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Porcine epidemic diarrhea virus infection: Inhibition by polysaccharide from *Ginkgo biloba* exocarp and mode of its action

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is the predominant cause of severe entero-pathogenic diarrhea in swine. Until now there is no recorded clinically effective antiviral chemotherapeutic agent for treatment of diseases caused by PEDV. This study aimed to investigate in vitro anti-PEDV effect of polysaccharide from *Ginkgo biloba* exocarp and mode of its action. The polysaccharide exhibited potent antiviral activity against PEDV reducing the formation of a visible CPE [a 50% inhibitory concentration (IC_{50}) = 1.7 ± 1.3 μg/mL], compared to positive control, ribavirin and it did not show cytotoxicity at 100 μg/mL [a 50% cytotoxicity concentration (CC_{50}) = 100 μg/mL]. Polysaccharide also showed effective inhibitory effects when added at the viral attachment and entry steps. Moreover, polysaccharide effectively inactivated PEDV infection in time-, dose- and temperature-dependent manners. Overall, this research revealed that polysaccharide could inhibit PEDV infection, and that polysaccharide may be involved in PEDV-Vero cell interactions, as the virus attachment and entry to the Vero cells was hindered by the polysaccharide. Therefore, polysaccharide possessing effective inhibitory effect on viral attachment and entry steps of PEDV life cycle is a good candidate for development of antivirals.

1. Introduction

Porcine epidemic diarrhea (PED) is an acute, highly contagious viral enteric disease of swine caused by PED virus (PEDV) (Bridgen et al., 1993). PEDV is a devastating enteric disease that is characterized by vomiting, acute severe watery diarrhea and dehydration, which results in significant high mortality in piglets under 7 days of age (Temeeyasen et al., 2013). At present, PED disease has continued to causing severe economic losses in many Asian countries, including China, Korea, Japan and Thailand (Gao et al., 2013; Li et al., 2012). Therefore, these problems demonstrate the need for the development of novel antiviral agents.

Medicinal plants have been traditionally used for the treatment of ailments (Mukhtar et al., 2008). *Ginkgo biloba* L. is cultivated because of its importance in traditional medicine and food value; and as an ornamental tree for its attractive shape and foliage (Yang et al., 2013). Recent studies have also implicated a use for *G. biloba* extract in prevention of Alzheimer's disease and dementia (Vellas et al., 2012; Weinmann et al., 2010). Beneficial actions of the extract against ischemia/reperfusion injury, hypoxia, cognitive deficits and dementia have also been described (Abdel-Salam et al., 2004).

In South Korea, *G. biloba* is the most tree species growing in the side of street. However, the citizens of South Korea did not use it because they think *G. biloba* contained materials such as heavy metals, air contaminants, etc., and although its effective value are emerging. Odor also was emitted by *G. biloba* exocarp of yellow color. *G. biloba* exocarp of yellow color was attached on road and rotten bad quickly. Therefore, it is still a challenge to satisfy the recycling requirement of *G. biloba.*

In this study, we investigated whether polysaccharide identified from *G. biloba* exocarp of yellow color exert has antiviral activity against PEDV in vitro. Furthermore, we elucidated the action of polysaccharide on PEDV multiplication in more detail.

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2. Materials and methods

2.1. Virus, cell and reagents

Vero (African green monkey kidney cell line; ATCC CCR-81) was kindly provided by ATCC (American Type Culture Collection, Manassas, VA, USA). PEDV CV 777 (porcine epidemic diarrhea virus) was obtained from national veterinary research & quarantine service in Korea. Vero cell lines were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic. Antibiotic-antimycotic, trypsin-EDTA, FBS and MEM were supplied by Gibco BRL (Grand Island, NY, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, NJ, USA). Ribavirin and sulforhodamine B (SRB) were purchased Sigma-Aldrich (St. Louis, MO). All other chemicals were of reagent grade.

2.2. Fractionation and isolation

G. biloba exocaps (1 kg) were extracted with 98% ethanol (3 L) at 90 °C. The ethanolic extract was suspended in H_2O and partitioned with hexane, ethyl acetate and butanol, sequentially. The water-soluble fraction showed anti-PEDV activity. The water-soluble fraction was chromatographed over HP-20-column chromatography using a stepwise gradient of MeOH-H_2O (from 0% to 100% with 20% increments; 500 mL for each step), to yield seven fractions (Fr. 1–Fr. 7). Of these, Fr. 3 showed the most potent anti-PEDV activity. Fr. 3 was further purified over a YMC gel column chromatography eluting with a gradient from 0% to 100% MeOH in H_2O, to yield six fractions. Fr. 3–2 (eluted with 10% MeOH in H_2O) showing anti-PEDV activity, conducted gel permeation chromatography and lyopilization. The purified compound 1 was verified as polysaccharide. Total sugar content in the purification polysaccharide was determined by the phenol-sulfuric acid method, using D-glucose as standard (Dubois et al., 1956).

