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Original Article

The antiPRRSV activity of the polysaccharide from enteromorpha prolifera

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Abstract: More and more studies on the antiviral activities of marine polysaccharides are catching more and more researcher's attention in recent years. From couple years ago, the enteromorpha prolifera outbreak, this is becoming a great problem to the marine environment. The anti-porcine reproductive and respiratory syndrome virus (PRRSV) activity of polysaccharide enteromorpha prolifera (PEP) was studied in this research. Firstly, we confirmed that PEP inhibited the proliferation of PRRSV in vitro, and the inhibitive efficiency was dose dependent. The minimum dose to inhibit PRRSV proliferation was 1.56 mg/mL. Furthermore, the mechanism of anti-PRRSV was exploited in several ways, including viralcides assay, attachment inhibition assay, biosynthetic process inhibition assay. The results showed that PEP inhibited the attachment of PRRSV to Marc145 cells, completely suppressed the biosynthesis when it existed continuously. On the other hand, we also testified that the virucidal effect does not seem to be involved in the PEP antiviral activity detected.

Keywords: Polysaccharide, enteromorpha prolifera, antiPRRSV, mechanism of antivirus

Introduction

In recent years, the constant outbreak of some emerging or reemerging viral diseases has caused serious harm to human health. During the last decade, the number of antivirals approved for clinical use has been increased from five to more than 30 drugs [1]. Despite these successes, drug efficacy, toxicity, and cost remained unresolved issues, which is particularly large in developing countries due to the relative unavailability of drugs and the continuous emergence of drug resistance [2, 3]. Hence, the development of novel antiviral agents that can be used alone or in combination with existing antivirals is of high importance.

Marine polysaccharides are very important biological macromolecules which widely exist in marine organisms. Marine polysaccharides present an enormous variety of structures and are still under-exploited, thus they should be considered as a novel source of natural compounds for drug discovery [4, 5]. Marine polysaccharides can be divided into different types such as marine animal polysaccharides, plant polysaccharides and microbial polysaccharides according to their different sources. Marine derived polysaccharides have been shown to have a variety of bioactivities such as antitumor, antiviral, anticoagulant, antioxidant, immuno-inflammatory effects and other medicinal properties. In particular, the studies on the antiviral actions of marine polysaccharides and their oligosaccharide derivatives are attracting increasing in the antiviral activity [6-11].

In this study, we aim to investigate the anti-porcine reproductive and respiratory syndrome virus (PRRSV) activity of the polysaccharide from enteromorpha prolifera and provide potent drug for treatment PRRSV-induced disease.

Material and methods

Enteromorpha prolifera powder

Enteromorpha prolifera was collected from Qingdao coast in Shandong Province of China. The plant was cleaned with distilled water for 8

times to remove the sand and other thing, then dried at room temperature and grinded into flour by Xibeile blender. The final powder was obtained by being filtered with 100-mesh sieve.

Extraction and purification of polysaccharides of enteromorpha prolifera

Eighty grams of dry enteromorpha prolifera powder was soaped in distilled water for 30 minutes, following with 10-minute ultrasonic disruption. The suspension was then blendered at 90°C for 3 h on the magnetic stirring apparatus. The suspension was centrifuged at 5000 rpm for 20 minutes. Proteinase was added in the supernatant and incubated at 37°C for 30 h to eliminate the proteins. The suspension with proteinase was centrafuged one more time and harvest the supernatant. Purification with ethanol precipation was conducted. The obtained was soluble polysaccharide and concentrated to the 40 percentage volume of the solution, and disignated as PEP. Sephadex G200 filtration to purify PEP, the solution of main peak was collected. Total sugar was measured with sulphuric acid-phenol colorimetry and, Folin-phenol method to detect the content of proteins as previously described. Infrared spectroscopy was conducted to analyze the characterization of polysaccharide of the PEP. Briefly, one microgram of PEP was used to prepared slices with potassium bromide. Scaning was done at 4000 cm^{-1} and 400 cm^{-1}. Ultraviolet spectrum analysis was used to detect the content of proteins.

Cells and virus

Marc145 Cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The PRRSV was used to inoculate Marc145 cells at a multiplicity of infection (MOI) of 0.05. The median tissue culture infection dose ($TCID_{50}$) was determined in Marc145 cells in a 96-well plate as previously described.

Cytotoxicity to Marc145 cells of PEP

The cytotoxicity of PEP was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, confluent Marc145 cells were exposed to different concentrations of PEP for 72 h. The medium was then replaced by the MTT solution and incubated for 4 h. After dissolution of formazan crystals, optical densities were read (540 nm).

