A novel antiviral protein, designated as Stellarmedin A, was purified from *Stellaria media* (L.) Vill. (Caryophyllaceae) by using ammonium sulfate precipitation, cation-exchange chromatography system. Gel electrophoresis analysis showed that Stellarmedin A is a highly basic glycoprotein with a molecular weight of 35.1 kDa and an isoelectric point of \( \sim 8.7 \). The N-terminal 14-amino acid sequence, MGNTGVLTGERNDR, is similar to those of other plant peroxidases. This protein inhibited herpes simplex virus type 2 (HSV-2) replication in vitro with an IC\(_{50}\) of 13.18 \( \mu \)g/ml and a therapeutic index exceeding 75.9. It was demonstrated that Stellarmedin A affects the initial stage of HSV-2 infection and is able to inhibit the proliferation of promyelocytic leukemia HL-60 and colon carcinoma LoVo cells with an IC\(_{50}\) of 9.09 and 12.32 \( \mu \)M, respectively. Moreover, Stellarmedin A has a peroxidase activity of 36.6 \( \mu \)mol/min/mg protein, when guaiacol was used as substrate. To our knowledge, this is the first report about an anti-HSV-2 protein with antiproliferative and peroxidase activities from *S. media*.

**Keywords**  *Stellaria media*; Stellarmedin A; anti-HSV-2; antiproliferative; peroxidase

Received: December 7, 2012  Accepted: March 18, 2013

**Introduction**

Herpes simplex virus (HSV) is a DNA-containing enveloped virus, which brings commonly viral infections in humans causing a variety of diseases. HSV-1 and HSV-2 can be distinguished on the basis of clinical manifestations and biochemical and serological characteristics. Generally, HSV infection is benign or asymptomatic in immune-competent individuals. However, in patients with an immature or weak immune system, such infections can be serious and even life-threatening [1,2]. The major antiviral therapy for HSV infection involves the use of nucleoside analogues, such as acyclovir. However, the increasing clinical use of this type of antiviral agents has been linked with the emergence of drug-resistant herpes virus strains [3–5]. Therefore, the development of anti-HSV agents with different modes of action is required.

The study of medicinal plants can lead to the identification of novel bioactive plant compounds which are suitable for drug discovery and development. Recently, research on herbs prescribed in traditional Chinese medicine (TCM) has attracted great attention as many have shown numerous biological activities including anti-HSV capability [6–9], and a number of anti-HSV proteins derived from herbs have been described [10–12].

*Stellaria media* (L.) Vill. (Caryophyllaceae) is a known invasive weed which disturbs grounds in the world. As one of the traditional medicinal plants, this plant has been used for hundreds of years in Chinese community, and shows very effective anti-inflammatory and antiviral activities [13]. In both Europe and North America, its herbal use also has a very long history, especially in the external treatment of skin itching [14]. However, not all of these uses are supported by scientific evidence; only those using phenolic acids, flavones [15,16], lipid [17], oligosaccharides [18], and cyclic dipeptides [19] have been reported. The composition of the effective constituents in this plant has not been adequately studied.

It has been reported that the extract of *S. media* is very useful for the treatment of HSV and is widely used in the eastern and southern regions of China. In this study, a series of experiments were conducted to investigate the anti-HSV-1 and anti-HSV-2 activity of *S. media* in vitro. A novel protein, designated as Stellarmedin A, was found to process antihSV-2 activity as well as strong antiproliferative and peroxidase activities. Our findings suggest that Stellarmedin A might have potential applications in agriculture and in medicine.

**Materials and Methods**

**Materials**

*Stellaria media* was obtained from Nanjing Botanical Garden, Mem. Sun Yat-sen (Nanjing, China) and authenticated by
Preparation of S. media extracts
The whole plants were rinsed, chopped, and homogenized in threes volumes of extraction buffer [50 mM Tris–Cl, pH 6.8, containing 0.2 M NaCl, 10 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpyrroldone]. The homogenate was stirred for 12 h and centrifuged at 8000 × g for 20 min. The supernatant (fraction 1) was lyophilized and resuspended in deionized water (final concentration of 20 mg/ml). Ammonium sulfate was slowly added to a final concentration of 85%, stirred for 2 h, and centrifuged at 8000 × g for 15 min. The precipitate (fraction 2) was resuspended in deionized water. Both fraction 2 and the supernatant (fraction 3) were dialyzed against water for 2 days (four changes of water). The dialysate (fraction 4) was lyophilized and stored at −70°C. All extraction procedures were conducted at 4°C. The antiviral activity of each fraction was determined by plaque reduction assay.

