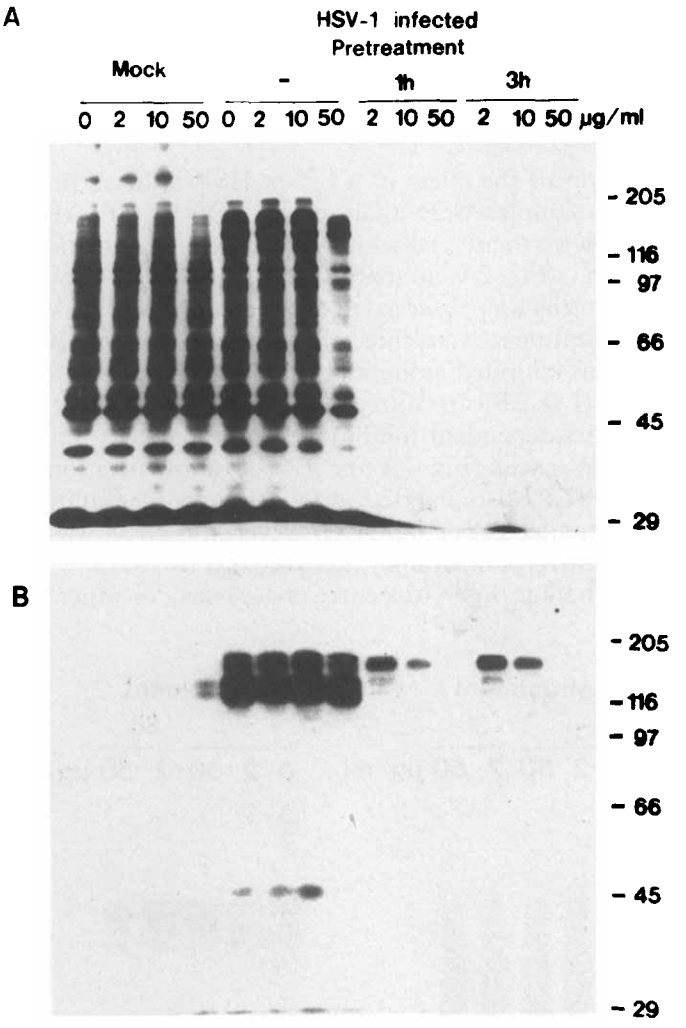

Fig. 2. Autoradiographic comparisons of electrophoretically separated ^{35}S-labeled proteins of Vero cells. Mock-infected or HSV-infected (MOI of 10) cells were incubated at 37°C in the absence or presence of 2, 10 and 50 μg/ml of YLP, and labeled with Tran ^{35}S-label from 0 to 4 h post-infection. When cells were pretreated with YLP, YLP was added from 1 h or 3 h before infection and throughout incubation. Solubilized cell lysates (A) and immunoprecipitates (B) prepared from the cell lysates using antiserum were subjected to electrophoresis on 8% polyacrylamide gel. The gels were then processed for fluorography.

Direct virucidal effect

In order to determine the effect of YLP on the inactivation of virus particles, YLP was diluted in MEM to provide final concentrations of 50 and 200 μg/ml. Virus suspension was added to the solution and incubated for 8 h at 37°C. Samples were harvested in small quantity of the mixtures every 2 h after

incubation and titered in HeLa cells. YLP exerted no effect on the infectivity of HSV-1 at 50 μg/ml. At 200 μg/ml the infectivity was reduced only slightly at 8-h incubation (0.27 log reduction).

