

Isolation, identification and function of a novel anti-HSV-1 protein from *Grifola frondosa*

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Abstract

A novel antiviral protein was purified from an extract of *Grifola frondosa* fruiting bodies using a procedure that included 40% ammonium sulfate precipitation and DEAE-cellulose ion exchange chromatography, and designated GFAHP. This protein inhibited herpes simplex virus type 1 (HSV-1) replication *in vitro* with an IC_{50} value of 4.1 $\mu\text{g/ml}$ and a therapeutic index >29.3 . Higher concentrations of GFAHP (125 and 500 $\mu\text{g/ml}$) also significantly reduced the severity of HSV-1 induced blepharitis, neovascularization, and stromal keratitis in a murine model. Topical administration of GFAHP to the mouse cornea resulted in a significant decrease in virus production (mean virus yields: 3.4 log₁₀ PFU in the treated group and 4.19 log₁₀ PFU in the control group). We proved that GFAHP directly inactivates HSV-1 while simultaneously inhibiting HSV-1 penetration into Vero cells. Gel electrophoresis showed that GFAHP had a molecular weight of 29.5 kDa. GFAHP was tryptic digested and analyzed from the PMF of matrix assisted desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) and nanoelectrospray ionization tandem mass spectrometry. The N-terminal sequence of GFAHP consisted of an 11 amino acid peptide, $\text{NH}_2\text{-REQDNAPCGLN-COOH}$ that did not match any known amino acid sequences, indicating that GFAHP is likely to be a novel antiviral protein. To our knowledge, this is the first report that characterizes an anti-HSV protein from *G. frondosa*.

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Keywords: *Grifola frondosa*; Protein; Antiviral activity; Identification; HSV-1

1. Introduction

Fungi are an important source of anti-microbial and immunosuppressive compounds. Many have also been tested for antibacterial activity and some mushrooms were recently shown to possess antiviral activity. The basidiomycete, *Fomes fomentarius*, for example, has antiviral activity against tobacco mosaic virus (Aoki et al., 1993) and an extract of the edible *Japanese Lentinus edodes* was found to inhibit replication of herpes simplex virus (HSV), western equine encephalitis virus, poliovirus, measles virus, and mumps virus (Sorimachi et al., 1990; Sarkar et al., 1993). The sterols from the edible mushroom, *Hypsizygus marmoreus*, have a potent inhibitory effect on Epstein-Barr virus activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (Akihisa et al., 2005) and ganodermediol and applanoxidic acid G from *G. pfeifferi* possess antiviral activity against influenza virus type

A (IVA) (Mothana et al., 2003). *In vitro* antiviral activity against IVA was also shown for mycelial extracts of *Kuehneromyces mutabilis* (Mentel et al., 1994). Screening other mushroom species may help to identify additional antiviral factors.

The edible mushroom, *G. frondosa*, is a basidiomycete fungus belonging to the *Polyporaceae* family. Fruit bodies and liquid-cultured mycelium from this mushroom are reported to contain useful antitumor polysaccharides (Borchers et al., 1999; Adachi et al., 1994), and show some promise as immunomodulatory agents. They may also be beneficial for the treatment of hyperlipidemia, hypertension, and hepatitis (Mayell, 2001). It is reported that D-Fraction, a polysaccharide extracted from the maitake mushroom, *G. frondosa*, activates macrophages, dendritic cells, and T cells, inhibits tumor cell growth (Kodama et al., 2002, 2005; Inoue et al., 2002), enhances the cytotoxicity of NK cells by inducing the production of IL-12 (Kodama et al., 2002), and improves the symptoms and secondary diseases caused by HIV (Nanba et al., 2000). We showed that the D-Fraction also has the ability to inhibit HBV *in vitro* (Gu et al., 2006).

In general, it is the polysaccharides from mushrooms, like *G. frondosa*, that contain antiviral activity (Collins and Ng, 1997;

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Lee et al., 2003, 2004; Shi et al., 2006; Hsieh et al., 2006; Nie et al., 2006; Cui et al., 2007). In this study, we describe a novel protein from *G. frondosa*, named GFAHP which has antiviral activity. To our knowledge, this is the first isolation and characterization of a protein from this mushroom species that has antiviral function.

2. Materials and methods

2.1. Virus and cell culture

High titer virus stocks were grown in Vero cells as described previously (Grau et al., 1989). Dr. Wang at the National Institute for Viral Disease, Chinese Center for Disease Control and Prevention provided the HSV-1 strain, KOS, and the Vero cells. Vero cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 8% fetal bovine serum (FBS) at 37 °C with 5% CO₂. Virus stocks were prepared in cells fed with 2% FBS.