2.3. Assays of antiviral and cytotoxicity

Assays of antiviral activity and cytotoxicity were evaluated by the SRB method using cytopathic effect (CPE) reduction recently reported (Choi et al., 2009).

Time-dependent inactivation activities of PEDV by polysaccharide were investigated by the following method. PEDV (50% cell culture infective dose, $CCID_{50}$) was incubated with polysaccharide at a final concentration of 10 μg/mL for 5, 10, 20, 30 and 60 min at 37 °C. At the indicated time, the PEDV-polysaccharide mixtures were added to Vero cell monolayers. After incubating at 37 °C for each time, the cultures were rinsed with complete MEM containing trypsin-EDTA and replenished with covering layer.

Dose- and temperature-dependent inactivation activities of PEDV by polysaccharide also were experimented. PEDV ($CCID_{50}$) was incubated with various concentrations of polysaccharide (1 μg/mL, 5 μg/mL, 10 μg/mL and 20 μg/mL) at 4, 25, 37 °C for 1 h. The PEDV-polysaccharide mixtures were added to Vero cell monolayers. After incubating at 37 °C for 1 h, the cultures were rinsed with complete MEM and replenished with covering layer. After 48 h postinfection, the antiviral activities were determined by CPE reduction assay.

2.4. Morphological changes

The effect of polysaccharide on PEDV-induced CPE was observed. Briefly, Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells per well. Next day, medium was removed and washed with PBS. Then, 0.09 mL of diluted virus suspension and 0.01 mL of medium supplemented with trypsin–EDTA containing polysaccharide of 1 μg/mL were added. After incubation at 37 °C in 5% CO_2 for 2 days, the morphology of cells was observed under microscope of 32 × 10 magnifications (AXIOVERT10, ZEISS, Germany), and images were recorded.

Table 1
Antiviral activity of polysaccharide from *Ginkgo biloba* exocarp against porcine epidemic diarrhea virus.

Test drugs	CC_{50}[a]	IC_{50}[b]	TI[c]
Polysaccharide	>100	1.7 ± 1.3	>58.8
Ribavirin	419.2	4.0 ± 3.1	103.7

Results are presented as mean IC_{50} values obtained from three independent experiments carried out in triplicate ± S.D.

[a] Concentration required to reduce cell growth by 50% (μg/mL).
[b] Concentration required to inhibit virus-induced CPE by 50% (μg/mL).
[c] Therapeutic index = CC_{50}/IC_{50}.

2.5. Antiviral mechanism

To investigate antiviral mechanism of polysaccharide against PEDV, viral inactivation assay, viral attachment assay, viral entry assay and postentry assay was conducted. To experiment viral inactivation assay, polysaccharides were added to the virus for 1 h at 37 °C, and then the mixtures were used to infect Vero cells for 1 h at 37 °C. The PEDV-polysaccharide mixtures were removed, and the cells were rinsed and replenished with cover layer without polysaccharide. After cultivation for 48 h, the inhibitory effects of polysaccharide were determined by CPE reduction assay. To conduct viral attachment assay, Vero cells were precooled at 4 °C for 30 min and polysaccharides were added together with PEDV for 1 h at 4 °C. At the indicated time the cells were rinsed and shifted at 37 °C for viral entry. To test viral entry assay, PEDV was attached to Vero cells for 1 h at 4 °C and the cells were rinsed before polysaccharides were added. The cultures were then shifted at 37 °C for viral entry. In postentry assay, Vero cells were infected with PEDV for 1 h at 37 °C, at which time the virus was removed. The cells were rinsed and replenished with cover layer containing polysaccharide throughout the experiment. After cultivation for 48 h, antiviral activity was determined by CPE reduction assay.

3. Results

3.1. Antiviral and cytotoxic activities of polysaccharide

Polysaccharide from *G. biloba* exocarp was investigated for its antiviral activity against PEDV. In our results, polysaccharide possessed strong antiviral spectrum against PEDV with a concentration required to inhibit PEDV-induced CPE by 50% (IC_{50}) of 1.7 μg/mL. It did not show toxicity to Vero cells with a concentration required to reduce cell growth by 50% (CC_{50}) of >100 μg/mL, thus showing lower antiviral therapeutic index (Table 1). Ribavirin also showed cytotoxicity in Vero cells with a CC_{50} value of 419.2 μg/mL. Its IC_{50} was 4.0 μg/mL and the therapeutic index was 103.7.

Trials of ribavirin in this study showed that it had favorable effects on antiviral activity in Vero cells infected with PEDV, showing it weak cytotoxicity in Vero cells. Therefore, we were able to ascertain that ribivirin do possess an antiviral property.

3.2. The effect of polysaccharide on morphological changes in PEDV-induced CPE

To confirm whether morphological changes of PEDV-induced CPE were blocked by the treatment of polysaccharide, we investigated effect of polysaccharide on the morphological changes in PEDV-induced CPE. As shown in Fig. 1, Mock cells (Fig. 1A) or ribavirin (Fig. 1C) or cells treated with 1 μg/mL polysaccharide (Fig. 1E)