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Isolation and characterization of an anti-HSV polysaccharide from *Prunella vulgaris*

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Abstract

A water soluble substance was isolated from a Chinese herb, *Prunella vulgaris*, by hot water extraction, ethanol precipitation and gel permeation column chromatography. Chemical tests showed that the substance was an anionic polysaccharide. Using a plaque reduction assay, the polysaccharide at 100 μg/ml was active against the herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), but was inactive against cytomegalovirus, the human influenza virus types A and B, the poliovirus type 1 or the vesicular stomatitis virus. The 50% plaque reduction dose of the polysaccharide for HSV-1 and HSV-2 was 10 μg/ml. Clinical isolates and known acyclovir-resistant (TK-deficient or polymerase-defective) strains of HSV-1 and HSV-2 were similarly inhibited by the polysaccharide. Pre-incubation of HSV-1 with the polysaccharide at 4, 25 or 37°C completely abrogated the infectivity of HSV-1, but pre-treatment of Vero cells with the polysaccharide did not protect cells from infection by the virus. The addition of the polysaccharide at 0, 2, 5.5 and 8 h post-infection of Vero cells with HSV-1 at a multiplicity of infection (MOI) of five reduced the 20 h-yield of intracellular infectious virus by 100, 99, 99 and 94%, respectively. In contrast, a similar addition of heparin showed 85, 63, 53 and 3% reduction of intracellular virus yield, respectively. These results suggest that the polysaccharide may inhibit HSV by competing for cell receptors as well as by some unknown mechanisms after the virus has penetrated the cells. The *Prunella* polysaccharide was not cytotoxic to mammalian cells up to the highest concentration tested, 0.5 mg/ml and did not show any anti-coagulant activity. In conclusion, the polysaccharide isolated from *P. vulgaris* has specific activity against HSV and its mode of action appears to be different from other anionic carbohydrates, such as heparin. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Prunella vulgaris*; Polysaccharide; Anti-viral activity; HSV

1. Introduction

Herpes infections are ubiquitous. Approximately 16–35%, 40–80% and >90% of the US

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population are sero-positive for, or infected by, herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2) and varicella zoster virus, respectively (Cassady and Whitley, 1997). More alarmingly, over the past decade, the incidence and severity of infections caused by HSV have increased due to the growth in number of immunocompromised patients produced by aggressive chemotherapy regimens, expanded organ transplantation and a greater occurrence of human immunodeficiency virus infections. With this change in disease pattern and the increase in drug use frequency, acyclovir-resistant HSV infections have emerged (Field and Biron, 1994). Thus, there is an urgent need for novel anti-HSV agents, especially those with a different mode of action than acyclovir.

Prunella vulgaris, a perennial plant commonly found in China, the British Isles and Europe, has been used as an astringent for internal and external purposes (Grieve, 1974), as a crude anti-cancer drug (Lee and Lin, 1988) and as a herbal remedy to lower high blood pressure (Namba, 1994). In western herbal remedies, the plant — which is better known as 'self heal' — is used in the form of a hot water infusion sweetened with honey to treat sores in the mouth and throat (Grieve, 1974). Zheng (1990) reported some success in the use of a crude aqueous extract of *P. vulgaris* in the clinical treatment of herpetic keratitis. Of the 78 patients who received eye drops containing crude extracts of *P. vulgaris* and *Pyrrosia lingua*, 38 were cured, 37 showed improvement and three did not respond. Kurokawa et al. (1993) previously reported on the inhibition of the replication of HSV-1 in Vero cells by the hot water extract from the spikes of *P. vulgaris*. Hence, there is evidence that *P. vulgaris* is an effective anti-HSV plant, however the active anti-HSV component(s) has not been described. Phytochemical studies indicate that the plant contains oleanolic acid, triterpene acids, triterpenoids, flavonoids, fenchone, tannins and prunellin (Namba, 1994). Prunellin is a 10-kDa anionic polysaccharide which inhibits the replication of human immunodeficiency virus-1 (Tabba et al., 1989; Yao et al., 1992).

In this study, we have used bioassay-directed fractionation to isolate a polysaccharide from the spike of *P. vulgaris* that displays anti-HSV activity. The isolation, purification, chemical nature and anti-viral activity of the water-soluble polysaccharide are described.