2.2. Isolation and identification of GFAHP

2.2.1. Preparation of *G. frondosa* extracts

Six hundred grams of *G. frondosa* mushrooms (Huayi Biotech Co., Henan, China) were homogenized with an equal amount of distilled water at room temperature (RT). The homogenate was heated at 60 °C for 12 h and centrifuged at 2500 × *g* for 20 min. The supernatant, designated as fraction 1, was lyophilized and resuspended in water to a concentration of 20 mg/ml. Ammonium sulfate was slowly added to make a final concentration of 40%, stirred at 4 °C for 2 h, and centrifuged at 2000 × *g* for 15 min. The precipitate was resuspended in deionized water, and both the precipitate (fraction 2) and the supernatant (fraction 3) were dialyzed against water for 2 days (four changes of water), lyophilized, and stored at –70 °C. The dialysate was designated as fraction 4. As shown in Table 1, the antiviral activity was predominantly in fraction 2.

Table 1
Anti-HSV-1 activity of fractions isolated from *Grifola frondosa*

Fraction	IC ₁₀₀ (μg/ml) ^a	Yield (%) ^b
Hot water extract	512	4.37
40% ammonium sulfate precipitate	64	0.75
40% ammonium sulfate supernatant	Inactive	0.24
Ammonium sulfate dialysate	Inactive	0.08
Unadsorbed material through column	Inactive	0.01
Eluate from 0.5 M NaCl through column ^c	11	0.06
Eluate form 1.0 M NaCl through column	Inactive	0.08
Eluate form 1.5 M NaCl through column	Inactive	0.02

^a The lowest concentration resulting in 100% inhibition of its cytopathic effect (mg/ml) against HSV-1 KOS. Vero cells were inoculated into wells of a 96-well plate and infected with HSV-1 KOS at 2 PFU/cell on day 2. Serial dilutions of each sample were added and the wells were scored at 2 days post-infection for the presence of a cytopathic effect.

^b Percent of starting material.

^c The material designated GFAHP.

2.2.2. Isolation of GFAHP

A 37 cm × 1.3 cm column was packed with DEAE-cellulose (Pharmacia Biotech) in distilled water, and an 8 ml sample of fraction 2 (3.5 mg/ml) in distilled water was passed through the column at a rate of 8 ml/h and eluted with a linear NaCl gradient at 4 °C. After removal of unabsorbed material (D1, designated fraction 5), the substance (D2, designated fraction 6) was eluted with 0.5M NaCl. Further substances were eluted with 1.0 M NaCl (designated fraction 7) and 1.5 M NaCl (designated fraction 8). The absorbance of each fraction was measured at 280 nm, and each was collected, dialyzed, and tested for anti-HSV-1 activity using the plaque reduction assay. The antiviral activity in fraction 6 was designated as *G. frondosa* antiviral protein, GFAHP.

2.2.3. Polyacrylamide gel electrophoresis

GFAHP was electrophoresed on a 15% denaturing-reducing sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) as described previously (Visalli and Brandt, 1993). The molecular weight was determined by comparing electrophoretic mobility with molecular weight marker proteins purchased from Takara Biotechnology Company (Dalian, China).

2.2.4. Identification of GFAHP by mass spectrometry

Acetone precipitated GFAHP was electrophoresed and transferred to a PVDF membrane. The 29 kDa band containing the active material was cut from the membrane and sequenced from the amino terminus using the trypsin digestion procedure (Wang et al., 2006).

All mass spectra of MALDI-TOF-MS were obtained on a Bruker REFLEX III MALDI-TOF-MS (Bruker-Franzen, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV with a α-cyano-4-hydroxy cinnamic acid matrix. The spectra were internally calibrated using trypsin autolysis products. The peptide mass fingerprint (PMF) obtained was used to search the SWISS-PROT and NCBIInr database using the Mascot search engine (<http://www.matrixscience.co.uk>) with a tolerance of ±0.1 Da and one missed cleavage site.

ESI-MS/MS experiments were performed on a Q-TOF2 hybrid quadrupole/TOF mass spectrometer (Micromass, UK) with a nano flow Z-spray source. Peptide sequencing was performed using a palladium-coated borosilicate electrospray needle (Protana, Denmark). The mass spectrometer was used in positive ion mode with a source temperature of 80 °C, and a potential of 800–1000 V was applied to the Nanospray probe. The amino acid sequences of the peptides were deduced using the peptide sequencing program, MasSeq. The database search done by the Mascot search engine (<http://www.matrixscience.co.uk>) was completed using the data processed through MaxEnt3 and MasSeq. Each palladium-coated borosilicate electrospray needle (Protana, Odense, Denmark) was only used once.

2.3. GFAHP antiviral activity in vitro

2.3.1. Therapeutic effect of GFAHP on HSV-1 infection

To measure GFAHP activity, Vero cells were inoculated into wells of a 24-well plate at 2 × 10⁵ cells/well and incubated

overnight. Once confluent, the cells were infected with HSV-1 at a multiplicity of infection (MOI) of 2 PFU/cell. Following a 1 h adsorption period, the cells were rinsed twice with PBS (pH 7.4) and mixed with media containing different concentrations of GFAHP. Virus infected GFAHP treated, uninfected untreated, and uninfected GFAHP treated cells were tested in triplicate. At 48 h post-infection (p.i.) the amount of infectious virus was measured by plaque assay and quantitated (Pirano and Brandt, 1999).

