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Structures of acidic polysaccharides from *Basella rubra* L. and their antiviral effects

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ABSTRACT

In this study four acidic polysaccharides (BRP-1, BRP-2, BPR-3 and BRP-4) were isolated from the aerial part of *Basella rubra* L. Sugar composition and methylation analyses indicated that both BRP-2 and BRP-4 were native pectins containing dominant homogalacturonan regions and minor ramified rhamnogalacturonan type I regions branched with arabinogalactan type II neutral side chains. They exhibited antiviral activity against herpes simplex virus type 2 by interfering with absorption and penetration of virus to host cells. The most abundant component, BRP-4, showed a high therapeutic efficacy in the mouse model infected intravaginally with herpes simplex virus type 2. Moreover, orally administered BRP-4 resulted in moderate therapeutic efficacy in the mice infected intranasally with influenza A virus, and was shown to stimulate the production of neutralizing antibody and the secretion of mucosal IgA in influenza virus-infected mice.

1. Introduction

Genital herpes, caused primarily by herpes simplex virus type 2 (HSV-2), is one of the most prevalent sexually transmitted diseases across the world. Epidemiological findings showed that HSV-2 is transmitted by direct body contact with infected lesions or body fluids, infects the mucosal tissues or lesions of the skin, and causes the characteristic lifelong latent infection. Importantly, HSV-2 has been recently shown to increase human immunodeficiency virus (HIV) acquisition and transmission (Freeman et al., 2006). When the immune system is impaired, HSV-2 invades immune surveillance and causes recurrent symptoms (Roizman, Knipe, & Whitley, 2007). In recent years, acyclovir (ACV), a representative of nucleoside analogues, has been clinically used for treating HSV-infection in immunocompetent patients. However, like other anti-herpetic agents for treating HSV-2 infection, acyclovir fails to eradicate the virus from infected cells and to prevent recurrence of HSV because it cannot counteract early HSV infection (Gnann, Barton, & Whitley, 1983). In addition, in recent studies drug-resistance has been observed, especially in immunocompromised persons, implicating an increasing risk of HSV recurrence (Chatis & Crumpacker, 1991; Posavad, Koelle, Shaughnessy, & Core, 1997; Swetter et al., 1998). In this regard, new drugs with novel mode of action are in high demand.

On the other hand, influenza is an acute viral infectious disease that results in high morbidity and mortality in humans and in animals (Thompson et al., 2003). The respiratory mucosal surface is the initial site of influenza virus (IFV) infection and proliferation. Therefore, the mucosal immune system of the respiratory tract is usually the first immunological barrier against IFV infection. Present medical methods for prevention and therapy include vaccination and anti-influenza medication, however, neither is entirely satisfactory (Suzuki, 2005). The current vaccines do not provide a complete solution because of their limited efficacy in preventing infection and the frequent emergence of genetic variant viruses every flu season. Oseltamivir (OSV), an IFV neuraminidase inhibitor which is the most widely used drug to suppress viral replication for the treatment of influenza, has recently been reported to have some adverse reactions (Nicholson et al., 2000) and complications (Maxwell, 2007; Okumura, Kubota, Kato, & Morishima, 2006). In addition, there are increasingly frequent reports on OSV-resistant viruses (Kiso et al., 2004). A recent study found that OSV administration significantly suppressed the secretion of mucosal immunoglobulin A (IgA) in the respiratory tract (Takahashi et al., 2010), suggesting a risk of re-infection in patients due to a low mucosal response following OSV treatment. Therefore, enhancing the mucosal immune function could be a promising strategy for the prevention of influenza.

In recent years, numerous polysaccharides from natural sources have been found to show anti-herpetic activity as reviewed (Ghosh et al., 2009). We also found several polysaccharides with antiviral characteristics, such as a sulfated polysaccharide, rhamnan sulfate,

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from the green alga *Monostroma nitidum* (Lee, Koizumi, Hayashi, & Hayashi, 2010), two acidic polysaccharides without a sulfate group isolated from an edible blue-green alga *Nostoc flagelliforme* (Kanekiyo, Hayashi, Takenaka, Lee, & Hayashi, 2007) and *Cordyceps militaris* (Ohta et al., 2007), and a pectin-type polysaccharide from *Portulaca oleracea* (Dong, Hayashi, Lee, & Hayashi, 2010). Therefore, in continuation of the study in search of novel types of antiviral drugs with low or even no toxicity, we evaluated the plant material *Basella* (*B.*) *rubra* L. (Basellaceae). This is a perennial plant originally found in tropical Asia, and extensively cultivated as an annual ornamental. Its leaves or aerial parts have been widely consumed as a vegetable and health food. Here, we report on the isolation and structural characterization of four polysaccharides (BRP-1, BRP-2, BRP-3 and BRP-4) isolated from *B. rubra* and their *in vitro* and *in vivo* anti-HSV-2 and anti- IFV-A activities.