2. Materials and methods

2.1. Viruses and cells

HSV-1 (strains BW-S, DM2.1 and PAAr^5) and HSV-2 (strains 8702 and Kost) were kindly provided by Jack Hill (Wellcome Research Laboratories, Burrough Wellcome, NC). Strain BW-S and 8702 are acyclovir-sensitive strain, while DM2.1 and Kost are acyclovir-resistant strains with thymidine kinase deficiency and altered phenotypes, respectively. PAAr^5 is also an acyclovir-resistant strain but is polymerase-defective. The clinical strains of HSV-1, HSV-2, cytomegalovirus, influenza A virus and influenza B virus were isolates from routine clinical specimens at the VG hospitals at Halifax, NS, Canada. The vesicular stomatitis virus (Indiana strain) and poliovirus type 1 (Sabin vaccine strain) were from our culture collection.

Vero cells were used as the host for HSV and poliovirus. Human foreskin cells, MDCK (dog kidney) cells and mouse L-929 cells were used for culturing cytomegalovirus, the influenza virus and the vesicular stomatitis virus, respectively. All the cells were grown in the Dulbecco's Modified Eagle's medium (MEM) with 10% fetal calf serum (FCS) (GIBCO/BRL, Burlington, ON, Canada).

2.2. Preparation of the Prunella extract

Dried spikes of *P. vulgaris* (1.4 kg) were imported from China and purchased locally in Halifax. The spikes were ground into small pieces with a warring blender and extracted with 12 l of distilled water at 95–100°C for 90 min. The extract was decanted to a clean container and the plant was extracted two more times. The pooled extract was clarified through a cotton cloth and condensed by a rotor evaporator to $\approx$1 l. The

extract was freeze-dried. A total of 85 g of dark brown dried powder was obtained. The active component in the aqueous extract was precipitated by ethanol. To achieve this, 60 g of the freeze-dried aqueous extract was dissolved in 300 ml of water and ethanol was added slowly while stirring, to a final concentration of 90% (v/v). The mixture was kept overnight at 4°C. The precipitate was recovered by filtration through filter paper and extracted with 4×1.5 l of butanol followed by 3×1.5 l of methanol. This yielded 31 g of dark brown powder named PVP (Fig. 1).

2.3. Gel filtration chromatography

An aqueous solution of PVP (450 mg in 10 ml) was applied to a Sephadex G-50 column (98×2.5 cm) and eluted with distilled water. Fractions of 5 ml were collected and the anti-HSV-1 activity was detected by the plaque reduction assay. To do this, fractions were freeze-dried and redissolved in distilled water to 1 mg/ml. These fractions were pooled to give the pooled fractions A–F. The amount of material recovered in each of the pooled fractions is indicated in Fig. 1.

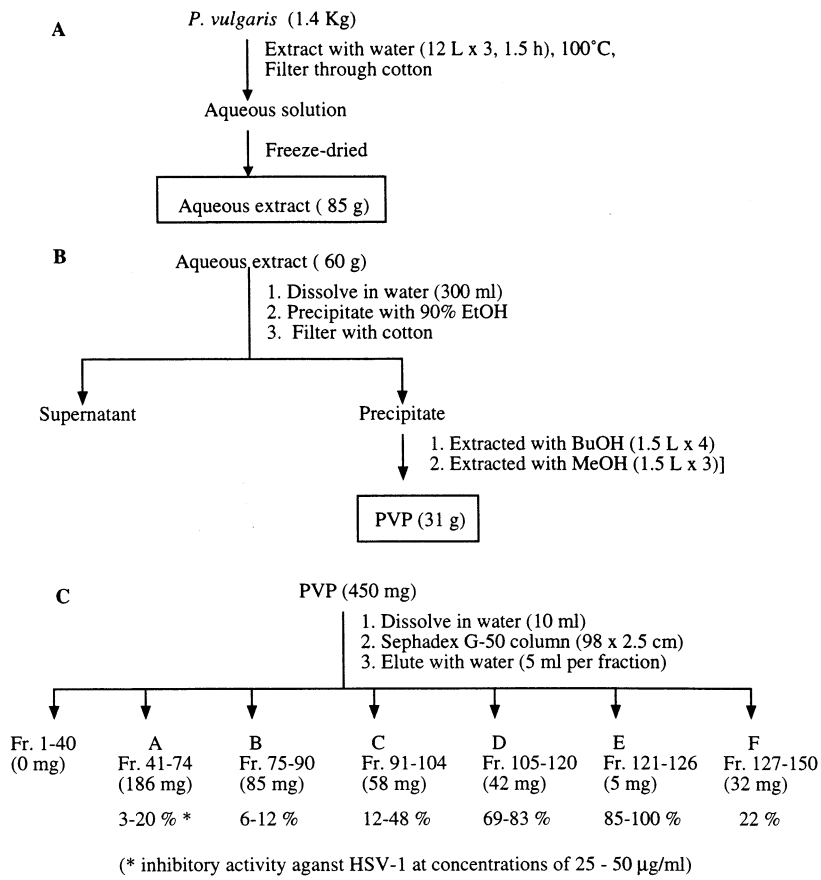

Fig. 1. Extraction and purification protocol for the anti-HSV polysaccharide from the spike of *Prunella vulgaris*. (A) Preparation of the hot water extract. (B) Ethanol precipitation of the active anti-HSV polysaccharide from the aqueous extract. (C) Purification by gel filtration chromatography. Percent inhibitory activity shown are percent reduction in plaque formation.