Purification of Stellarmedin A
The fraction containing the antiviral activity (fraction 2) was subjected to column chromatography by using a CM Sepharose column (15 × 2.6 cm; GE). Equilibration, loading, and washing were carried out in 20 mM sodium phosphate (pH 6.8) and elution was performed with a linear NaCl gradient at a flow rate of 5 ml/min at 4°C. After removal of unabsorbed material (fraction 5), the column was eluted consecutively with 0.1 M NaCl (fraction 6), 0.2 M NaCl (fraction 7), and 1.0 M NaCl (fraction 8). The absorbance at 280 nm of each fraction was measured. Each fraction was collected, concentrated, and desalted using 10 kDa ultrafiltration devices (Millipore, Bedford, USA), and tested for anti-HSV-2 activity by using plaque reduction assay.

The active fraction (fraction 6) was dialyzed against water at 4°C, adjusted to 20 mM sodium phosphate (pH 6.8) containing 150 mM NaCl and loaded onto a Superdex 75 column (120 × 2.0 cm; GE) which was previously equilibrated with same buffer by using a AKTA-fast protein liquid chromatography (FPLC) system (Pharmacia Amersham Biotech, Uppsala, Sweden). The proteins were eluted with an isocratic gradient of 20 mM sodium phosphate (pH 6.8) containing 150 mM NaCl, at a flow rate of 0.2 ml/min. Each fraction (2 ml) was monitored at 280 nm and analyzed for anti-HSV-2 activity. The active fractions were then pooled, analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The purified antiviral protein exhibited a single band and was designated as Stellarmedin A.

Determination of molecular weight and isoelectric point
Stellarmedin A was electrophoresed on a 15% denaturing–reducing SDS–PAGE as described previously [20]. The apparent molecular weight of the purified Stellarmedin A was determined by using an FPLC system (TSK-Gel G4000 PWXL column) equilibrated and eluted with 50 mM phosphate-buffered saline (PBS; pH 6.8) at a flow rate of 0.8 ml/min. The isoelectric point (pl) of the purified protein was determined by isoelectric focusing under non-denaturing condition on 5% polyacrylamide gel containing ampholines (pH 8.0–10.5) using the method of Righetti and Chellemi [21].

Carbohydrate-specific periodic acid schiff staining
The staining assay was carried out according to a previously published paper [22]. After SDS–PAGE, the gel was incubated in fixation solution for 2 h and then in periodic solution for another 1 h. After rinsing with water, the gel was incubated in metabisulfite solution for 10 min and then in Schiff’s reagent in dark for 3 h. After glycoprotein bands were developed to bright red bands, the gel was washed twice with metabisulfite solution in dark for 1 h and finally washed four times with recovery solution.

Analysis of N-terminal amino acid sequence
The purified Stellarmedin A was electrophoresed and transferred to a polyvinyl fluoride membrane. The band containing the active material was excised from the membrane and sequenced from the amino terminus using the trypsin digestion procedure [23]. The N-terminal sequence was determined by automated Edman degradation using a Procise Protein Sequencer 492 (Applied Biosystems, Carlsbad, USA).

Cytotoxicity evaluation
As a preliminary test of toxicity, replicate wells of uninfected cells were exposed to various concentrations of test substances and incubated for 16–18 h at 37°C. Cell survival rate was measured by using MTT assay as previously described [24]. Cytotoxicity was expressed as 50% of cytotoxic concentration (CC₅₀) at which a substance inhibited up to 50% of cell growth.
Therapeutic effect of Stellarmedin A on HSV infection
To determine anti-HSV activity, Vero cells were grown in 24-well culture plates at $2 \times 10^5$ cells/well for 48 h to reach at least 95% confluence. The medium was then discarded and the cell monolayer was infected with 100 plaque-forming units (PFU) of HSV-1 or HSV-2. After an adsorption period of 1 h, the cells were rinsed twice with PBS (pH 7.4) and mixed with media containing different concentrations of Stellarmedin A. The amount of infectious virus was quantified by plaque assay until 48 h post-infection (p.i.) [25]. Test was carried out in triplicate.