2.3.2. GFAHP pretreatment and direct virucidal activity

To determine the preventive effect of GFAHP on virus infected Vero cells, monolayers were incubated with 30 or 60 $\mu\text{g/ml}$ GFAHP for 24 h at 37 °C. After incubation, the cells were washed three times with PBS to remove unused GFAHP and infected with HSV-1 at an MOI of 2 to allow plaque formation. Untreated monolayers were used as controls (Chiang et al., 2002).

To investigate the direct virucidal effect of GFAHP on HSV-1, 1.5×10^6 PFU virus (0.1 ml volume) was mixed with 0.1 ml 60 $\mu\text{g/ml}$ GFAHP or 0.1 ml medium and incubated at 37 °C for 1 h. The mixture was diluted 1000 \times and assayed for residual virus by adsorbing for 1 h at 37 °C. Inocula were removed and Vero cell cultures were washed twice prior to the addition of overlay medium.

2.3.3. HSV-1 cell attachment and penetration

Vero monolayers grown in 24-well plates were pre-chilled at 4 °C for 15 min and infected with HSV-1 diluted in serum-free MEM to 200 PFU/ml for 1.0 h at 4 °C in the presence of GFAHP with 30 and 60 $\mu\text{g/ml}$ or absence. Unadsorbed virus was then removed and plaques were allowed to develop at 37 °C for 72 h (De Logu et al., 2000).

The penetration assay was performed as previously described with some modifications (Rosenthal and Peres, 1985; De Logu et al., 2000). HSV-1 stock was added to Vero cells in a 24-well culture plate and incubated at 4 °C for 2 h, and the temperature was abruptly increased to 37 °C to maximize penetration of the virus. Penetration proceeded for 1 h in the presence and absence of GFAHP. Monolayers were then treated with PBS, pH 3 for 1 min to neutralize any remaining attached virus and after several washes with serum-free medium, overlay media was added to promote plaque formation.

2.4. GFAHP antiviral activity in vivo

2.4.1. Experimental design of HSV-1 infection in mice and disease scoring

Five-week-old female BALB/c mice (Center of Animals, Academy of Military Medical Sciences, Beijing, China) were used for all studies. Mice were anesthetized by halothane inhalation. While under anesthesia, the right cornea was scratched three times vertically and four times horizontally with a sterile 27-gauge needle. A 10 μl drop of DMEM containing 10^6 PFU of HSV-1 KOS was placed on the damaged cornea for 30 s. Excess inoculum was removed by adsorption with a sterile swab. The mice were examined microscopically for ocular disease using a

scoring system that was described previously (Grau et al., 1989; Brandt et al., 1992). In brief, eyelid inflammation, blepharitis, was scored as follows: (1+) noticeably puffy eyelids, (2+) puffy eyelids with moderate crusting, (3+) eye swollen, 50% shut with severe crusting, and (4+) eye totally swollen and crusted shut. Vascularization was scored as follows: (1+) 5% of the cornea involved, (2+) <25 to 50% of the cornea involved, and (3+) >50% of the cornea involved. Stromal keratitis was scored as follows: (1+) cloudiness, some iris detail visible, (2+) iris detail obscured, (3+) cornea totally opaque, and (4+) cornea perforated. Ten mice in each group were scored for ocular disease.

Data were reported as the mean disease score for each mouse group on the day of observation, or as the mean peak disease score (MPDS). The MPDS was calculated by averaging the most severe disease score for each animal in a group regardless of the day when the peak score was observed. This model is not reliable for scoring epithelial keratitis because of difficulties in distinguishing virus-induced damage from scarification during infection. Thus, the corneas were not stained with fluorescein, and disease was scored in a masked fashion.

2.4.2. Treatment protocol

A high (GFAHP-HD; 150 $\mu\text{g/ml}$), medium (GFAHP-MD; 37.5 $\mu\text{g/ml}$), and low (GFAHP-LD; 3.8 $\mu\text{g/ml}$) dose of GFAHP, or 1000 $\mu\text{g/ml}$ Acyclovir (ACV, Pusheng BioTech. Co., Wuhan, China) was administered topically in 10 μl saline onto the right cornea. The mice were treated six times per day for the first 3 days, beginning 3–4 h p.i., and four times per day for the next 4 days. Mice receiving six treatments per day were treated at 9:00 a.m., 12:00 p.m., 3:00 p.m., 6:00 p.m., 9:00 p.m., and midnight. Mice receiving four treatments per day were treated at 9:00 a.m., 1:00 p.m., 5:00 p.m., and 9:00 p.m. Ocular disease scores were recorded until day 18 p.i. In control animals, typical dendritic keratitis usually appeared between days 3 and 5 p.i.

