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Antiviral activity of sulfated *Chuanminshen violaceum* polysaccharide against duck enteritis virus *in vitro*

Xu Song [a,1], Zhongqiong Yin [a,b,1,*], Li Li [a,1], Anchun Cheng [a,b], Renyong Jia [a,b], Jiao Xu [a], Yin Wang [a], Xueping Yao [a], Cheng Lv [a], Xinghong Zhao [a]

[a] College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, China
[b] Institute of Prevention Veterinary Medicine, Sichuan Agricultural University, Chengdu 611103, China

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ABSTRACT

Duck enteritis virus (DEV) of the family *Herpesviridae* is one of the main diseases in waterfowl. Despite the wide use of vaccines to control the disease, infection with the virus cannot be completely prevented. Therefore, antiviral agents against DEV should be developed. This study presents a novel sulfated polysaccharide from *Chuanminshen violaceum* (sCVPS), which exhibits significant antiviral activity against DEV with 50% inhibitory concentrations (IC_{50}) ranging from 77.12 μg/mL to 104.81 μg/mL. sCVPS is more effective than heparan sulfate (HS, as a positive control) with IC_{50} = 132.61 μg/mL. sCVPS and HS inhibit viral activity by preventing virus adsorption with IC_{50} values ranging from 82.83 μg/mL to 109.28 μg/mL for sCVPS and 150.22 μg/mL for HS. Direct immunofluorescence assay and transmission electron microscopy demonstrated that the mechanism of action was the interference with virus adsorption. The amount of inhibited virus during adsorption was quantified using fluorescent quantitative polymerase chain reaction, which revealed that both sCVPS and HS can significantly reduce all viruses attached to cells. sCVPS also prevented the cell-to-cell spread of DEV. These results indicated that sCVPSs perform more effectively than does HS as antiviral agents against DEV and can be further examined for potential effects as an alternative control measure for DEV infection.

1. Introduction

The *Herpesviridae* are a large family of double-stranded DNA viruses that cause serious diseases in humans and animals (Siakallis et al., 2009). Duck enteritis virus (DEV), a member of the herpesvirus, can cause an acute infectious viral disease called duck virus enteritis (DVE) or duck plague in ducks, geese, swans, and other waterfowl species (Mondal et al., 2010; Qi et al., 2009). DEV infection can result in high mortality, carcass condemnation, and reduced egg production or hatchability (Wang et al., 2011a; Wu et al., 2012a). Despite the use of attenuated vaccines that can provide protection against DEV, infection is not prevented completely (Li et al., 2009; Mondal et al., 2010; Wang and Osterrieder, 2011; Wang et al., 2011a). Moreover, no antiviral agents against DEV are available.

The antiviral activity of sulfated polysaccharides was first reported in 1958 by Gerber (Gerber et al., 1958), who found that polysaccharides extracted from *Gelidium cartilagenium* prevented influenza B and mumps virus infection in embryonated eggs. Many sulfated polysaccharides have been found to possess a broad spectrum of antiviral activity since the report (Han et al., 2010). The inhibitory effect of sulfated polysaccharides can potentially block early stages of viral life, including initial attachment to the target cell and viral entry (Damonte et al., 2004; Harden et al., 2009). Therefore, sulfated polysaccharides exhibit an inhibitory activity mainly by interaction with viral envelope proteins involved in virus infection of susceptible cells. In DEV, glycoprotein C significantly affects virus adsorption and transmission, binding to heparan sulfate proteoglycans present on the cell surface, leading to initial attachment (Lian et al., 2010). DEV entry is mediated by the coordinated effects of glycoprotein B, D, and H/L with cell receptors, resulting in fusion and penetration (Heldwein and Krummenacher, 2008; Li et al., 2009; Spear and Longnecker, 2003). These glycoproteins may act as candidate target receptors in anti-DEV drug design.

Chuanminshen violaceum is a traditional Chinese medicinal herb used as a tonic. *C. violaceum* polysaccharides (CVPSs) mainly constitute (almost 28%) *C. violaceum* (Lei and Zhang, 2012). CVPS is composed of D-carubinose and D-glucose with a ratio of 1:16.2 (Zhang et al., 2007). The weight average molecular weight and the number average molecular weight of CVPS are 9.7632×10^5 Da and 5.2270×10^4 Da, respectively (Zhang et al., 2007). CVPS can strengthen specific and nonspecific humoral immunity in mice and exhibits an anti-mutagenic effect (Li and Shao, 1996; Zhang et al., 2007).

* Corresponding author at: College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, China. Tel.: +86 835 2885614; fax: +86 835 2885302.
E-mail address: yinzhongq@163.com (Z. Yin).
[1] These authors contribute equally to this work and should be considered as the first author.

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Sulfated modification has been widely used to improve polysaccharide structure and enhance antiviral activity (Huang et al., 2008a; Lu et al., 2008; Wang et al., 2010a; Zhao et al., 2011). Moreover, some polysaccharides that originally showed no antiviral activity exhibited antiviral activity after sulfated modification (Huang et al., 2008a). In this paper, we report on the sulfated modification of CVPSs by the chlorosulfonic acid-pyridine method and the characterization of its antiviral efficacy against DEV. This investigation aims to provide a new anti-herpesvirus drug candidate with development potential and a new alternative control measure for DEV infection.