Anti-PPRS evaluation

To evaluate the potentially anti-PRRSV activity of PEP, a CPE inhibitory test was conducted. Briefly, Marc145 cells grown in 96-well plates to 90% confluency, were treated with PEP at different concentrations. The inoculum was replaced with maintenance media with PRRSV of 0.1 MOI at 2 h post treatment. After incubation for 30 h, the cells and supernatant were harvested by freezing-thaw. Virus was collected by centrifuge and then titrated.

Evaluation of the mechanism of anti-PRRSV action

Mixtures of equal sample volumes (20 μg/mL) and different concentration of PRRSV in serum-free MEM were co-incubated for 20 min at 4°C. Samples were then diluted to non-inhibitory concentrations (1:1000) to determine the residual infectivity by titer decrease assay as described above. Ethanol 70% (v/v) served as a positive control. The attachment assay to evaluate the inhibitory activity of PEP to the attachment of PRRSV to Marc145 cells, experiment was conducted as above. Different concentrations of PEP were added to the confluent cells for 2 h, then the solution was replaced with maintenance medium. PRRSV of 0.01MOI was inoculuated to the cells post or previous to the treatment. Cells then were incubated at 37°C and harvested at 30 hpi. Virus was titrated with Reed-Muench method. Time-of-addition study Confluent Marc145 cells was treated with certain concentration of PEP. PEP solution was replaced with maintenance medium or not at 2 h post treatment. PRRSV of 0.01 MOI was seeded and cells were incubated for further 30 h. Cells and the supernatant were harvested by freezing-thaw and virus were titrated.

Indirect immunofluorescence assay (IFA)

Cells treated with PEP without being replaced with medium was inoculated with virus for 30 h, and fixed with acetone. IFA was carried out as reported previously with an N-specific monoclonal antibody. Specific reactions between and N protein were detected with goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma, St Louis, MO) and observed under fluo-

Table 1. Effects of PEP on the toxicity of Marc145 cell line

Concentration of PEP (mg/mL)	0.78	1.56	3.13	6.25	12.5	25	50	Blank
Value of OD	0.585±0.01^{b}	0.499±0.01^{b}	0.591±0.01^{b}	0.599±0.01^{b}	0.601±0.01^{b}	0.612±0.01^{b}	0.630±0.01^{a}	0.645±0.01^{b}

Note: Values with different superscripts in the same line differ significantly (P<0.05), a<0.01, b<0.001.

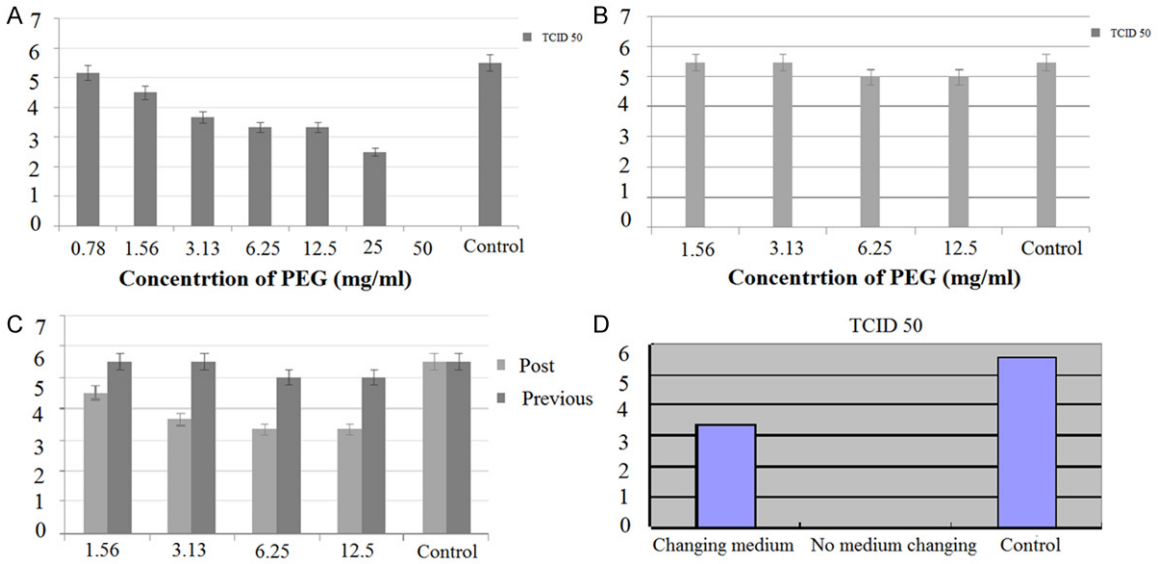

Figure 1. A: The inhibitive effect of PEP to PRRSV; B: The virucidal activity of PEP to PRRSV; C: Anti-attachment activity of PEP to PRRSV; D: The inhibitive effects of PEP on the titer of PRRSV with continuing action. Concentrtion of PEG (mg/ml).

rescence microscopy. Cells inoculated with PRRSV only or treated with PEP only were included as controls.