2.4.3. Measuring ocular HSV-1 replication

To quantitate virus in the mouse corneas, mice were sacrificed with halothane 24 and 48 h p.i. and the right eyeballs were obtained. The eyeballs were homogenized in 4 ml PBS containing 0.2% BSA at 48 °C and the homogenate supernatants were prepared as described previously (Grau et al., 1989). The supernatants from two different eyeballs were pooled, and the virus was quantified by plaque assay (Pirano and Brandt, 1999). The results of groups given GFAHP or ACV were compared to those from the untreated groups.

2.5. GFAHP toxicity

2.5.1. Cytotoxicity assay

As a preliminary test of toxicity, replicate wells of uninfected cells were exposed to various concentrations of each substance and incubated for 16–18 h at 37 °C. Viable cell yield was determined using the MTT reduction assay as previously described (Scubiero et al., 1988). Cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}) at which a substance inhibited up to 50% of cell growth, as shown by MTT viability 48 h later.

2.5.2. Toxicity in vivo

Uninfected mice were treated with 10 μ l 150 μ g/ml GFAHP using the protocol described above. Eight hours after the last treatment, the corneas were stained with 1% fluorescein and examined with a cobalt blue light source for evidence of punctate keratopathy, ulceration, or other epithelial defects. A total of 10 mice were examined.

2.6. Stability of GFAHP

Equal volumes of GFAHP in phosphate buffered saline (PBS) were mixed with DMSO ether, or 80% acetone for 15 min and incubated for an additional 15 min at room temperature. For organic solvents, the aqueous layers were removed and the samples were air dried or lyophilized, resuspended at the original concentration (0.1 mg/ml) in DMEM, and tested for antiviral activity. To measure heat stability, GFAHP samples in PBS were boiled for 10 min, cooled to room temperature, and tested for antiviral activity *in vitro*. To test protease sensitivity, 10 mg/ml GFAHP was incubated with 1.25% trypsinase overnight at 37 °C. 0.5% Soybean trypsinase inhibitor (STI) was added to inactivate the trypsinase and the samples were tested for antiviral activity *in vitro*. The percent reduction in antiviral activity was determined by comparing the amount of GFAHP required to reduce viral titers [(untreated/treated) \times 100]. Control cultures were exposed to a solution containing STI and no GFAHP and STI was found to have no effect (data not shown).

2.7. Statistical analysis

Student's *t*-test, analysis of variance (ANOVA), and calculation of correlation coefficients were carried out using SPSS 10. Statistically significant differences were calculated at the 5% significance level using the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Isolation and purification of GFAHP from *G. frondosa*

To investigate the antiviral activity of *G. frondosa* extracts, a protein designated GFAHP was isolated from water-soluble fractions of *G. frondosa* by ammonium sulfate precipitation and DEAE-cellulose column chromatography. The anti-HSV-1 activity of GFAHP was examined by plaque reduction assay and found to exhibit potent antiviral activity. As shown in Table 1, the IC₁₀₀ for the crude extract against HSV-1 was 512 μ g/ml. The 40% ammonium sulfate precipitate was found to contain all of the antiviral activity (IC₁₀₀, 64 μ g/ml). The active material was chromatographed on a DEAE-cellulose column and the antiviral activity of each fraction was tested. We got four fractions from the chromatograph, and only the second fraction (total number was fraction 6) had an IC₁₀₀ of 11 μ g/ml against HSV-1 KOS (Table 1). Altogether, the protocol resulted in approximately 70 \times purification with a 0.06% yield. The IC₅₀ value of GFAHP was 4.1 μ g/ml, and its CC₅₀ value was more than 120 μ g/ml

Table 2

Anti-HSV activity of GFAHP by plaque reduction assay

Antiviral substances	IC ₅₀ (μ g/ml) ^a	CC ₅₀ (μ g/ml) ^b	TI ₅₀ (CC ₅₀ /IC ₅₀) ^c
GFAHP	4.10 \pm 1.50	>120.0	>29.3
Acyclovir	0.42 \pm 0.13	>300.0	>714.0

^a IC₅₀, 50% inhibition concentration, defined as a drug concentration that induced 50% inhibition of HSV-1 (as compared to the untreated culture).

^b CC₅₀, 50% cytotoxic concentration, defined as a drug concentration that induced 50% inhibition of Vero cells (as compared to the untreated culture).

^c TI₅₀, therapeutic index, calculated as the ratio of CC₅₀ over IC₅₀.

Table 3

GFAHP pretreatment of cells and virucidal activity

Cell pretreatment (μ g/ml)	PFU/well inhibition (%)			
	Virucidal		Pretreatment	
Control (0)	105 \pm 12		82 \pm 11	
15	13 \pm 7	87.6	–	–
30	2 \pm 2	98.1	71 \pm 9	13.4
60	–	–	65 \pm 7	20.7

(Table 2). The therapeutic index (CC₅₀/IC₅₀) of GFAHP was more than 29.3.