2.4. High pressure liquid chromatography (HPLC)

For the analysis of the anti-HSV fractions from *P. vulgaris* by HPLC (Beckman System Gold, Beckman Instruments, Mississauga, ON, Canada), the samples were dissolved in water (0.2 mg/ml) and 25 μl was injected into a reversed-phase C18 column (Supelcosil LC-18, 25 cm × 4.6 mm ID, 5 μ, Sigma, St. Louis, MO). Compounds were eluted with water:acetonitrile (5:95) at a flow rate of 0.3 ml/min and detected with a UV detector at 210 nm. For the analysis of the samples on a gel permeation column (TSK G3000PWxl, 7.8 mm × 30 cm, 6 μ, Sigma), the mobile phase was water at a flow rate of 0.8 ml/min. In the estimation of the molecular weight of the *Prunella* polysaccharide by gel permeation HPLC, dextran sulfate standards (MW 50 000, 10 000, 5000 and 1000, Sigma and CalBiochem, La Jolla, CA) and trehalose (MW 378, Sigma) were used under the conditions described above.

2.5. Plaque reduction assay

Plaque reduction assay was performed according to the standard method described by Hill et al. (1991). Briefly, monolayers of Vero cells grown on 6-well culture plates were infected with 100–200 pfu (plaque-forming unit) of virus. After incubation for 1 h to allow viral adsorption, the inoculum was aspirated and the cultures were overlaid with maintenance medium (MM [MEM with 2% FCS]) with 0.8% methylcellulose containing dilutions of the *P. vulgaris* extract. After 72 h of incubation at 37°C, the plates were fixed with formalin, stained with crystal violet, air-dried and the number of plaques counted. Plates overlaid with methylcellulose medium without the extract were used as controls. The percentage of inhibition of plaque formation was calculated as follows: [(mean no. of plaques in control − mean no. of plaques in test)]/(mean no. of plaques in control) × 100.

2.6. Effects of pre-treatment by Prunella polysaccharide on Vero cells and virus

To determine the effects of the polysaccharide on Vero cells, monolayers were incubated with the polysaccharide at 100, 50, 25, 12.5 and 6.125 μg/ml for 24 h at 37°C. After incubation, the cells were washed three times with medium to remove unreacted polysaccharide and infected with HSV-1 to allow plaque formation. Monolayers that were not treated with the polysaccharide were used as controls.

For the study of the direct effect of polysaccharide on HSV-1, 0.1 ml of virus containing 1.5×10^6 pfu was mixed with 100 μg of polysaccharide in 0.1 ml or with 0.1 ml of medium (control) and incubated at 37°C for 1 h. The mixture was promptly diluted (10 000 folds) and assayed for residual virus. Diluted virus was adsorbed for 1 h at 37°C. Inoculum was removed and Vero cell cultures were washed twice prior to receiving overlaid medium to allow plaque formation.

2.7. Viral binding assay

HSV-1 stock was diluted to 200 pfu/ml in pre-chilled medium and mixed with an equal volume of pre-chilled medium containing 50, 25, 12.5, 6.25 and 3.125 μg of the polysaccharide or with medium (control). The mixtures in 0.5-ml volumes were immediately inoculated onto Vero cell cultures at 4, 25 and 37°C. After 1 h adsorption, inocula were removed from the cultures followed by two washes with medium and plaques were allowed to form at 37°C in methylcellulose medium.

2.8. Effects of Prunella polysaccharide on viral growth

A single-cycle (one-step) growth of HSV-1 in Vero cells was performed to determine: (a) whether the polysaccharide inhibited virus replication; and (b) the stage (s) of the replication cycle affected by the polysaccharide. Vero cells were pre-chilled at 4°C and infected with virus at a multiplicity of infection (MOI) of five for 1 h at 4°C to synchronize the initial stage of infection

and adsorption. Virus inoculum was then removed and cultures were washed three times with pre-chilled medium prior to incubation with MM at 37°C. At 0, 2, 5.5 and 8 h post-infection, the polysaccharide was added to duplicate cultures and gave a final concentration of 100 μg/ml. At each time interval, when culture medium was replaced with medium containing the polysaccharide, one set of cultures were removed for assay of extracellular and intracellular virus as a measure of base level of virus present at the time the polysaccharide was added. The second set of cultures were further incubated to 20 h post-infection, at which time, the culture media were removed for assay of extracellular virus. The cultures were gently washed twice with 5 ml of maintenance medium and were harvested for assay of intracellular virus. Cell-free intracellular virus was measured following three frozen-thawed cycles and centrifugation. For comparison purposes, a similar experiment was carried out with heparin (10 kDa, sodium salt, Sigma) at a final concentration of 1 mg/ml.