2. Materials and methods

2.1. Materials and general methods

B. rubra was collected in July, 2007, at Toyama in Japan. A voucher specimen has been deposited at the Laboratory of Pharmacognosy, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Japan. Toyopearl DEAE 650 M and TSK $GMPW_{XL}$ columns were purchased from Tosoh (Tokyo, Japan). Sepharose 6 B, Sephacryl S-500 HR were from GE Healthcare (Amersham Biosciences AB, Uppsala, Sweden). ACV was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oseltamivir phosphate was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Eagle's minimal essential medium (MEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Gas chromatography (GC) analysis was carried out on a GL-Science GC-353 gas chromatograph equipped with a FID detector, and nitrogen was used as carrier gas. GC–MS analysis was performed on a Shimadzu GCMS-QP5000, and helium was used as carrier gas.

2.2. Extraction, isolation and purification of polysaccharides

Fresh aerial parts of *B. rubra* (5.4 kg) were cut into pieces and extracted with hot H_2O under reflux three times for 2 h. After filtration through defatted cotton, concentration in vacuo and centrifugation at 3000 rpm for 20 min to remove the insoluble portion, the supernatant was subjected to dialysis and the non-dialysate was lyophilized to yield a crude polysaccharide fraction (BH, 1.4%).

BH (78 g) was suspended in H_2O again while stirring overnight at room temperature and the insoluble portion (33.6%) removed by centrifugation at 3000 rpm for 20 min. The supernatant was fractionated into a neutral fraction (BH1: 15.8%) and an acidic fraction (BH2: 26.2%) by ion-exchange chromatography on Toyopearl DEAE 650 M (5 i.d. × 90 cm), which were successively eluted with H_2O and 0.5 M NaCl.

BH2 (20.4 g) was subjected again to a Toyopearl DEAE 650 M column (5 i.d. × 20 cm) and eluted first with H_2O and then stepwisely with 0.1 M, 0.15 M and 0.2 M NaCl, successively, to give four subfractions (BH2A: 8.5%, BH2B: 16.2%, BH2C: 15.3%, BH2D: 1.1%). BH2B (3.31 g) was applied to a gel filtration (4.4 i.d. × 90 cm) on Sepharose 6 B and eluted with 0.01 M citrate buffer (pH 7) containing 0.1 M NaCl to yield four subfractions (BH2B-1, BH2B-2, BH2B-3, BH2B-4). Fractions of 15 mL were collected and monitored by phenol–H_2SO_4 method and UV absorbance at 280 nm. The major fraction BH2B-2 was subjected to gel filtration (2.2 i.d. × 90 cm) on Sephacryl S-500 HR and eluted with 0.1 M NaCl to give three fractions (BH2B-2a, BH2B-2b, BH2B-2c). The fraction BH2B-2b obtained as the most abundant fraction was purified by using the same chromatographic procedure to yield an acidic polysaccharide BRP-1 (256 mg). BH2B-4 was rechromatographed on Sepharose 6 B column to give BRP-2 (121 mg).

BH2C (3.13 g) was applied to a gel filtration (4.4 i.d. × 90 cm) on Sepharose 6 B and eluted with 0.01 M citrate buffer (pH 7) containing 0.1 M NaCl to give four fractions (BH2C-1, BH2C-2, BH2C-3, BH2C-4). BH2C-1 was rechromatographed on Sepharose 6 B column to yield a major fraction (BH2C-1a) which was applied to gel filtration (2.2 i.d. × 90 cm) on Sephacryl S-500 HR and eluted with 0.1 M NaCl to give three fractions (BH2C-1a-1, BH2C-1a-2, BH2C-1a-3). BH2C-1a-2 obtained as the most abundant fraction was rechromatographed on Sephacryl S-500 HR to yield an acidic polysaccharide BRP-3 (130 mg). The major fraction BH2C-4 was further purified by using gel filtration chromatography on Sepharose 6 B to give a polysaccharide BRP-4 (937 mg).

2.3. Estimation of homogeneity and colorimetric analyses of the polysaccharides

The apparent molecular weights of BRP-1–BRP-4 were estimated by HPLC analysis. The samples were applied to TSK $GMPW_{XL}$ gel filtration columns (7.6 × 300 mm × 2) and eluted with 0.1 M $NaNO_3$ at 0.6 mL/min. Commercially available pululans (Shodex P-52; Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers. Electrophoresis analysis was performed on a cellulose acetate membrane (Separax; Jokoh Co. Ltd., Tokyo, Japan) in 0.1 M pyridine/0.47 M formic acid buffer (pH 3) and run at 1 mA/cm. The membrane was stained with 0.25% toluidine blue.

Total carbohydrate and uronic acid were determined by phenol–sulfuric acid and m-hydroxydiphenyl methods with galactose and galacturonic acid as standards, respectively (Blumenkrantz & Asboe-Hansen, 1973; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content analyses were conducted using a Bio-Rad protein assay kit.