3.2. Possible mechanism of GFAHP antiviral activity in vitro

The preventive and virucidal effects of GFAHP, in addition to its effect on HSV-1 attachment and penetration, were determined in order to define its potential mechanism of action. The number of plaques formed by HSV-1 in GFAHP pretreated Vero cells was not significantly different from the number formed in untreated Vero cells (Table 3). This suggested that GFAHP may not protect cells from infection. GFAHP was found to significantly decrease viral titer compared with the control group, however, suggesting that it does have virucidal activity. This effect was assumed to be irreversible (Table 3).

Viruses were allowed to attach to cells in the presence or absence of GFAHP for 3 h. The assay was terminated by removing the virus with PBS (pH 7.4). Treatment with 30 or 60 μ g/ml GFAHP was not found to inhibit HSV-1 attachment (Table 4). To examine the effect of GFAHP on penetration, viruses were allowed to attach at 4 °C for 2 h, a temperature sufficient for attachment but not penetration, after which the temperature was abruptly increased to 37 °C to facilitate penetration. 30 μ g/ml GFAHP prevented 83.7% HSV-1 penetration into cells and 60 μ g/ml GFAHP inhibited 93.5% HSV-1 penetration (Table 4).

Table 4

Inhibitory effect of GFAHP on cell attachment and penetration by HSV-1

Concentration (μ g/ml)	PFU/well inhibition (%)			
	Attachment		Penetration	
Control (0)	117 \pm 6		92 \pm 9	
30	105 \pm 10	10.2	15 \pm 2	83.7
60	111 \pm 9	5.1	6 \pm 3	93.5

Thus, GFAHP may exert its antiviral activity by preventing HSV penetration into Vero cells.

Based on the above results, GFAHP may have a direct virucidal effect on viral activity *in vitro* and inhibits viral penetration into cells.

3.3. GFAHP antiviral activity *in vivo*

3.3.1. Effect of GFAHP treatment on the development of ocular disease

To determine if GFAHP treatment was effective against ocular disease, mice were infected and treated with ACV, GFAHP, or placebo (Fig. 1A–C). Disease development in the placebo group was similar to that observed in untreated infected mice (data not shown). Blepharitis was evident in all groups by day four, with scores of approximately 1 (Fig. 1A). Blepharitis continued to increase in the placebo and GFAHP-LD treated groups, peaking on day 12 with scores ranging from 1.92 to 2.5. In the GFAHP-HD, MD, and ACV treated groups, blepharitis peaked on day eight with scores of approximately 1, and began to heal soon thereafter. By day 18, when scoring was halted, blepharitis had essentially healed in the GFAHP-HD and MD treatment groups and had nearly healed in the ACV group. In the GFAHP-LD and placebo groups, blepharitis had not healed by this time point.

Corneal neovascularization was evident by day six through eight in all groups (Fig. 1B). In the placebo group, vascularization continued to increase in severity, reaching a score of 2.2 on day 14, when scoring was halted. In the GFAHP-HD, GFAHP-MD, and ACV treatment groups, vascularization also increased, but peaked on day 18 with a score between 0.2 and 0.5, substantially less than that observed in the placebo group. In the group treated with GFAHP-LD, vascularization peaked on day 10 with a score of 1.45 and declined to 0.65 on day 16.

Stromal keratitis was evident by day four and peaked on day eight in all groups (Fig. 1C). In the placebo group, stromal disease increased in severity, reaching a score of 1.75 on day eight, when scoring was halted. In the ACV and GFAHP treated groups, stromal disease was not as severe as in the placebo group. On day 18, the stromal disease scores were 0.4, 0.33, and 0.36 for the GFAHP-HD, GFAHP-MD, and ACV treated groups, respectively, and 1.6 for the placebo group.

3.3.2. Effect of GFAHP on disease incidence

GFAHP treatment had very little effect on the incidence of blepharitis. In the placebo, ACV, GFAHP-LD, and GFAHP-MD treated groups, 100% of the mice developed disease. In the GFAHP-HD treated groups, all but two of the mice developed blepharitis (77.8%). Peak incidence was reached between days 4 and 8 by all groups. The incidence declined after day 8 in the GFAHP-HD treated group, and reached 22.2% by day 18. GFAHP also reduced the incidence of vascularization from 100% in the placebo group to between 22% and 56% in the GFAHP-HD and GFAHP-MD treated groups. Treatment further reduced the incidence of stromal disease from 100% in the placebo group to approximately 70% in the GFAHP-HD and GFAHP-MD treated groups. In the GFAHP-HD treated groups,