Results

Charaterization of the polysaccharide of enteromorpha prolifera

After Sephadex G200 column chromatographic separation, 50 tubes of solution was obtained and the OD values at 490 nm. There were 3 peaks and the second peak had most area. The polysaccharide of enteromorpha prolifera was collected, concentrated, precipated and freeze-dried. The weight of polysaccharide was 12.8 grams, and yeild of polysaccharide is 16.0%. Total sugar measured with sulphuric acid-phenol colorimetry was 75.2%. And there was no protein in it.

Anti-PRRSV activity

The data of cytotoxicity assay was listed as (**Table 1**). As the data showed that the OD values of wells with PEP at the following concentrations are all lower than control, and this indicated that PEP has no damage to the cells.

Anti-PPRS evaluation

PEP at different concentrations of 0.78 mg/mL, 1.56 mg/mL, 3.13 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL was used to test the inhibitive effect to PRRSV (**Figure 1A**). The data showed that when the concentration of PEP was equal to or higher than 1.56 mg/mL, the $TCID_{50}$ of PRRSV was decreased to more than 10^{-1}/mL. As the data in (**Figure 1B**) showed that PEP presented no virucidal activity.

Anti-attachment assay

PEP pretreated can reduce the titer of PRRSV, while post treatment did not have this effect (**Figure 1C**). This indicated that PEP can inhibit the attachment of PRRSV to Marc145 cells.

The inhibitory effect of PEP on PRRSV of sustaining action

When PEP existed in the wells continuously, CPE did not appeared (**Figures 1D** and **2**). This

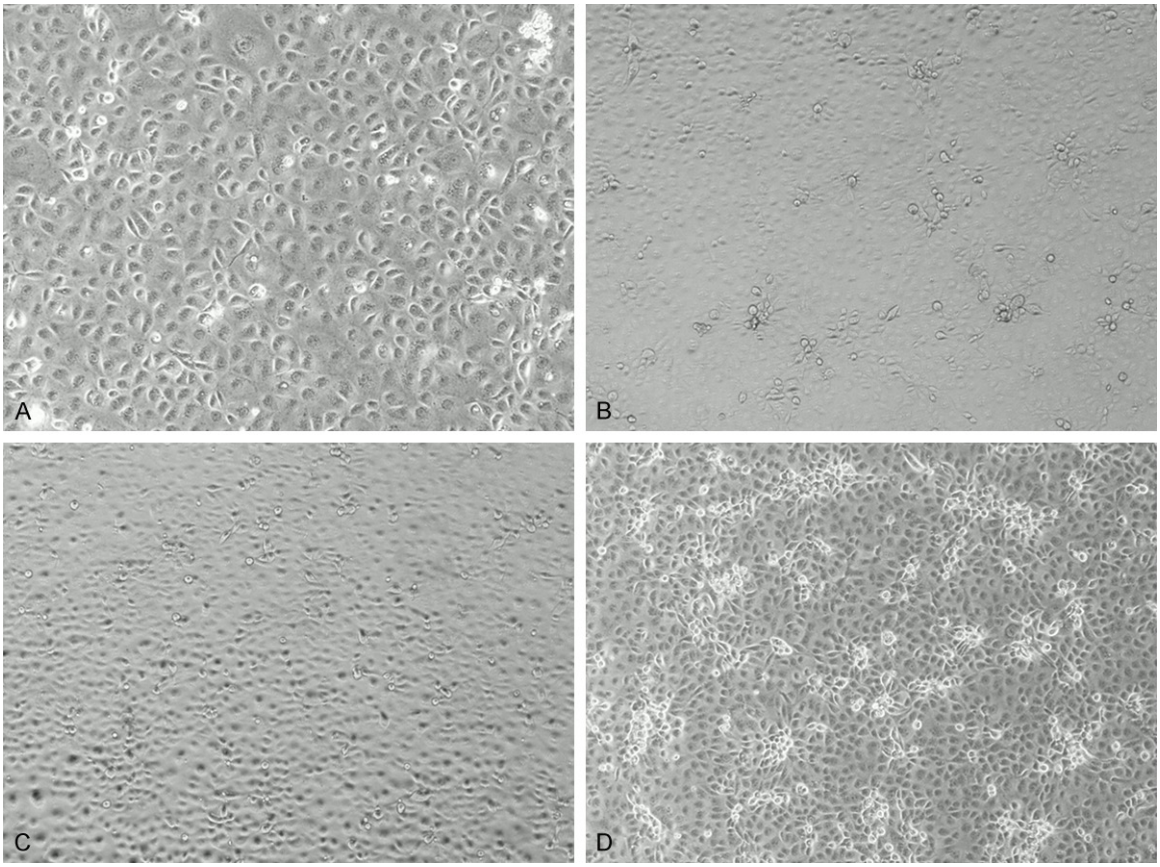

Figure 2. CPE in the test of inhibitive effects of PEP to PRRSV with continuing action. A: Normal Marc145 cells; B: Cells treated with PEP being replaced at 2 h post PEP-treatment; C: Cells treated with PEP without being replaced; D: Cells inoculated with PRRSV only.