Time-of-addition study
The time-of-addition effect on HSV-2 was examined according to previously described procedures with minor modifications [26]. Briefly, Vero cells were grown in 24-well culture plates at a density of $2 \times 10^5$ cells/well for 48 h to reach at least 95% confluence. For pre-infection groups, Stellarmedin A (62.5 µg/ml) was added to plates 6 or 2 h before infection and then cells were washed twice in PBS to eliminate Stellarmedin A and exposed to the virus. For p.i. groups, Stellarmedin A was added at the 0th, 2nd, 4th, and 8th h of infection. The inhibitory rates of all groups were calculated until 48 h p.i.

Peroxidase activity assay
Peroxidase activity assays were carried out as previously described [27]. Guaiacol peroxidase (GPrx) activity was determined and expressed as µmol/min/mg of total protein.

Antiproliferative activity assay
The cytotoxic effect of Stellarmedin A on tumor cells was determined by using MTT staining. Briefly, $5 \times 10^5$ cells/well were plated in 96-well plates with 100 µl of culture medium for 24 h, and then exposed to different concentrations of Stellarmedin A. After 72 h, the culture medium was replaced with 100 µl of fresh medium containing 0.5 mg/ml MTT and incubated for another 4 h at 37°C. This medium was then removed, and 100 µl of DMSO was added to each well to dissolve the purple formazan crystals. IC$_{50}$ (50% of inhibitory concentration) was calculated by measuring the absorbance at 570 nm with an automatic plate reader (Bio-Tek Instrument Inc., Winooski, USA). Each experiment was repeated three times.

Statistical analysis
Data were expressed as mean ± SD of three independent experiments. The Student’s t-test was used to analyze data. $P < 0.05$ was considered to be statistically significant.

### Table 1 Anti-HSV-2 activity of fractions isolated from S. media

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Chromatographic fraction</th>
<th>IC$_{50}$ (µg/ml)$^a$</th>
<th>Yield (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water extract</td>
<td>378</td>
<td>4.17</td>
</tr>
<tr>
<td>2</td>
<td>85% ammonium sulfate</td>
<td>238</td>
<td>0.72</td>
</tr>
<tr>
<td>3</td>
<td>precipitate</td>
<td>Inactive</td>
<td>0.21</td>
</tr>
<tr>
<td>4</td>
<td>85% ammonium sulfate</td>
<td>Inactive</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>supernatant</td>
<td>Inactive</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>ammonium sulfate</td>
<td>Inactive</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
<td>diaylsate</td>
<td>Inactive</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>through column</td>
<td>Inactive</td>
<td>0.01</td>
</tr>
<tr>
<td>9</td>
<td>through column</td>
<td>Inactive</td>
<td>0.01</td>
</tr>
</tbody>
</table>

$^a$The lowest concentration resulted in 50% inhibition of its cytopathic effect (mg/ml) against HSV-2. Vero cells were inoculated into wells of a 96-well plate and infected with HSV-2 at 2 PFU/cell at 48th h. Serial dilutions of each sample were added and the wells were scored at 48 h p.i. for the presence of a cytopathic effect.

$^b$Percent of starting material.
pattern showed that the protein migrated towards the cathode end of focused gel and the pI was calculated to be ~8.7.

The N-terminal sequence of Stellarmedin A, MGNTGVLGGERNDR, was compared with several peroxidase sequences available in SWISS-PROT and NCBInr databases. Results are listed in Table 3. It was shown that Stellarmedin A was not identical to any protein published so far. However, it exhibited 64.3% similarity to the N-terminal sequences of peroxidases from other plants.

Cytotoxic effect on viability of Vero cells and anti-HSV activities of Stellarmedin A

The cytotoxic activity of Stellarmedin A was investigated by MTT assay. Results showed that Stellarmedin A had no cytotoxic effects at concentrations below 1000 μg/ml. The CC50 and CC0 values of Stellarmedin A exceeded 1000 μg/ml (Table 2). Therefore, the anti-HSV activity of Stellarmedin A was examined at a concentration of 250 μg/ml or lower.

The inhibitory effect of Stellarmedin A on HSV-1 and HSV-2 was investigated by the plaque reduction assay (Fig. 3). Results showed that Stellarmedin A inhibited HSV-2 infection in a dose-dependent manner but did not inhibit HSV-1 infection (Table 2). The IC50 was 13.18 μg/ml and the therapeutic index (CC50/IC50) was >75.9.