2.9. Carbohydrate assays

Total carbohydrates were determined by the phenol-sulfuric acid assay (Dubois et al., 1956), using glucuronic acid (Sigma) as the standard. Uronic acids were measured with the method described by Blumenkrantz and Asboe-Hansen (1973) using glucuronic acid as the standard. Total hexosamines were determined by the Molgan-Elson reagent using N-acetylglucosamine (Sigma) as the standard (Ghuysen et al., 1966). Polyanionic polysaccharides were measured by the Alcian blue 8GX dye binding assay, described by Whitman (1973) using heparin (Sigma) as the standard.

2.10. Acid hydrolysis of Prunella polysaccharide

The purified compound (3 mg) was hydrolyzed in 2 N trifluoroacetic acid at 121°C for 1 h. The hydrolysate was dried by a rotary evaporator under reduced pressure at 40°C and residues obtained were dissolved in water and analyzed by ascending paper chromatography on Whatman no. 1 chromatography paper using the solvent system: pyridine:ethyl acetate:water = 4:10:3. Glucose, galactose, xylose, mannose and rhamose were used as the standards. Sugars were revealed by staining the paper with alkaline silver oxide reagents (Menzies and Seakins, 1969).

2.11. Cytotoxicity

The cytotoxic effect of the aqueous extract on mammalian cells was tested using a rat intestinal epithelial cell line (RIE-1), according to a published method (Blay and Poon, 1995). The dried aqueous extract was dissolved in dimethylsulfoxide (DMSO) and diluted in culture medium (MEM with 5% (v/v) heat-inactivated calf serum) before added directly to RIE-1 cells. The cultures were incubated for 48 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to the culture wells to give a final concentration of 0.5 mg/ml. The cultures were incubated for 3 h at 37°C to allow the conversion of MTT to formazan dye by mitochondrial dehydrogenase. The dye was measured at A_{492} with a Titertek Multiscan plate reader. Percent cytotoxicity was calculated using A_{492} readings from control wells which contained the same amounts of DMSO but no aqueous extract.

The cytotoxic effect of the purified polysaccharide was also determined on Vero cells by the Trypan blue dye exclusion test. Following 3-days incubation of Vero cell monolayer in maintenance medium with the polysaccharide at 100, 250 and 500 μg/ml or without the polysaccharide as controls, single cell suspensions were prepared by trypsinization and stained with 0.5% Trypan blue dye in phosphate buffered saline, prior to enumeration of viable cells in a Newbaner hemocytometer.

2.12. Anti-coagulant activity

The anti-coagulant activity of the compound was measured by the prothrombin time test. The prothrombin times were measured at 37°C using a BBL fibrometer (Becton Dickinson). Blood (9 ml), collected from the rabbit marginal ear vein, was mixed with 3.8% sodium citrate (1 ml) and cen-

trifuged at 1500 × *g* for 10 min at room temperature to obtain a clear supernatant as the testing plasma. In a typical assay, the mixture containing 50 μl of the testing solution (1 mg/ml), 150 μl of 50 mM Tris buffer, 0.1 M HCl, pH 7.5, 100 μl of plasma and 100 μl of thromboplastin with calcium was incubated at 37°C. The prothrombin times were recorded in seconds as the fibrometer stopped due to clotting.

3. Results

3.1. Extraction and isolation of the Prunella polysaccharide

During the initial screening of herbal extracts for anti-viral activity, we noted that the hot water extract from the spike *P. vulgaris* displayed good activity in inhibiting HSV-1 plaque formation. Since little is known about the anti-HSV activity of *P. vulgaris*, we initiated experiments to isolate the active anti-HSV principle from this plant. Fig. 1 outlines the protocol used in fractionation of the *P. vulgaris* aqueous extract. Following ethanol precipitation, the anti-HSV activity was found associated with the dark brown precipitate (PVP) by the plaque reduction assay. Materials recovered from the supernatant by rotary evaporation contained no detectable anti-HSV activity. The anti-HSV compound present in PVP was further fractionated by gel filtration chromatography on a Sephadex G-50 column. Upon assaying, the active compound was detected in pooled fractions C, D, E and F, with the highest activity found in fraction E (Fig. 1). The purity of the isolated anti-HSV compound was analyzed by HPLC with a reversed-phase (ODS-2) column and a gel filtration column. As shown in Fig. 2, samples from fraction E contained essentially one peak on both the ODS-2 and gel filtration columns indicating the anti-HSV compound is relatively pure.