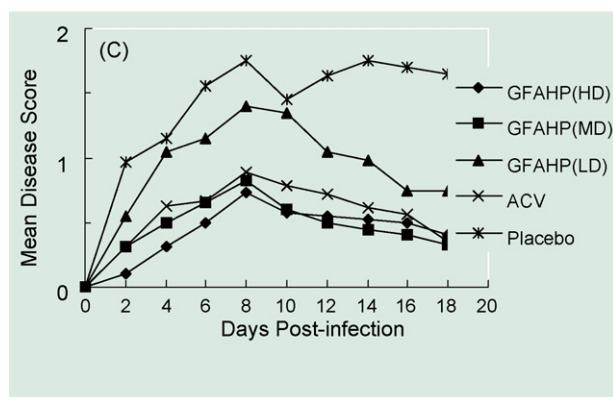
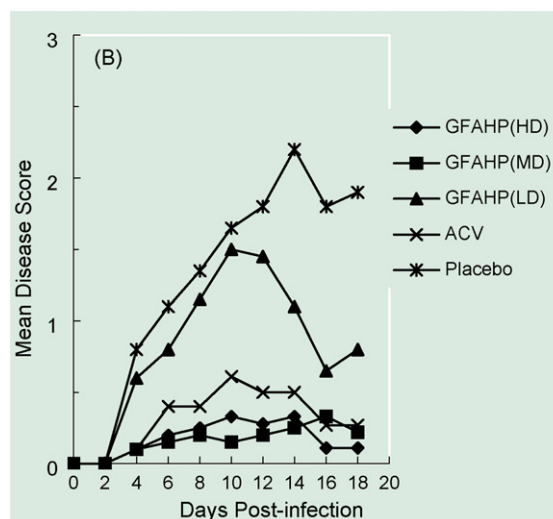
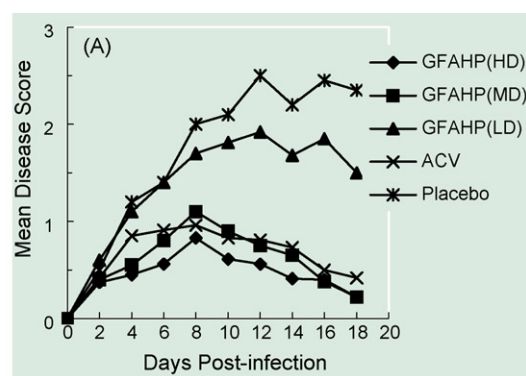


Fig. 1. Effect of GFAHP treatment on the severity of HSV-1 ocular disease. Mice were treated with GFAHP, ACV, or placebo. The severity of blepharitis (A), corneal vascularization (B), and stromal keratitis (C) were scored at various time points. GFAHP(HD), GFAHP(MD) and GFAHP(LD) represent the high dosage group, medium dosage group and low dosage group, respectively.

only two mice (22%) had permanent corneal clouding on day 16 (data not shown).

3.3.3. MPDSs

Mean peak disease score (MPDS) is used to compare the disease scores of multiple groups (Grau et al., 1989). Statistical analysis of the MPDSs revealed that GFAHP-LD did not significantly reduce blepharitis, vascularization, or stromal

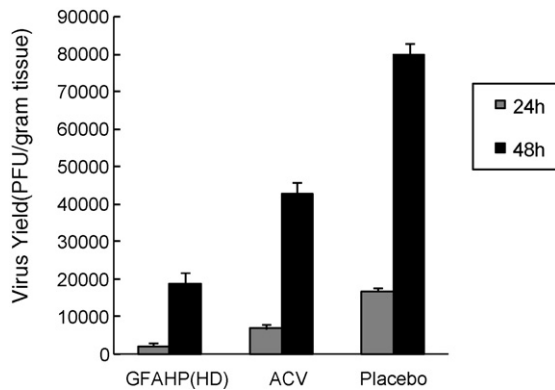


Fig. 2. Antiviral effects of GFAHP and ACV on HSV-1 replication in mouse corneas. GFAHP and ACV were administered to mice after virus inoculation. The right eyeball from each mouse was obtained at 24 and 48 h p.i., and the virus yield was quantitated by plaque-forming assay.

disease, with p values of 0.08, 0.12, and 0.09, respectively. Treatment with GFAHP-HD, GFAHP-MD, and ACV had a significant effect on blepharitis, however ($p < 0.005$). GFAHP-HD and GFAHP-MD also significantly reduced the MPDSs for vascularization and stromal disease ($p < 0.01$) (data not shown).

3.3.4. Antiviral effect of GFAHP-I on HSV-1 replication in the mouse cornea

We quantitated the amount of HSV-1 recovered from the eyeballs of mice treated with GFAHP-HD or ACV after virus inoculation. The mean virus titer in GFAHP-HD treated mice was 12.7% of that observed in the control group ($p < 0.01$) 24 h p.i. After 48 h, the mean virus yield in the GFAHP-HD group was 23.5% of that observed in the control group ($p < 0.01$). The virus yield after GFAHP-HD treatment was significantly lower than after ACV treatment ($p < 0.05$). ACV treatment also suppressed viral replication in the cornea, reaching 40.38% and 53.5% of the control groups at 12 and 24 h, respectively ($p < 0.05$ versus control group) (Fig. 2).