Effect of YLP on protein synthesis

In order to determine the effect of YLP on HSV-induced protein synthesis, ^{35}S-labeled protein samples were analyzed by SDS-PAGE. Mock-infected or virus-infected cells were incubated with YLP at the concentrations of 2, 10 and 50 μg/ml. As shown in Fig. 2A, no marked inhibition of the protein synthesis of mock-infected host cells was observed at these concentrations. When Vero cells without YLP pretreatment were infected with virus at an MOI of 10, virus protein synthesis was inhibited moderately at 50 μg/ml but not so markedly at 2 and 10 μg/ml YLP (Fig. 2B). In contrast, in the cells pretreated with YLP for 1 h or 3 h, strong dose-dependent inhibition of both cellular and virus-induced protein syntheses was seen (Fig. 2A and 2B). At a low titration of virus (MOI of 0.2) infected, YLP also exerted dose-dependent inhibition of protein synthesis in the pretreated cells (Fig. 3). However, this effect was not so striking as that in the cells infected with high titer of MOI of 10.

To make clear whether only virus entry is necessary or other virus functions

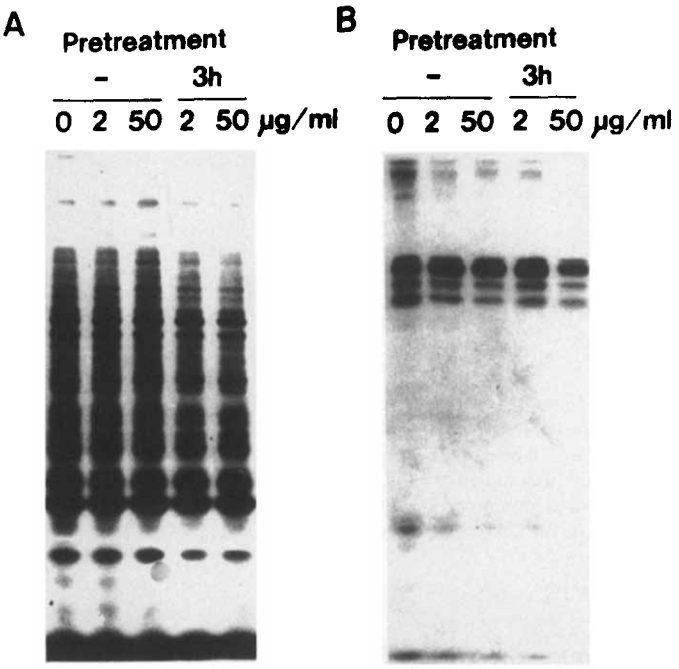

Fig. 3. SDS-PAGE analysis of ^{35}S-labeled proteins of Vero cells. The cells were infected at an MOI of 0.2. Some cultures were pretreated with 2 or 50 μg/ml YLP for 3 h. The protein samples were subjected to electrophoresis as described for Fig. 2. (A) Cell lysates; (B) immunoprecipitates.

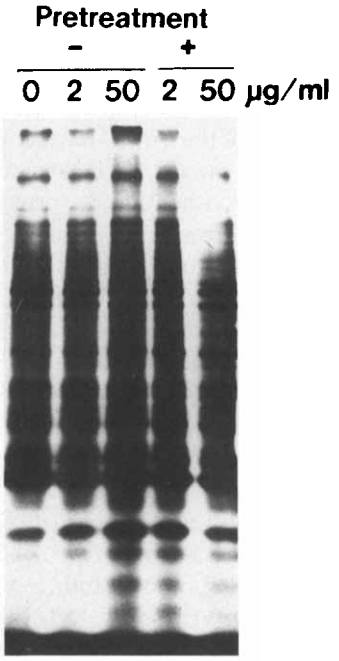

Fig. 4. SDS-PAGE analysis of ^{35}S-labeled proteins of Vero cells infected with UV-inactivated HSV-1. The same stock of virus as that used in Fig. 2 was inactivated by UV irradiation for 5 min and ascertained to have no infectious virus particle by plaque assay. Vero cells with or without pretreatment with YLP were inoculated with the inactivated virus at the amount corresponding to an MOI of 10. After radiolabeling of the cells for 4 h in the absence or presence of YLP, the cell lysates prepared were subjected to electrophoresis as described for Fig. 2.

are required for YLP to exert the inhibitory effect on protein synthesis, UV-inactivated virus was inoculated to Vero cells instead of infectious virus. As shown in Fig. 4, no inhibition of protein synthesis was observed even in the cells pretreated with 50 μg/ml YLP.