3.2. Chemical nature of the anti-herpes compound

The nature of the anti-HSV compound was investigated by different chemical tests. Results from the phenol-sulfuric acid assay showed that the purified anti-HSV compound contained 42% carbohydrate (expressed as milligram glucuronic acid per milligram of compound). The compound contained only 0.75% uronic acid (expressed as milligram glucuronic acid per milligram). Hexasomines and proteins were not detected. Elemental analysis showed the compound contained 30.78% carbon, 3.05% hydrogen, 0.66% nitrogen and 2.69% sulfur. These results collectively indicated that the anti-HSV compound is a polysaccharide. The molecular weight of this polysaccharide is estimated to be 3500 by HPLC using a gel filtration column with dextran sulfates as standards.

The polysaccharide was found to be precipitated by the cationic dye Alcian blue 8GX and bound to DEAE-Sepharose at neutral pH. The bound polysaccharide could be eluted by 2 M NaCl from the DEAE-Sepharose, suggesting its polyanionic nature.

The polysaccharide was water soluble but was insoluble in methanol, ethanol, butanol, acetone or chloroform. An aqueous solution (1 mg/ml) of the polysaccharide had a pH of 5.5. Spectrophotometry showed a strong absorption peak at 202 nm and a shoulder at 280 nm, which extended to 380 nm.

The purified polysaccharide was hydrolyzed in 2 N trifluoroacetic acid at 121°C for 1 h. When the hydrolysate was analyzed by paper chromatography, three spots, which had the same R_f values of glucose, galactose and xylose standards, were observed. By comparing the intensity of the spots, glucose was the major constituent sugar. Galactose and xylose were minor components. These results indicate that the purified anti-herpes polysaccharide is composed mainly of glucose with some galactose and xylose as the constituent monosaccharides.

3.3. Anti-HSV activity of the Prunella polysaccharide

PVP and fraction E gave a dose-dependent response in inhibiting HSV-1 plaque formation (Table 1). The IC_{50} (concentration that gave 50% inhibition of plaque formation) against HSV-1 (strain BW-S) was estimated as 18 and 10 μg/ml for PVP and fraction E, respectively. In contrast,