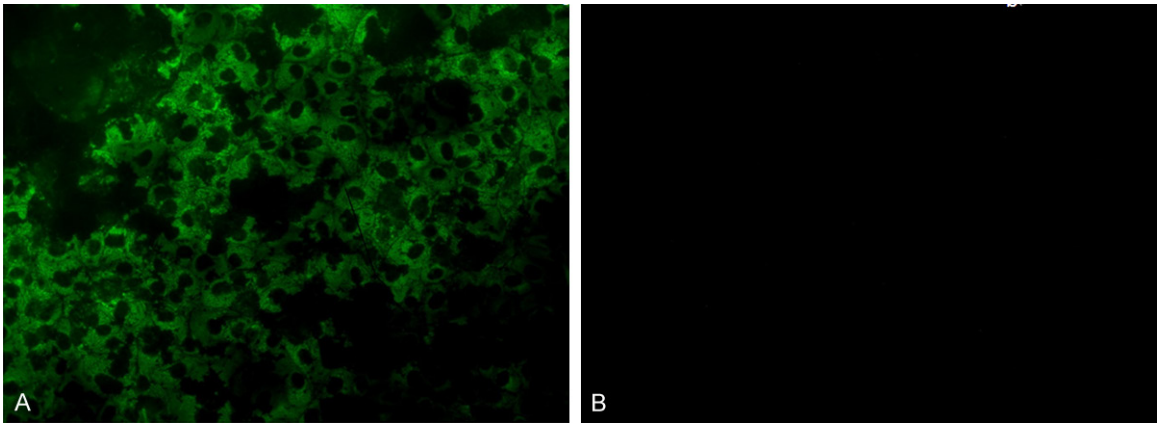

Figure 3. IFA to detect the virus in the test of inhibitive effects of PEP to PRRSV with continuing action. A: A virus control, strong fluorescence signal in the cytoplasm; B: PEP continuing role of holefluorescence signal was observed.

is significantly different with the situation when PEP was removed at 2 h post treatment. And the indirect influorescent assay showed that there was no signal in the wells which PEP exited continuously (**Figure 3**).

Discussion

For couple years, the pollution of enteromorpha prolifera (EP) to the marine environment was focused by the public and the government [12].

Many methods were adopted to treat this pollution. The utilization of enteromorpha prolifera is further study area. The enteromorpha prolifera has abundant polysaccharide as the other sea alga, and in the previous reports, the polysaccharide from sea algae shows activity of antimicrobe. The exploitation of the activity antivirus activity of EP should open a new path to its utilization [12-15].

We have detected the potential antiPRRSV activity of the polysaccharide of enteromorpha prolifera (PEP), and the data showed that PEP has definite antiPRRSV effect in vitro, and the antiPRRSV activity was dose dependent [16-18]. The minimum inhibitive dose was 1.56 mg/mL. In the attachment assay, pretreatment could obviously reduce the titer of PRRSV, while in the situation of post infection did not get the same effect. This indicated that PEP can block the virus mutiplication by inhibit the attachment of virus to the cells. We also conducted a continuous existance assay. That is put PEP in the cells and did not remove during the whole virus incubation period [19, 20]. Surprisingly, there was no virus growing in these wells. An indirect fluorescence assay was use to confirm this phenomina, and there was no signal in the testing well. These results testified definitely that the continuously existance in the wells can fully inhibit the virus amplication. The reason could be that PEP can not only inhibit the attachment of the virus to cells, it also block the biosynthetic process [20-24].

The virucidal assay was conducted as described by Francielle Tramontini Gomes de Sousa Cardozo et al. Preincubation of virus suspensions with PEP at 4 and 37°C, had no significant inactivating effects (data not shown) on PRRSV at the tested concentrations (50 mg/mL), which suppressed 100% of viral replication in the CPE reduction assay [25-27]. These results indicate that the virucidal effect does not seem to be involved in the PEP antiviral activity detected.

It is the first study about antiPRRSV activity of the polysaccharide of enteromorpha prolifera, and this should break a new path to utilize and treat enteromorpha prelifera.

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Disclosure of conflict of interest

None.

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