Discussion

The results obtained in the present study show that the YLP protein, isolated from *Yucca recurvifolia* Salisb., exerts a significant inhibitory effect in vitro on the replication of herpes simplex virus type 1. This activity was demonstrated by virus yield reduction at concentrations well below the cytotoxic dose, making the therapeutic index (ID_{50}/ED_{50}) considerably high. YLP also inhibited the replication of herpes simplex virus type 2 and human cytomegalovirus.

In order to elucidate the mode of the action in the inhibition of HSV-1 replication, the effect of YLP was studied under various experimental conditions. It was shown that direct inactivation of viruses could not be

attributed to the antiviral effect of YLP. In the studies of timing of addition, YLP was found to exert a strong antiviral activity especially when host cells were pretreated with it before virus infection. The results suggest that one of the modes of action may be inhibition of attachment and/or penetration of viruses. The fact that virus adsorption to the cells treated with YLP was almost the same as that to the untreated cells means YLP does not act at this early stage in the virus replication cycle. On the other hand, virus penetration into cells was reduced dose-dependently by pretreating the cells with YLP. That is, this protein inhibits virus penetration into host cells but not attachment to cells.

Pokeweed antiviral protein (PAP) is another protein of plant origin and is a basic protein of molecular weight 29 000 (Aron and Irvin, 1980). The antiviral action of PAP has been shown to be general in that it inhibits the multiplication of such viruses as influenza virus (Tomlinson et al., 1974), poliovirus (Ussery et al., 1977), and herpes simplex virus type 1 (Aron and Irvin, 1980) in cell culture. The mechanism of the antiviral activity of PAP has been suggested to involve its transport into intact cells by virus followed by inactivation of host cell ribosomes (Ussery et al., 1977; Irvin and Aron, 1982). Furthermore, liposomes containing fragment A of diphtheria toxin (M_r = 21 150) have been reported to kill selectively subacute sclerosing panencephalitis virus-infected (Ueda et al., 1981) or human immunodeficiency virus-infected (Ikuta et al., 1987) cells. YLP was also found to have no effect on protein synthesis in uninfected cells but inhibit strongly protein synthesis in virus-infected cells pretreated with YLP. The degree of this inhibition was dependent on the dose of YLP and the amount of virus infected. The time of pretreatment which is sufficient to exert this effect was one hour. This may mean that it is important for YLP to be present at the time of virus infection. Moreover, in the experiment using UV-inactivated virus, it was shown that some functions of infectious virus other than virus entry might be needed for this inhibition of protein synthesis. From the results in present study, it is suggested that YLP cannot enter intact cells but can enter virus-infected cells, and that inhibition of protein synthesis by YLP occurs along with virus replication. Thus, it can be expected that not only HSV-1 infected cells but also cells infected with other kinds of virus are affected by YLP. In fact, YLP inhibits the production of diverse viruses such as TMV (Hiramatsu et al., 1987), HSV-1, HSV-2 and HCMV (present data).

When host cells are infected with virus at room temperature, a part of virus particles may penetrate into the cells during infection, and after penetration the addition of YLP is thought to have little inhibitory effect on the virus replication. This may explain the difference between the ED_{50}s of cultures with and without pretreatment with YLP before infection as shown in Table 1.

References

Aron, G.M. and Irvin, J.D. (1980) Inhibition of herpes simplex virus multiplication by the pokeweed antiviral protein. Antimicrob. Agents Chemother. 17, 1032–1033.