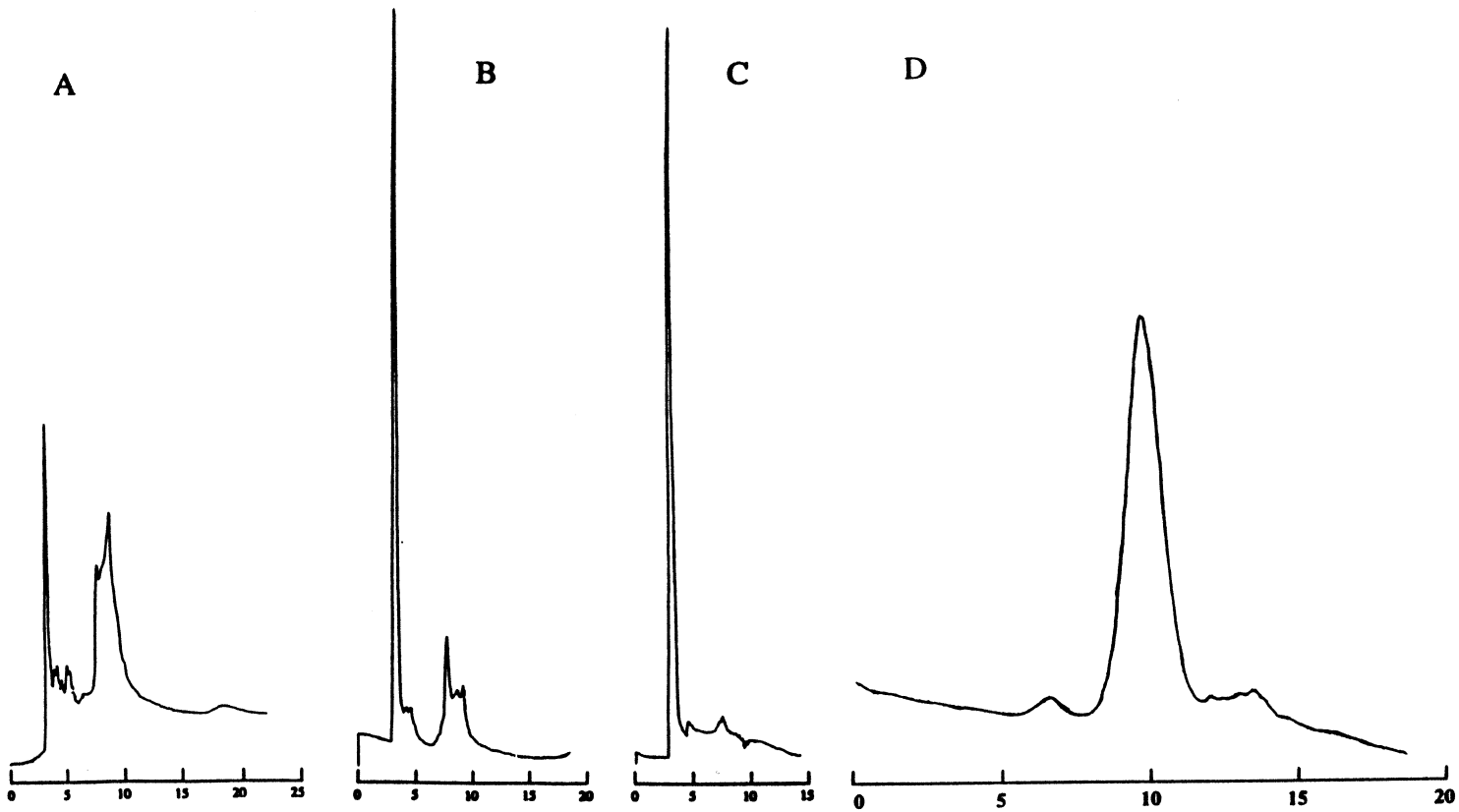

Fig. 2. HPLC chromatograms of the *Prunella* anti-HSV polysaccharide. Panels A, B and C are chromatograms of aqueous extract, PVP and Fraction E analyzed by a reverse phase column (Supelcosil LC-18). Panel D is Fraction E analyzed by a gel filtration column (TSK G30000PWxl). Conditions of HPLC and detection of the polysaccharide are described in Section 2. Scales shown are elution times in minutes.

Table 1
Dose-dependent inhibition of HSV-1 plaque formation by PVP and Fraction E

PVP (μg/ml)	Average no. of plaques	% Plaque inhibition	Fraction E (μg/ml)	Average no. of plaques	% Plaque inhibition
50	2	96.9	50	0	100
25	25	61.1	25	0	100
12.5	41.5	35.4	12.5	28.5	55.6
6.25	45.5	29.2	6.25	41.5	35.4
3.125	65.5	0	3.125	55.5	13.6
Control (0)	67	–	Control (0)	57	–

Table 2
Effect of *Prunella* polysaccharide on HSV-1 attachment and plaque formation on Vero cells at different temperatures

Temp. (°C)	No. of plaques per well					
	Concentrations of polysaccharide, μg/ml					
	50	25	12.5	6.25	3.125	Control (0)
4	1 (99)[a]	0 (100)	4 (95)	1 (99)	10 (88)	83
25	0 (100)	2 (98)	4 (96)	5 (95)	6 (94)	107
37	0 (100)	1 (99)	2 (98)	16 (84)	70 (29)	99

[a] % Plaque inhibition is indicated in parenthesis.

the IC_{50} against this HSV-1 strain for heparin and acyclovir were estimated as 750 and 0.1 μg/ml, respectively.

The mode of action of the anti-HSV activity of the polysaccharide was studied. When Vero cells were pre-treated with the polysaccharide followed by infection with HSV-1, no protective effect of the polysaccharide was observed. However, when HSV-1 (1.5×10^6 pfu) was pre-incubated with 100 μg of polysaccharide for 1 h before infecting Vero cells, $< 1\%$ of the virus was recovered in comparison to the medium-treated control. In contrast, when the same amount of HSV-1 was pre-incubated with 1 mg of heparin, 30% of the virus could be recovered.

The effect of the polysaccharide on virus attachment at different temperatures (37, 25 and 4°C) were tested in the viral binding assay. As shown in Table 2, at high concentrations (12.5 μg/ml or higher) the polysaccharide was equally effective in preventing plaque formation at the three temperatures used for viral adsorption. However, at a lower concentration (3.13 μg/ml) the polysaccharide was a better inhibitor at low temperatures than at 37°C.

The effect of the polysaccharide on HSV-1 growth in Vero cells was further investigated in a one-step growth study, in which the polysaccharide was added at 0, 2, 5.5 and 8 h post-infection (p.i.). The extracellular and intracellular viral yields were measured. As expected, the polysaccharide reduced more than 99% of the extracellular viral yield for each of the time points as compared to controls, where cells were not treated with the polysaccharide. More interestingly, the intracellular viral yield was also reduced dramatically by 100, 99, 99 and 94% for the respective time points (Table 3). A parallel experiment carried out using heparin showed that the extracellular viral yield was also reduced by more than 99%. However, the intracellular viral yield was reduced by 85, 63, 53 and 3% of the controls for the respective time points. These results indicated a clear difference between the effect of the *Prunella* polysaccharide and heparin on HSV-1 growth.