2.4. Sugar composition

Sugar composition of polysaccharides was determined by methanolysis with 1 M methanolic HCl followed by trimethylsilylation with TMSI-H. Then, the partially methylated trimethylsilyated derivatives were analyzed on GC using a SPB-1 fused silica capillary column (30 m × 0.32 mm i.d., Supelco, PA, USA). The temperature program was set starting at 150 °C followed by 2 °C/min to 250 °C, then 5 °C/min to 300 °C, and the injector temperature was kept at 250 °C.

2.5. Determination of D,L-configuration

The absolute configuration of the glycosyl residues was determined as described in the literature (Gerwig, Jamerling, & Vliegenthart, 1979) with minor modification. Each sample (2 mg) was subjected to methanolysis as described above before formation of the (*S*)-(+)-2-butyl glycosides followed by trimethylsilylation with TMSI-H. Then, the partially methylated and trimethylsilyated derivatives were analyzed on GC using a SPB-1 fused silica capillary column (30 m × 0.32 mm i.d., Supelco, PA, USA) with a column temperature program starting at 135 °C followed by 1 °C/min to 200 °C, then 5 °C/min to 250 °C, and the injector temperature was kept at 200 °C.

2.6. Methylation analysis

Prior to methylation, the uronic acids of BRP-2 and BRP-4 were reduced to the corresponding neutral sugars. The carboxyl esters were firstly reduced with sodium borodeuteride ($NaBD_4$) in

imidazole buffer to generate 6,6-dideuteriosugars. Following the primary reduction, the free uronic acids were activated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMC) and reduced with $NaBH_4$ (Kim & Carpita, 1992). The equal amounts of native and reduced BRP-2 and BRP-4 were then methylated according to Ciucanu's method (Ciucanu & Kerek, 1984). The methylated products were then hydrolyzed, reduced and acetylated to form partially methylated alditol acetates. As for BRP-1 and BRP-3, dried samples were firstly methylated following Hakomori's method (Hakomori, 1964), and the resultants were divided into two equal portions. One portion was reduced with $LiBH_4$ in tetrahydrofuran (THF) at 70 °C for 1.5 h followed by post-treatment with 10% acetic acid in methanol and evaporation to give the reduced production, which together with another portion were converted into partially methylated alditol acetates. The partially methylated alditol acetates were analyzed by GC–MS using a DB-5 MS fused silica capillary column (30 m × 0.32 mm i.d., J&W Scientific Inc., CA, USA). The compound corresponding to each peak was identified by an interpretation of the characteristic mass spectra and relative retention to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (Carpita & Shea, 1988). Meanwhile, the derivatives were quantified by GC apparatus with a SP-2330 fused silica capillary column with a temperature program starting at 160 °C followed by 2 °C/min to 210 °C, then 5 °C/min to 240 °C. Peak areas were corrected using published molar response factors (Sweet, Shapiro, & Albersheim, 1975).

2.7. Cells and viruses

Vero and MDCK cells were grown in MEM containing 5% fetal bovine serum (FBS). HSV-2 (UW268 strain) and IFV (A/NWS/33, H1N1) were propagated on Vero and MDCK cells, respectively.

2.8. Cytotoxicities and in vitro antiviral activities

Evaluation of cytotoxicities and antiviral assays of the polysaccharides were performed according to the previous report (Dong et al., 2010). Briefly, as for cytotoxicities, cells were incubated for 72 h in the presence of increasing concentrations of test compound. The 50% cytotoxic concentration (CC_{50}) was calculated from concentration–response curves after viable cells were counted using the Trypan blue exclusion test. In the antiviral assays, cells were infected with virus at 0.1 plaque-forming units (PFU) per cell for 1 h at room temperature, and then incubated at 37 °C. Samples were added during infection and throughout the incubation thereafter (A) or immediately after virus infection (B). Virus yields were determined by plaque assay at 1-day incubation. The 50% inhibitory concentration (IC_{50}) was obtained from concentration–response curves. Antiviral activities were estimated by selectivity indices calculated from CC_{50} and IC_{50} values.

2.9. Time-of-addition experiments

Vero cell monolayers were infected with HSV-2 at 10 PFU per cell. BRP-2 or BRP-4 was added at a concentration of 100 or 500 μg/mL before viral infection for 3 h, during viral infection for 1 h, during viral infection and throughout the incubation thereafter, at 0 h post-infection (p.i.), 2 h p.i., at 4 h p.i., and at 6 h p.i. At 10 h p.i., the cell cultures were harvested and plaque assay was applied.