De Clercq, E., Descamps, J., De Somer, P., Barr, P.J. and Walker, R.T. (1979) (*E*)-5-(2-

Bromovinyl)-2'-deoxyuridine: a potent and selective anti-herpes agent. Proc. Natl. Acad. Sci. USA 76, 2947–2951.

De Clercq, E., Descamps, J., Verhelst, G., Walker, R.T., Jones, A.S., Torrence, P.F. and Shugar, D. (1980) Comparative efficacy of antiherpes drugs against different strains of herpes simplex virus. J. Infect. Dis. 141, 563–574.

Elion, G.B., Furman, P.A., Fyfe, J.A., de Miranda, P., Beauchamp, L. and Schaeffer, H.J. (1977) Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA 74, 5716–5720.

Ellis, M.N., Keller, P.M., Fyfe, J.A., Martin, J.L., Rooney, J.F., Straus, S.E., Lehrman, S.N. and Barry, D.W. (1987) Clinical isolate of herpes simplex virus type 2 that induces a thymidine kinase with altered substrate specificity. Antimicrob. Agents Chemother. 31, 1117–1125.

Highlander, S.L., Sutherland, S.L., Gage, P.J., Johnson, D.C., Levine, M. and Glorioso, J.C. (1987) Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J.Virol. 61, 3356–3364.

Hiramatsu, A., Kobayashi, N. and Osawa, N. (1987) Properties of two inhibitors of plant virus infection from fruiting bodies of *Lentinus edodes* and from leaves of *Yucca recurvifolia* Salisb. Agric. Biol. Chem. 51, 897–904.

Huang, A.S. and Wagner, R.R. (1964) Penetration of herpes simplex virus into human epidermoid cells. Proc. Soc. Exp. Biol. Med. 116, 863–869.

Ikuta, K., Ueda, S., Uchida, T., Okada, Y. and Kato, S. (1987) Selective killing of human immunodeficiency virus-infected and -producing cells by liposomes containing diphtheria toxin fragment A. Jpn. J. Cancer Res. (Gann) 78, 1159–1163.

Irvin, J.D. and Aron, G.M. (1982) Chemical modifications of pokeweed antiviral protein: effects upon ribosome inactivation, antiviral activity and cytotoxicity. FEBS Lett. 148, 127–130.

McLaren, C., Chen, M.S., Ghazzouli, J., Saral, R. and Burns, W.H. (1985) Drug resistance patterns of herpes simplex virus isolates from patients treated with acyclovir. Antimicrob. Agents Chemother. 28, 740–744.

Mclimans, W.F., Davis, E.V., Glover, F.L. and Pake, G.W. (1957) The submerged culture of mammalian cells: The spinner culture. J. Immunol. 79, 428–433.

Osawa, N. and Hiramatsu, A. (1987) Purification and chemical properties of an inhibitor of plant virus infection from leaves of *Yucca recurvifolia* Salisb. Agric. Biol. Chem. 51, 891–896.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Showalter, S.D., Sweig, M. and Hampar, B. (1981) Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP4. Infect. Immun. 34, 684–692.

Tomlinson, J.A., Walker, V.M., Flewett, T.H. and Barclay, G.R. (1974) The inhibition of infection by cucumber mosaic virus and influenza virus by extracts from *Phytolacca americana*. J. Gen. Virol. 22, 225–232.

Ueda, S., Uchida, T. and Okada, Y. (1981) Selective killing of subacute sclerosing panencephalitis virus-infected cells by liposomes containing fragment A of diphtheria toxin. Exp. Cell Res. 132, 259–263.

Ussery, M.A., Irvin, J.D. and Hardesty, B. (1977) Inhibition of poliovirus replication by a plant antiviral peptide. Ann. NY Acad. Sci. 284, 431–440.

Wade, J.C., McLaren, C. and Meyers, J.D. (1983) Frequency and significance of acyclovir-resistant herpes simplex virus isolated from marrow transplant patients receiving multiple courses of treatment with acyclovir. J. Infect. Dis. 148, 1077–1082.