Table 3
Effect of *Prunella* polysaccharide and heparin on intracellular HSV-1 viral yield

Time of addition of test compound p.i. (h)	Intracellular virus yield at 20 h p.i. (pfu/ml)		% Inhibition[a]	
	P. vulgaris polysaccharide	Heparin	*P. vulgaris* polysaccharide	Heparin
0	5.6×10^4	8.9×10^6	99.9	85
2	4.0×10^5	2.2×10^7	99.3	63
5.5	5.0×10^5	2.8×10^7	99.2	53
8	3.6×10^6	5.8×10^7	94.0	3

[a] % Inhibition = [(no. of intracellular virus in untreated control − no. of intracellular virus in treated sample)/no. of intracellular virus in control] × 100. Untreated control Vero cells produced 6.0×10^7 pfu/ml of intracellular virus at 20 h p.i.

3.4. Spectrum of anti-viral activity

The spectrum of anti-viral activity of the *Prunella* polysaccharide was investigated. The polysaccharide, at 100 μg/ml, was found to inhibit plaque formation in Vero cells by laboratory strains (BW-S and 8702) and two clinical isolates each of HSV-1 and HSV-2 as well as acyclovir-resistant strains of HSV-1 (DM2.1 and PAAr[5]) and HSV-2 (Kost). However, the polysaccharide had no activity against cytomegalovirus, human influenza virus types A and B, poliovirus type 1 and vesicular stomatitis virus at the same concentration.

3.5. Cytotoxicity and anti-coagulant activity

The aqueous extract of *P. vulgaris* was tested for cytotoxicity against a rat intestinal epithelial cell line (RIE-1). As shown in Fig. 3, no cytotoxic effect was noted with the aqueous extract up to the highest concentration tested, 500 μg/ml. The Trypan blue dye exclusion test showed similar numbers of unstained (viable) Vero cells in cultures incubated in the presence or absence of the polysaccharide at the concentrations tested, suggesting the polysaccharide was not toxic.

The *Prunella* polysaccharide was tested for the presence of anti-coagulant activity by the prothrombin time test. The average prothrombin time for the *Prunella* polysaccharide was 25.9 ± 1.5 s, a value similar to the water control 29.9 ± 1.4 s. This is in contrast to the prothrombin time of > 300 s for the methanol extract of another Chinese herb, *Geum japonicum*, which has been shown to contain anti-coagulant compounds (Zeng, 1996).

4. Discussion

In this study, the active anti-HSV principle was isolated from the herbal plant *P. vulgaris* by hot water extraction, precipitation and gel permeation chromatography. The active principle is a polysaccharide, as indicated from the results of the phenol-sulfuric acid assay, paper chromatography analysis of the acid hydrolysate and elemental analysis. The fact that the polysaccharide binds to the cationic dye Alcian blue 8GX and DEAE-Sepharose and can be eluted from DEAE-

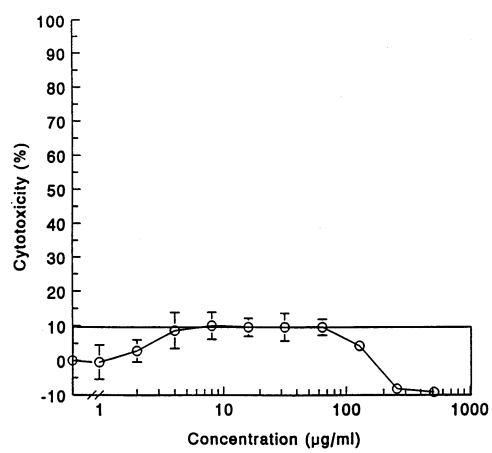

Fig. 3. Cytotoxicity of the aqueous extract from *Prunella vulgaris*.

Sepharose with salt suggests that the polysaccharide is anionic in nature. The presence of sulfur suggests that some of the sugar residues may contain SO_4 to confer the anionic nature of the polysaccharide. Polysaccharides are known to affect the growth of animal viruses (Shannan, 1984). In particular, anionic polysaccharides, such as heparin, dextran sulfate, carrageenans, pentosan polysulfate, fucoidan and sulfated xylogalactans, are potent inhibitors of herpes virus binding to host cells (Gonzalez et al., 1987; Baba et al., 1988; Damonte et al., 1996). These polysaccharides are a competitor of receptors (heparan sulfate) to viral glycoproteins. Herold et al. (1996) showed that N-sulfations and the presence of carboxy groups on heparin are key determinants for HSV-1 and HSV-2 interactions with host cells, since N-desulfation and carboxyl reduction abolished heparin's anti-viral activity. Hence, our finding is consistent with the literature.