3.3.5. Histopathology of infected corneas

Corneal sections from day 18 infected mice were substantially thicker and edematous after placebo than GFAHP-HD treatment. The epithelium showed more evidence of necrosis and polymorphonuclear leukocyte infiltration after the placebo, and intranuclear inclusion bodies usually associated with HSV-1 infection were present. There was also a stromal infiltrate consisting of polymorphonuclear leukocytes and epithelioid cells. The corneal stroma was vascularized, and there were typical signs of both acute and chronic interstitial keratitis typical of HSV-1 corneal infection. The corneas from infected mice in the GFAHP-HD treatment group were almost normal, showing no evidence of inflammation, necrosis, or cells containing inclusion bodies (data not shown).

3.4. GFAHP toxicity in vivo

To determine if GFAHP was toxic *in vivo*, uninfected mice were treated with either placebo or GFAHP and at the end of the

treatment period the corneas were stained with fluorescein and examined for epithelial defects. No evidence of corneal damage such as punctate keratopathy was observed in either placebo or GFAHP treated mice (data not shown). Importantly, injured corneas of mice treated with and without GFAHP healed in a similar period of time, and corneal ulceration produced by needle scratching healed within 2 days regardless of GFAHP treatment. Histopathological analysis of the corneas revealed no discernible differences between GFAHP treated and untreated corneas (data not shown).

3.5. Identification of GFAHP

GFAHP was resuspended at 0.1 mg/ml in distilled water and treated with lipid solvents, ether, DMSO, acetone, or trypsinase, to determine the nature of its antiviral activity. Ether, acetone and DMSO had no effect on the antiviral activity of GFAHP. Treatment with trypsinase digestion reduced GFAHP activity to 27% after overnight incubation. These results illustrate that GFAHP is likely to be a protein or to have a proteinaceous component. Boiling GFAHP for 10 min did not reduce its antiviral activity.

GFAHP was isolated from the 40% ammonium sulfate precipitate of *G. frondosa* and its structure was identified using both physical and chemical analyses. Analysis of the second peak (fraction 6-GFAHP) with SDS-PAGE indicated that GFAHP contained one band with a molecular weight of approximately 29.5 kDa (Fig. 3). A piece of the filter containing the 29.5 kDa protein was then cut from the SDS-PAGE gel, and sequenced by MALDI-TOF-MS and nano ESI-MS/MS. The N-terminal sequence of GFAHP consisted of an 11 amino acid peptide, $\text{NH}_2\text{-REQDNAPCGLN-COOH}$. The data was processed using Mascot software and a database search was performed with the MS/MS ion on the Mascot engine. The sequences were not found to match any known amino acid sequences. The

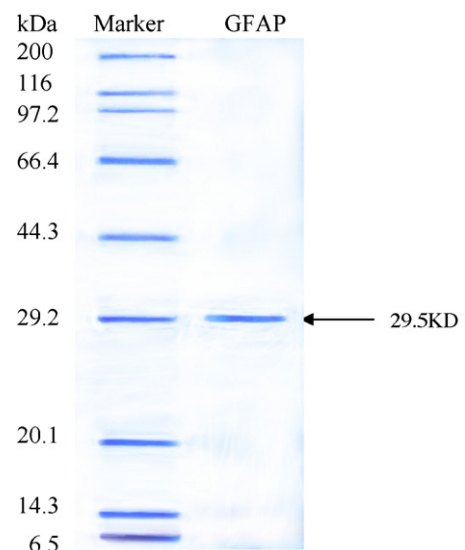


Fig. 3. Denaturing polyacrylamide gel electrophoresis of GFAHP. The material loaded in each lane is denoted in the figure. Samples were electrophoresed on a 15% gel and stained with Coomassie blue.

molecular weight of this protein from the MALDI-TOF-MS was 29.529 kDa.

4. Discussion

The present study described the isolation, partial structural characterization, and *in vitro* and *in vivo* activity of an anti-HSV-1 substance isolated from the edible mushroom, *G. frondosa*. GFAHP was isolated from water-soluble fractions of *G. frondosa* by ammonium sulfate precipitation and DEAE-cellulose column chromatography. Based on the anti-HSV-1 activity, the 40% ammonium sulfate precipitate had kept all of the antiviral activity. The active material was isolated and purified through DEAE-cellulose chromatographic column, and finally we got the antiviral activation fraction.