To investigate the inhibitory effect of Stellarmedin A on the stage of HSV-2 infection, Stellarmedin A was added at different times of HSV-2 infection. Figure 4 showed that Stellarmedin A effectively inhibited HSV-2 infection with an inhibitory rate >89% when added concurrently. However, the inhibitory rate declined to 32% or lower in 2, 4, and 8 h p.i. groups. In contrast, pre-treatment of cells with Stellarmedin A did not reduce HSV-2 infectivity (Fig. 4), suggesting that Stellarmedin A affected the initial stage of HSV-2 infection.

Table 2 Anti-HSV activity of Stellarmedin A by plaque reduction assay

<table>
<thead>
<tr>
<th>Antiviral substances</th>
<th>Anti-HSV-1 activity</th>
<th>Anti-HSV-2 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (μg/ml)a</td>
<td>CC50 (μg/ml)b</td>
</tr>
<tr>
<td>Stellarmedin A</td>
<td>&gt;250</td>
<td>&gt;1000.0</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>0.67 ± 0.11</td>
<td>&gt;300.0</td>
</tr>
<tr>
<td></td>
<td>13.18 ± 1.50</td>
<td>&gt;1000.0</td>
</tr>
</tbody>
</table>

aIC50, 50% inhibition concentration, defined as a drug concentration that induced 50% inhibition of HSV-1 or HSV-2 (as compared to the untreated culture).
bCC50, 50% cytotoxic concentration, defined as a drug concentration that induced 50% inhibition of Vero cells (as compared to the untreated culture).
cTI50, therapeutic index, calculated as the ratio of CC50 over IC50.

Figure 2 Determination of molecular weight of Stellarmedin A M, standard marker; 1, Stellarmedin A from Superdex 75 column; 2, Stellarmedin A with PAS staining. Lactoferrin (71 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa) were used as standard markers.
Peroxidase activity of Stellarmedin A was investigated in vitro using the substrate, guaiacol (o-methoxyphenol), which is often used in peroxidase enzyme assays [28]. Peroxidase activity of Stellarmedin A was 36.6 μmol/min/mg protein.

Antiproliferative activity of Stellarmedin A

Two human cancer cell lines HL-60 and LoVo and one human normal cell line HFB were used to test the effect of Stellarmedin A on cell proliferation by using MTT assay. Stellarmedin A exerted potent and concentration-dependent cytotoxic effects on HL-60 and LoVo cells with an IC50 of 9.09 and 12.32 μM, respectively (Fig. 5). In contrast, Stellarmedin A barely had inhibitory effects on the proliferation of HFB cells.

Discussion

In the present study, an anti-HSV-2 protein, Stellarmedin A, was isolated from S. media. The N-terminus of the protein was used to search SWISS-PROT and NCBIInr databases by using Mascot search engine (http://www.matrixscience.co.uk) with a tolerance of ±0.1 Da and a missed cleavage site. Five proteins with high similarity were chosen for further analysis (Table 3). Acyl-CoA dehydrogenase-like protein (YP_523632.1) shared high similarity with Stellarmedin A (9/14), and the 247th amino acid in its N-terminus matched the 5th amino acid in that of Stellarmedin A. However, the source of this protein is Rhodoferax ferrireducens T118 and the relativeness between two species is too distant. At least four plant peroxidase isozymes (AAC98519.1, AAB41810.1, AAD37427.1, and BAD97439.1) shared 64.3% similarity with Stellarmedin A N-terminus, suggesting that Stellarmedin A was a novel plant protein with peroxidase activity. Subsequently, enzymatic activity assay confirmed that Stellarmedin A had guaiacol peroxidase activity.

Peroxidase is involved in many significant processes in plant physiology [29]. As a commercial reagent, peroxidases are often applied in immunohistochemical, enzyme-linked immunosorbent assay (ELISA), and immunoblotting methods. Besides, they have medicinal applications. It has been reported that a 34 kDa peroxidase with antifungal activity against Fusarium solani (IC50 = 76 μM), Mycosphaerella arachidicola (IC50 = 103 μM), and Pythium aphanidermatum (IC50 = 119 μM) is isolated from lima bean seeds.