2.10. Virus adsorption and penetration assays

Inhibitory effects of BRP-2 and BRP-4 on HSV-2 adsorption to and penetration into Vero cells were estimated as described previously (Kanekiyo et al., 2007). Briefly, for virus adsorption assay, Vero cells were infected with HSV-2 at 4 °C for 1 h in the presence of the sample, and a plaque assay was applied. For virus penetration assay, Vero cells were infected with HSV-2 at 4 °C for 1 h in the absence of sample, shifted to 37 °C in the presence of sample, and treated with 40 mM citrate buffer (pH 3.0) for 1 min at 1, 2, 3 and 6 h p.i. to inactivate any unpenetrated viruses.

2.11. Virucidal assay

To determine the effect of BRP-2 and BRP-4 on direct inactivation of virus particles, HSV-2 (2×10^4 PFU/100 μL) was treated with an equal volume of samples at 37 °C. After 0, 1, 3, or 6 h, 100-fold dilutions of the mixture were added to Vero cell monolayers for 1 h at room temperature. The cell monolayers were overlaid with media containing 0.8% methylcellulose and 2% FBS to be plaque-assayed.

2.12. Evaluation of in vivo anti-HSV-2 activity

Six-week-old female BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). All animal experiments were conducted in accordance with the animal experimentation guidelines of the University of Toyama with the approval of the Animal Care Committee at University of Toyama. Mice ($n = 5$ in each group) were subcutaneously injected with 3 mg medroxyprogesterone 17-acetate (Sigma Chemical Co.) on 6 days and 1 day before intravaginal inoculation with HSV-2 (1×10^4 PFU/20 μL/mouse) to increase the susceptibility to HSV-2 infection. BRP-4 (1 mg/20 μL) or ACV (0.2 mg/20 μL) was intravaginally administered at 1 h before infection, 1 h and 8 h after infection and then twice a day (every 12 h) until 7 days post-inoculation. In the control group, mice were treated with 20 μL PBS alone. The scores of the lesions were graded as follows: 0, no lesion; 1, slight and local erosion; 2, moderate genital inflammation; 3, severe exudative genital lesions; 4, hind limb paralysis; and 5, death. The average lesion score was calculated as the mean severity for all animals in a treatment group. Viral shedding was determined by washing the vaginal cavity with 100 μL PBS on day 3 after infection and titerating the virus by plaque assay on Vero cell monolayers.

2.13. Evaluation of in vivo anti-IFV activity

Six-week-old female BALB/c mice ($n = 10$ in each group) were intranasally inoculated with 2×10^4 PFU of IFV in 50 μL of PBS at day 0. BRP-4 and OSV were given by oral administration at a dose of 5 mg/day and 0.2 mg/day, respectively, twice a day from 7 days before virus inoculation to 7 days after virus inoculation. Control mice were treated orally with distilled water. After viral infection, loss of body weight and survival of mice were recorded every day for 2 weeks post-inoculation. At day 3 after virus inoculation, bronchoalveolar lavage fluids (BALFs) and the lungs of 5 mice in each group were individually collected. Bronchoalveolar lavage fluids were prepared by four washes with 0.8 mL of ice-cold PBS *via* a tracheal cannula and centrifuged at 1500 rpm for 10 min to obtain the supernatants, which were then stored at −80 °C. Lung samples were sonicated for 10 s after the addition of 1 μL of PBS per mg of lung tissue and centrifuged at 1000 rpm for 30 min to separate the supernatants, which were then stored at −80 °C. Virus titers in these samples were determined by plaque assay on MDCK cell monolayers.

2.14. Assay for neutralizing anti-IFV antibody and mucosal IgA titers

Blood, BALF, and fecal samples were individually collected from 5 mice of each group at 14 days. Blood samples were centrifuged at 3000 rpm for 10 min, and sera were stored at −20 °C. BALFs

Table 1
Chemical composition (%, w/w), monosaccharide composition (mol%) and yields (%, w/w) of the polysaccharides from *B. rubra* L.

Polysaccharides	BRP-1	BRP-2	BRP-3	BRP-4
Chemical composition (%, w/w)				
Total carbohydrate	89	63	69	50
Uronic acid content	19.1	49.5	10.4	70.8
Protein content	1.6	2.0	1.1	1.7
Monosaccharide composition (mol%)				
Ara	57.8	13.6	64.6	8.0
Rha	14.5	18.2	13.3	5.5
Gal	14.6	13.7	11.8	7.8
Glc	1.1	2.3	1.3	3.6
GalA	12.1	52.1	8.9	75.1
Yield (%, w/w)[a]	0.16	0.33	0.17	1.20

[a] Based on the crude polysaccharides.

were prepared as described above. Fecal extracts were prepared by adding PBS at 10 μL per mg of feces. IFV-specific IgA levels were determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with purified IFV (1 μg/mL), blocked with 2% bovine serum albumin (BSA), added with BALF samples or fecal extracts, and then treated with HRP-conjugated anti-mouse IgA antibody.