The IC₅₀ value of GFAHP was 4.1 and 0.42 µg/ml was for ACV. We investigated the mechanism of GFAHP antiviral activity *in vitro* by evaluating its ability to prevent infection and directly inhibit HSV-1 by preventing replication and cell adsorption and penetration. We found that GFAHP had direct virucidal effect on HSV, and inhibited viral entry, but could not prevent virus infection, or inhibit adsorption and replication. In a previous study, we reported that the D-Fraction (GF-D) extracted from *G. frondosa* had a significant inhibitory effect on hepatitis B virus (HBV) in HepG2 2.2.15 cells, and may also inhibit HBV replication (Gu et al., 2006).

When mice were infected with HSV-1 KOS and treated with various concentrations of GFAHP, the severity of ocular disease, as measured by scoring blepharitis, corneal vascularization, and stromal keratitis, was reduced in a dose-dependent manner. With the increase on GFAHP concentration, the severity of blepharitis, vascularization and stromal disease was reduced. The medium and high concentrations of GFAHP significantly reduced the severity of these diseases compared with the control and low GFAHP groups.

With the exception of ACV, many currently available nucleoside analog antiviral agents exhibit toxicity even when used topically (Squires, 2001). In the present study we showed that application of the high dose of GFAHP (150 µg/ml) for seven consecutive days (34 treatments) did not cause conjunctivitis or blepharitis, indicating that this protein has little if any toxicity, at least when applied topically. Histopathological examination of treated corneas also showed no evidence that the high dose of GFAHP is toxic to cells.

Active GFAHP was heat stable and partially sensitive to trypsinase digestion. Trypsinase was able to reduce GFAHP activity by 73% but unable to completely eliminate it. These findings illustrated that GFAHP is likely to be a protein or to have a proteinaceous component. Analysis using gel electrophoresis, MALDI-TOF-MS, and nano ESI-MS/MS suggested that it had a molecular weight of 29.5 kDa, and an N-terminal sequence with 11 amino acid peptides, NH₂-REQDNAPCGLN-COOH. The GFAHP N-terminus was used to search the SWISS-PORT and NCBIInr databases using the Mascot search engine (<http://www.matrixscience.co.uk>) with a tolerance of ±0.1 Da and one missed cleavage site. Three proteins with high similarity were chosen to analyze. The hypothetical pro-

tein, THERM_00324530, shared 87% similarity with GFAHP (7/8), and the 632nd amino acid in its N-terminus matched the first amino acid of GFAHP, however the function of this protein is unknown. One serine proteinase shared 82% similarity with the GFAHP N-terminus (9/11), and the 92nd amino acid in its N-terminus matched the first amino acid of GFAHP. Serine proteinases are involved in many processes of critical medical significance and are often involved in severe pathophysiological diseases (Czapinska and Otlewski, 1999). However, there is no report about that this protease has antiviral activity. Hypothetical protein, THERM_00637630 had 87% (7/8) similarity with GFAHP and its 206th amino acid matched with the 4th amino acid of GFAHP, however the function of this protein is also unknown. Thus, it is likely that GFAHP is a novel protein with antiviral activity.

A number of antiviral proteins have been described. Three different proteins with similar antiviral activity have been isolated from pokeweed, *Phytolacca Americana* (Hartley et al., 1991; Irvin and Uckun, 1992; Wang et al., 2006). *Trichosanthes kirilowii* contains a 29-kDa material that inhibits HIV (Lee-Huang et al., 1991). A protein of 10,425 Da purified from the edible mushroom, *Rozites caperata*, was shown to inhibit HSV-1 and -2 replication (Pirano and Brandt, 1999). A proteinaceous material with broad spectrum antiviral activity was isolated from the cactus, *Opuntia streptocantha* (Ahmad et al., 1996), and lectins are known to have activity against many different viruses (Ooi et al., 2004; Charan et al., 2000; Ye et al., 2001). A protein from the edible mushroom, *R. caperata*, has antiviral activity against HSV, varicella zoster, IVA, and respiratory syncytial virus (Pirano and Brandt, 1999). Velutin, a ribosome inactivating protein from *Flammulina velutipes*, is shown to inhibit HIV-1 reverse transcriptase (Wang and Ng, 2001), and a protein from *G. frondosa* inhibits tobacco mosaic virus infection (Chen et al., 2004). Recently, Pirano (2006) observed on the characterization and antiviral properties of a novel antiviral protein, RC 28, isolated from the mushroom *Rozites caperatus*. It is unlikely that GFAHP is related to a previously reported antiviral protein since its amino acid sequence did not match proteins with known antiviral activity. Thus, it is probable that GFAHP is a novel structure.

In summary, we have isolated and partially characterized a novel protein from the edible mushroom, *G. frondosa*, with potent antiviral activity. The antiviral substance, GFAHP which has a favorable IC₅₀ value of 4.1 µg/ml, is active against HSV-1 both *in vitro* and *in vivo*. Confirming the structure and determining the mechanism of GFAHP antiviral function should aid development of a new antiviral and help to identify novel features of the viral life cycle.

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