The anti-HSV polysaccharide we have isolated showed some similarities, but not identical, to the polyanionic polysaccharide, prunellin, previously isolated by Tabba et al. (1989) which has anti-HIV activity (Tabba et al., 1989; Yao et al., 1992). prunellin was described to be 10 kDa in size, had a pH of 7.4 in aqueous solution, showed an adsorption peak at 370 nm which extended to 500 nm, contained 0.14% sulfur and glucose and galactose as the major constituent sugars and xylose and galactosamine as minor sugars. In contrast, our anti-HSV polysaccharide is 3500 Da, has a pH of 5.5 in aqueous solution, showed an adsorption peak at 202 nm which extended to 380 nm, contained 2.69% sulfur and glucose as the major constituent sugar and galactose and xylose as minor sugars. It may be possible that the anti-HSV polysaccharide is a fragment of prunellin or it can also be a completely different polysaccharide synthesized by *P. vulgaris*. This question can only be answered by comparing the structure of prunellin and the anti-HSV polysaccharide, which is not available. Assaying our polysaccharide for anti-HIV activity will not provide conclusive evidence that it originated from prunellin.

The results from the virus pre-treatment experiment suggest that the *Prunella* polysaccharide exerts its effect by binding to the viral particles and preventing them binding and infecting Vero cells. In the viral binding assay, the polysaccharide at low concentrations was a better inhibitor at 4°C than at 37°C. Since HSV can bind to Vero cell receptors but cannot penetrate the cells at 4°C, the results suggest that the polysaccharide is competing with Vero cell receptors for the virus. At 37°C, the competition is complicated by the fact that some of the initially bound viral particles have penetrated the cells.

The results of the single-cycle growth of HSV-1 is most interesting. As expected, both the *Prunella* polysaccharide and heparin are excellent inhibitors to extracellular viral yield, presumably via binding to the virions. However, the *Prunella* polysaccharide is a better inhibitor for intracellular viral yield than heparin, particularly at 8 h post-infection. Although the polysaccharide and heparin were used at different concentrations relative to their IC_{50} values, the observed difference in intracellular viral yield is unlikely due to the relative difference in concentrations of the two inhibitors. Over the time course, there was a clear decrease in intracellular viral yield (85–3%) while the reduction in extracellular viral yield remained constant at $>99\%$, suggesting that the observation is due to the inability of heparin to effect intracellular inhibition at the later stage of infection. This result is in good agreement with that reported in the literature that heparin is effective in HSV inhibition only at the early stage (binding and penetration) of virus replication (Nahmias and Kibrick, 1964; Herold, et al., 1996). The dramatic reduction of intracellular viral yield by the polysaccharide at 8 h post-infection is most intriguing because at this time point the virus should have penetrated the cell and is well into its replication cycle. It is tempting to speculate that the *Prunella* polysaccharide may have an intracellular mode of action, although pre-treatment of Vero cells with the polysaccharide confers no protection. Perhaps, uninfected cells are impermeable to the polysaccharide and infected cells, which are known to have an altered membrane permeability (Carrasco, 1978; Palu, et al., 1994), may allow the entrance of the polysaccharide to effect its intracellular action. However, this remains to be proven.

Unlike heparin and other known anionic polysaccharides, the *Prunella* polysaccharide contains no detectable anti-coagulant activity. In addition to the fact that the *Prunella* polysaccharide can inhibit HSV not only at the early stage but also at a later stage of virus replication, the *Prunella* polysaccharide is a more suitable candidate as an anti-HSV drug.

The *Prunella* polysaccharide was examined for its inhibitory effect on the replication of several different RNA and DNA viruses. The results showed that it is quite specific for HSV with activity against HSV-1 and HSV-2 including acyclovir-resistant strains. This is again in contrast to other polyanionic polysaccharides, which are active against a number of viruses including cytomegalovirus and vesicular stomatitis virus (Baba et al., 1988).

In summary, we have isolated an anionic polysaccharide from the spike of *P. vulgaris* that exhibits strong activity against HSV and acyclovir-resistant HSV. This polysaccharide lacks anti-coagulant activity and cytotoxicity. To the best of our knowledge, this is the first report of the isolation of a polysaccharide from this plant with anti-HSV activity. Our results suggest that this polysaccharide may be a potential anti-HSV drug candidate.

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