Table 3 Comparison of the N-terminal amino acid sequences (14 amino acids) of Stellarmedin A and other plant peroxidase proteins

<table>
<thead>
<tr>
<th>Source</th>
<th>Name</th>
<th>N-terminal sequence</th>
<th>Residue sites</th>
<th>Percent identity (%)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stellaria media</td>
<td>Stellarmedin A</td>
<td>MGNTGVLTGERNDR</td>
<td>1–14</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Glycine max</td>
<td>Peroxidase precursor</td>
<td>MGNI GVTGKG EiKr</td>
<td>308–322</td>
<td>64.3</td>
<td>AAC98519.1</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>Peroxidase</td>
<td>MGNI GVTGTKGEiR</td>
<td>300–314</td>
<td>64.3</td>
<td>AAB41810.1</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>Peroxidase 1 precursor</td>
<td>MGNI GVTGSQGEiR</td>
<td>297–311</td>
<td>64.3</td>
<td>AAD37427.1</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>Peroxidase</td>
<td>MGNI GVTGNKGEiR</td>
<td>309–323</td>
<td>64.3</td>
<td>BAD97439.1</td>
</tr>
<tr>
<td>Rhodoferax ferrireducens T118</td>
<td>Acyl-CoA dehydrogenase-like</td>
<td>VNEAGVTGERNDV</td>
<td>243–256</td>
<td>64.3</td>
<td>YP_523632.1</td>
</tr>
</tbody>
</table>

Figure 3 Concentration effect of Stellarmedin A on HSV-1 and HSV-2 replication in Vero cells. The IC50 was calculated using regression line. Data were expressed as mean ± SD from three independent experiments.

Figure 4 Time-of-addition effect of Stellarmedin A on HSV-2 replication in Vero cells. Stellarmedin A (62.5 μg/ml) was added at different times of virus infection. Data were expressed as mean ± SD from three independent experiments.
French bean legumes produce a 37 kDa peroxidase with inhibitory activity against mycelia growth of Botrytis cinerea, Fusarium oxysporum, and M. arachidicola [32]. However, there is no report showing that this protease has antiviral activity. Stellarmedin A is the first peroxidase with antiviral activity isolated from S. media.

A number of antiviral proteins have been described, which can be classified into three groups: ribosome-inactivating proteins (RIP), lectins, and proteins isolated from fungi. RIPs are toxic N-glycosides that inactivate ribosomes and block protein synthesis [33]. Examples of this group include trichosanthin (TCS) which is isolated from Trichosanthes kirilowii and inhibits replication of HIV-1 [34], and MAP30 from the seeds of Momordica charantia, which exhibits potent anti-HSV and anti-HIV activities [35]. Lectins are defined by their ability of clumping erythrocytes and other types of animal and human cells [36]. Typhonium divaricatum lectin (TDL) is isolated from Typhonium divaricatum [37] and Polygonatum odoratum lectin (POL) from rhizomes of Polygonatum odoratum [11], both of which have antiviral activity against HSV-2 and antiproliferative effects on human cancer cell lines. The last type comprises proteins isolated from fungi, especially edible mushrooms, such as RC-183 purified from the edible mushroom, Rozites caperata, which was shown to inhibit HSV-1 and HSV-2 replication [25]. GFAHP from Grifola frondosa exhibits antiviral activity against HSV-1 both in vitro and in vivo [38]. In comparison, the lack of identity with any other proteins with known antiviral activity indicated that Stellarmedin A had a novel structure.

The anti-HSV activity assay showed that Stellarmedin A of S. media inhibited HSV-2 but not HSV-1 infection, suggesting some differences between the antiviral profiles of Stellarmedin A from S. media and currently available anti-HSV drugs, most of which have broad-spectrum anti-HSV activity [39]. The effect of addition time on the antiviral activity indicated that the initial stage of HSV-2 infection was inhibited by Stellarmedin A. Currently available anti-HSV drugs mainly inhibit the initial stage of HSV-2 infection by the virus attaching, penetrating cell membrane, and transferring viral DNA into cell nucleus [40]. In addition, treatment of supercoiled plasmid pUC18 revealed that Stellarmedin A exerted DNA-cleavage activity (Supplementary Fig. S1), which might involve in the mechanism of Stellarmedin A antiviral activity. Further studies are required to investigate the detailed mechanism of this activity and to validate the feasibility of developing Stellarmedin A as an anti-HSV agent.

Acknowledgement

The authors are grateful to Associate Prof. Xinyu Li (Dermatosis Research Center, China Medical Academy of Science, Nanjing, China) for providing the viruses.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (31100251).

References