Neutralizing IFV-specific antibody titers were determined using 50% plaque reduction assay as described previously (Hayashi, Narutaki, Nagaoka, Hayashi, & Uesato, 2010). Briefly, serum and BALF samples were diluted from 20- to 10,000-fold. Each dilution was mixed with approximately 200 PFU of virus, and incubated at 37 °C for 1 h. Samples were added onto MDCK cell monolayers to measure the residual viruses by plaque assay. The neutralizing antibody titer was defined as the highest dilution that reduced the plaque numbers by 50% compared to the control without the sample.

2.15. Statistical analysis

The data were expressed as mean ± SD, and analyzed by Student's *t* test. $P < 0.05$ was considered as significant.

3. Results and discussion

3.1. Extraction and isolation of BRP-1–BRP-4

The hot water extract obtained from aerial parts of *B. rubra* was fractionated by dialysis to give crude polysaccharide fraction (BH). BH was separated into neutral fraction (BH1) and acidic fraction (BH2) by using anion-exchange chromatography. BH2 was subjected again to anion-exchange column chromatography and two abundant fractions (BH2B and BH2C) were obtained by elution with 0.1 M and 0.15 M NaCl, respectively. From BH2B, BRP-1 and BRP-2 were isolated by combination of gel filtration on Sepharose 6 B and Sephacryl S-500 HR. On the other hand, BRP-3 and BRP-4 were isolated from BH2C by the same manner.

BRP-1, BRP-2, BRP-3 and BRP-4 were all eluted as a single and symmetrically sharp peak on HPLC chromatogram, and their apparent molecular weights were estimated to be 4.9×10^5 (Mw/Mn = 1.35), 1.7×10^4 (Mw/Mn = 1.24), 4.6×10^5 (Mw/Mn = 1.27) and 1.4×10^4 (Mw/Mn = 1.38), respectively. Additionally, they were detected as a single band on the cellulose acetate membrane electrophoresis (data not shown). These results revealed that the four polysaccharides might be homogeneous polysaccharides based on the molecular weight and charge distribution. Their yields are summarized in Table 1.

3.2. Chemical characterization of the polysaccharides

The contents of total carbohydrate, uronic acid and protein are shown in Table 1. Sugar composition of the four acidic polysaccharides revealed that both BRP-1 and BRP-3 mainly consist of large amounts of arabinose (Ara), relatively high amounts of galactose (Gal), and almost equal amounts of rhamnose (Rha) and galacturonic acid (GalA) (Table 1). BRP-2 and BRP-4 were speculated to contain the same types of monosaccharides, but there are clear differences regarding to the relative amounts of the different monosaccharides present, especially dominant amounts of GalA residues in BRP-4 (75.1%). The absolute configurations of Gal, glucose (Glc), and GalA in the four polysaccharides were identified to be D-configurations while that of Ara and Rha be L-configurations as determined by GC of trimethylsilylated (+)-2-butyl glycosides compared with the corresponding derivatives of the authentic standard sugars.

According to methylation analysis, BRP-1 and BRP-3 were found to contain the same types and similar amounts of glycosidic linkages (Table 2). Ara detected as the major sugar component in BRP-1 and BRP-3 was considered to exist mainly as non-reducing terminal and linear chains of (1 → 5)- and (1 → 2)-linked furanosyl units in addition to small amounts of (1 → 2,5)- and (1 → 2,3,5)-linked residues. The high amount of terminal linked arabinofuranose (Ara*f*) residues does not correlate with the low amount of branched Ara*f* residues, only 4.8% for BRP-1 and 11.9% for BRP-3. This indicates that some terminal Ara*f* residues could be linked to other branched glycosyl residues, most probably to the Gal units. In supporting this findings, Gal residues were detected mainly as (1 → 3,4,6)-linked residues (78.5% in BRP-1 and 76.4% in BRP-3), along with small amounts of (1 → 4)- and (1 → 4,6)-linked residues. This suggested that the branched Gal residues might be substituted by terminal-linked Ara*f* residues at *O*-3,6 positions in addition to small amounts at *O*-6 position. Due to the distribution of Ara and Gal residues and their linkages as described above, the presence of arabinan and arabinogalactan type I (AG-I) are suggested (O'Neill, Albersheim, & Darvill, 1990). In addition, Rha was found to be (1 → 2)-linked, of which more than 80% were branched at *O*-4 position and the rest were branched at *O*-3 position. Generally, the (1 → 2)-linked Rha is branched at *O*-4 position, while it was also reported to be branched at *O*-3 position (Mondal, Das, Maiti, Roy, & Islam, 2009). GalA exists entirely as (1 → 4)-linked units calculated from the amounts of increased (1 → 4,6)-linked Gal residues from reduced polysaccharides compared with those from native forms. The almost equal amounts of (1 → 2,4)-linked Rha and (1 → 4)-linked GalA residues indicate one typical for pectic polysaccharide, suggesting that BRP-1 and BRP-3 might contain a rhamnogalacturonan type I (RG-I) core (O'Neill et al., 1990) which mainly branched at *O*-4 position of Rha residues. Taken together, the large amounts of neutral chains composed of arabinans and AG-Is might attach to *O*-4 and *O*-3 position of Rha in minor amounts of RG-I core, suggesting a very complex structure with high degree of branching.