2. Materials and methods

2.1. Sulfated modification of CVPS

CVPS was extracted and purified in our laboratory (Song et al., 2013). Sulfated CVPS (sCVPS) was prepared by the chlorosulfonic acid-pyridine method (Guo et al., 2009b). Chlorosulfonic acid was added dropwise to pyridine (1:4, 1:6, and 1:8) in an ice-water bath, with stirring. Polysaccharides (300 mg) dispersed in dry *N,N*-dimethylformamide (20 mL) were then added to the mixtures and stirred in a water bath at 60 °C for 2 h. The solution was subsequently neutralized with NaOH, dialyzed, and lyophilized to yield sulfated CVPS. The sulfur content of three sCVPSs was measured by the barium chloride–gelatin method (Dodgson and Price, 1962).

2.2. Cell culture and virus infection

A duck embryo fibroblast (DEF) was grown in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) bovine calf serum (Gibco®), 100 U/mL penicillin, and 100 μg/mL streptomycin. For the maintenance medium (MM), serum concentration was reduced to 2%.

DEV (CH virulent strain) was provided by the Institute of Prevention Veterinary Medicine (Chengdu, China). Virus stocks were propagated in DEF and prepared as described in a previous report (Guo et al., 2009a).

2.3. Cytotoxicity assays

The cytotoxicity of CVPS, sCVPS, and HS (H7640; sulfur content: 5–7%; Sigma–Aldrich, USA) was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich, USA) method (Wang et al., 2011b). Thereafter, 2.5×10^4 DEF cells were added to each well of 96-well plates and incubated at 37 °C for 24 h. The growth medium was withdrawn; MM containing twofold dilutions of compounds was added in triplicate. After 72 h of incubation at 37 °C, 10 μL of PBS containing MTT (final concentration: 0.5 mg/mL) was added to each well. Plates were reincubated at 37 °C for 4 h, the supernate in each well was aspirated, and 200 μL of dimethyl sulfoxide was added to solubilize the formazan crystals. After vigorous shaking, absorbance values were measured in a microplate reader (Bio-Rad, USA) at 570 nm. The 50% cytotoxic concentration (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.4. Antiviral activity of sCVPS and HS

Antiviral activity was evaluated by plaque reduction assay (Talarico et al., 2005). The DEF monolayer in 24-well plates was infected with about 50 plaque-forming units (PFU)/well with or without various concentrations of test polysaccharides. After adsorption for 1 h at 37 °C, the medium was replaced with MM containing 1% methylcellulose and a corresponding dose of each compound. After incubation for 72 h at 37 °C, the cells were stained with 0.1% crystal violet in 20% methanol. Plaques were counted, and the 50% inhibitory concentration (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50% according to the Reed–Muench method (Reed and Muench, 1938).

2.5. Inhibitory action assays

2.5.1. Pretreatment assay

Dilutions of test polysaccharides were added to each well of 24-well plates containing a DEF monolayer, and the plates were incubated at 37 °C for 1 h. The compounds were removed, and the monolayer was washed thrice with PBS (pH = 7.4). Virus suspensions (50 PFU/well) were then added to each well. After incubation at 37 °C for 1 h, the cells were rinsed thrice with PBS and overlaid with MM containing 1% methylcellulose. The plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.2. Virus adsorption assay

A DEF monolayer grown in 24-well plates was infected with about 50 PFU/well with or without various concentrations of test polysaccharides. After incubation at 4 °C for 1 h, the monolayer was washed thrice with cold PBS to remove unadsorbed viruses and then overlaid with MM containing 1% methylcellulose. Plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.3. Virus penetration assay

A DEF monolayer grown in 24-well plates was infected with about 50 PFU/well and incubated at 4 °C for 1 h. Virus inocula were aspirated and washed twice to remove unadsorbed viruses. Dilutions of test polysaccharides were added to each well, and plates were incubated at 37 °C for 1 h to allow penetration. After removal of compounds, the monolayer was rinsed with a citrate buffer (pH 3.0) to inactivate virions that had not penetrated the cells. MM containing 1% methylcellulose was then added to each well, and plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.4. Virus replication assay

A DEF monolayer grown in 24-well plates was infected with about 50 PFU/well and incubated at 37 °C for 2 h to allow adsorption and penetration. MM containing 1% methylcellulose and dilutions of test polysaccharides was then added to each well, and plates were incubated at 37 °C for 72 h. Plaques were counted, and the IC_{50} was calculated.

2.5.5. Virus inactivation assay

MM containing dilutions of test polysaccharides was incubated with an equal volume of virus suspension (5×10^5 PFU) at 37 °C for 1 h. The solution was then diluted 10^4 times, and the titer of residual infectious virus was quantified by plaque assay.

2.6. Plaque size assay

A DEF monolayer grown in 6-well plates was infected with about 100 PFU/well. After incubation at 37 °C for 2 h to allow penetration, the inocula were removed. MM containing 1% methylcellulose and $sCVPS_{1.37}$ (50, 100, and 200 μg/mL) was then added, and plates were incubated at 37 °C for 72 h. The plaque size was calculated using Nikon Imaging Software-Elements. The average plaque