Both in BRP-2 and BRP-4, GalA exist as the main sugar component, which were calculated from the amounts of increased (1 → 4)-, (1 → 2,4)-, (1 → 3,4)- and terminal-linked Gal residues in reduced polysaccharides compared with those in native forms. Most of GalA residues are (1 → 4)-linked (69.2% and 83.2%, respectively), as summarized in Table 2. Due to the dominant feature consisting of a linear chain of (1 → 4)-linked GalA units (smooth region), BRP-2 and BRP-4 were deduced to be pectin-type polysaccharides (O'Neill et al., 1990). In addition, substitution at *O*-2 and *O*-3 position of (1 → 4)-linked GalA residues were also detected (14.1% and 6.2%, respectively), which may be attached with neutral sugars in side chains or acetylated by acetyl groups (Aspinall, 1980). Most commonly reported in pectins are 2-*O*- or 3-*O*-acetylated GalA

Table 2
The linkages of the acidic polysaccharides BRP-1–BRP-4 determined by reduction, methylation, and GC-MS.

Sugar	Type of linkage	BRP-1		BRP-2		BRP-3		BRP-4	
		mol%	%	mol%	%	mol%	%	mol%	%
Ara	T*f*	41.1	70.4	3.6	29.5	46.0	70.1	2.3	27.1
	T*p*	–		3.1	25.4	–		1.0	11.8
	1,2*f*	5.2	8.9	–		5.0	7.6	–	
	1,5*f*	9.2	15.8	2.1	17.2	6.8	10.4	2.8	32.9
	1,2,5*f*	2.2	3.8	3.4	27.9	4.8	7.3	2.4	28.2
	1,2,3,5*f*	0.7	1.0	–		3.0	4.6	–	
Rha	T	–		2.4	13.3	–		1.2	24.5
	1,2	–		5.2	28.9	–		1.0	20.4
	1,3	–		4.7	26.1	–		0.6	12.2
	1,2,3	1.8	12.9	2.8	16.1	2.3	19.0	1.3	26.5
	1,2,4	12.2	87.1	2.9	15.6	9.8	81.0	0.7	14.4
Gal	T	–		12.5	77.2	–		7.5	61.5
	1,3	–		3.7	22.8	–		1.7	13.9
	1,4	2.6	15	–		2.5	17.9	–	
	1,3,6	–		–		–		1.2	9.8
	1,3,4	Tr.		–		Tr.		–	
	1,4,6	1.1	6.4	–		0.8	5.7	0.9	7.4
	1,3,4,6	13.5	78.5	–		10.7	76.4	0.9	7.4
Glc	T	Tr.		2.7	100.0	Tr.		1.9	100
GalA	T	–		9.0	16.7	–		7.8	10.6
	1,4	10.4	100.0	37.2	69.2	8.3	100.0	61.1	83.2
	1,2,4	–		3.5	6.5	–		1.8	2.5
	1,3,4	–		4.1	7.6	–		2.7	3.7

Tr.: trace, –: not detected.

residues, mainly in the homogalacturonan (HG) regions. Furthermore, (1 → 2)- and (1 → 2,4)-linked Rha (8.1 mol% and 1.7 mol%, respectively) were also found in BRP-2 and BRP-4, suggesting the presence of RG-I region. As for the neutral sugars, Ara are present in non-reducing terminal-, (1 → 5)-, and (1 → 2,5)-linked furanosyl form and Gal are mainly present in non-reducing terminal- and (1 → 3)-linked pyranosyl form in these two polysaccharides in addition to small amounts of (1 → 3,6)-linked residues in BRP-4, suggesting the presence of arabinogalactan type II (AG-II) (O'Neill et al., 1990). These neutral side chains may be attached to *O*-4 position of Rha residues but have also been found at *O*-2 or *O*-3 position of GalA residues as mentioned above. In together, BRP-2 and BRP-4 might be native pectins containing a backbone in which HG regions of α-(1 → 4)-linked D-Gal residues are interrupted by RG-I regions consisting of alternating α-(1 → 2)-linked L-Rha and α-(1 → 4)-linked D-GalA residues. Glc residues are present in small amounts which can also be found in pectins (Rolin, Neilsen, & Glahn, 1998).

3.3. In vitro antiviral assay

In this study, two pectic polysaccharides (BRP-1 and BRP-3) and two pectin-type polysaccharides (BRP-2 and BRP-4) obtained from *B. rubra* were screened for antiviral activities against HSV-2 and IFV *in vitro*. As shown in Table 3, the cytotoxicities of the four samples were low, with CC_{50} values more than 1000 μg/mL for both Vero and MDCK cells. All samples did not show anti-IFV activities. However, mild anti-HSV-2 activity was observed in two pectins but not two pectic polysaccharides with selectivity index (SI) of about 10, suggesting that the dominant HG region might correlate with the exhibition of anti-HSV-2 activity. Supporting this outcome, another pectin-type polysaccharide (named RP) isolated from *P. oleracea* L., which was composed of predominant HG regions but not pectic regions (named AP), showed potential anti-HSV-2 activity (Dong et al., 2010). As seen from a structural point of view, the highly charged HG regions might contribute to the interference with electrostatic interactions between positive-charged regions of viral glycoproteins and negative-charged heparin sulfate chains of the cell-surface glycoprotein receptor, while the high branching characteristic of RG-I regions might shield the electrostatic interactions.

3.4. Mode of anti-HSV-2 action in vitro

To determine the most sensitive phase of HSV-2 replication to BRP-2 and BRP-4, time-of-addition experiments were performed.

Table 3
Antiviral activities of polysaccharides obtained from *B. rubra*.

Virus	Samples	Cytotoxicity (CC_{50}, μg/mL)	Antiviral activity (IC_{50}, μg/mL)		Selectivity index (CC_{50}/IC_{50})	
			A[a]	B[b]	A	B
HSV-2	BRP-1	1600	810	610	3	2
	BRP-2	1300	110	130	12	10
	BRP-3	2100	>1000	>1000	<2	<2
	BRP-4	1800	210	210	9	8
IFV-A	BRP-1	2100	>1000	>1000	<2.1	<2.1
	BRP-2	1600	>1000	>1000	<1.6	<1.6
	BRP-3	2400	>1000	>1000	<2.4	<2.4
	BRP-4	2300	>1000	>1000	<2.3	<2.3

[a] A: Sample was added during infection and throughout the incubation thereafter.
[b] B: Sample was added immediately after viral infection.

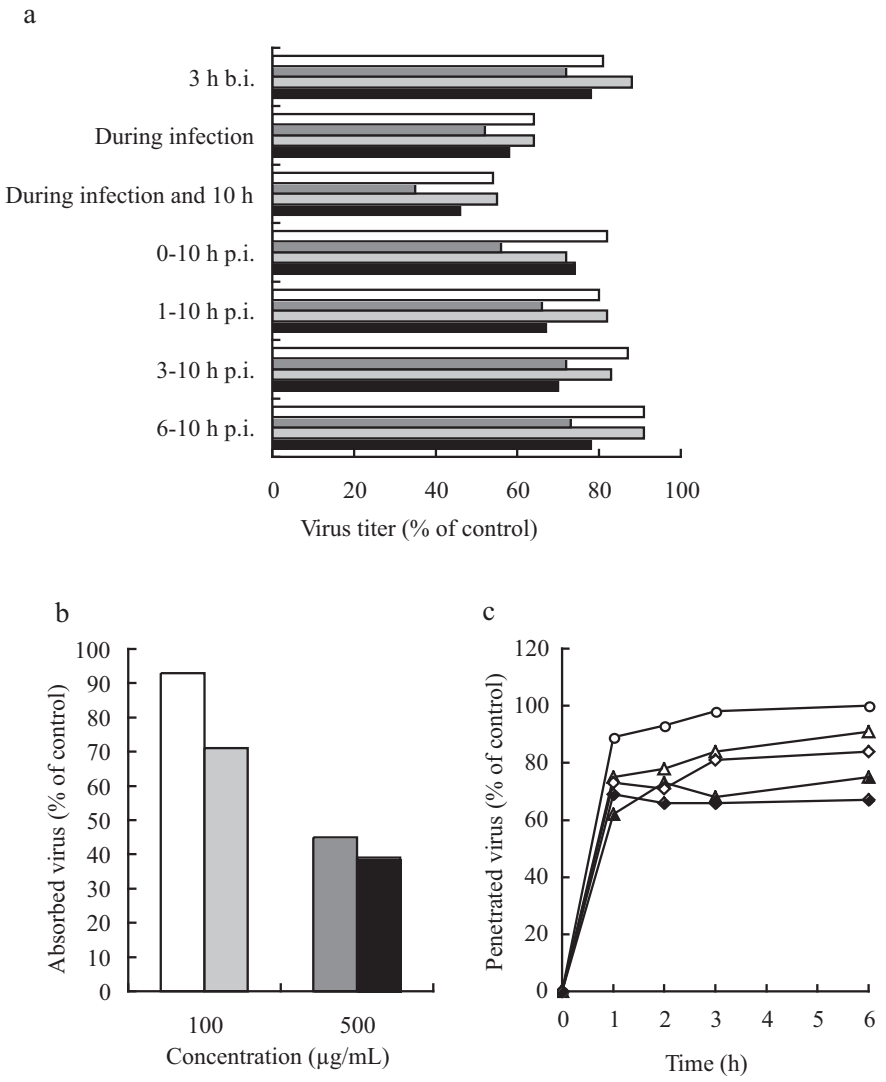

Fig. 1. Mode of anti-HSV-2 action of BRP-2 and BRP-4. (a) Time-of-addition experiment: Vero cells were infected with HSV-2 at 10 PFU/cell. Sample was added at various time intervals: 3 h before infection (3 h b.i.), during infection for 1 h, during viral infection and throughout the incubation, and at 0 h, 1 h, 3 h and 6 h post-infection (p.i.). No test compound was added to the control. At 10 h p.i., virus yields were determined by plaque assay. The plaque number of control was taken as 100%. ■, BRP-2 (100 μg/mL); ■, BRP-2 (500 μg/mL); □, BRP-4 (100 μg/mL); and ■, BRP-4 (500 μg/mL). (b) Adsorption experiment: Vero cell suspensions were infected with HSV-2 (1 PFU/cell) in the absence or presence of sample at 4 °C. Virus-adsorbing cells were counted by an infection center assay. ■, BRP-2 (100 μg/mL); ■, BRP-2 (500 μg/mL); □, BRP-4 (100 μg/mL); and ■, BRP-4 (500 μg/mL). (c) Penetration experiment: Cell monolayers were infected at 4 °C with HSV-2 in the absence of sample, and then shifted to 37 °C to penetrate the adsorbed virus in the presence of compound. The penetrated viruses were titrated by plaque assay. The plaque number of control at 6 h after temperature shift was taken as 100%. Each value is the mean from triplicate assays. ○, no drug control; ◊, BRP-2 (100 μg/mL); ♦, BRP-2 (500 μg/mL); △, BRP-4 (100 μg/mL); and ▲, BRP-4 (500 μg/mL).

In these experiments, Vero cells were infected with HSV-2 at a high multiplicity of infection of 10. As shown in Fig. 1(a), BRP-2 and BPR-4 suppressed virus production most efficiently in a dose-dependent manner when added at the same time of virus infection and throughout the incubation thereafter. In addition, the presence of test compounds only during viral infection for 1 h also produced relatively potent inhibitory effects on HSV-2 replication. However, these compounds were less effective when the host cells were pretreated for 3 h or they were added at various times after viral infection.

These results of time-of-addition experiments suggested that the antiviral targets might be early events including virus adsorption to the host cell surface and/or virus penetration into host cells. Firstly, the effect of test compounds on HSV-2 adsorption to host cells was evaluated by infection center assay, which determined the number of cells binding the virus particles at low temperature (4 °C) in the presence of different concentrations of the compounds. As shown in Fig. 1(b), both BRP-2 and BRP-4 interfered significantly with virus adsorption in a dose-dependent manner. Then, after HSV-2 was adsorbed to Vero cells under the conditions that caused no virus penetration, the kinetics of virus penetration in the presence of different concentrations of test compounds were studied for 6 h post-infection. As shown in Fig. 1(c), the test compounds exerted a moderate inhibitory effect on virus penetration in dose- and time-dependent manners.

Furthermore, the virucidal effects of the test compounds on HSV-2 infectivity were examined. As a result, both BRP-2 and BRP-4 showed very weak inactivation after 6 h of incubation at 37 °C (data not shown). These results were corresponding to the data from the time-of-addition experiments, suggesting that the virucidal step was not the major antiviral target of BRP-2 and BRP-4.

It is well known that sulfated polysaccharides have been reported to be endowed with the capacity to inhibit viral infection by blocking the adsorption, entry and cell-to-cell spread of viruses (Ghosh et al., 2009). In the present study, the main anti-HSV-2 target of these two pectins was demonstrated to be viral

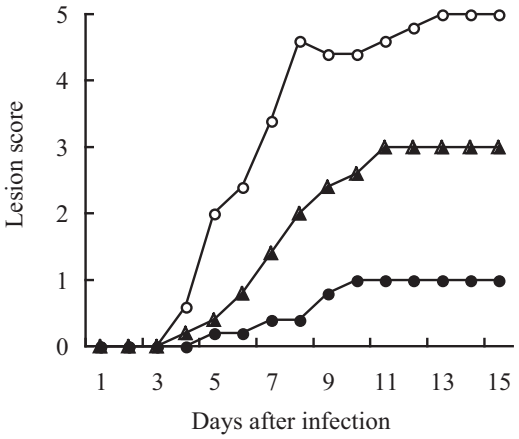

Fig. 2. Effects of intravaginal treatment with BPR-4 on herpetic lesions caused by HSV-2 infection. Mice were given samples intravaginally from 1 h before virus infection to 7 days after infection and analyzed severity of genital infection by lesion scores as described in Section 2. ○, no drug control; ●, ACV (0.4 mg/day); and ▲, BRP-4 (2 mg/day).

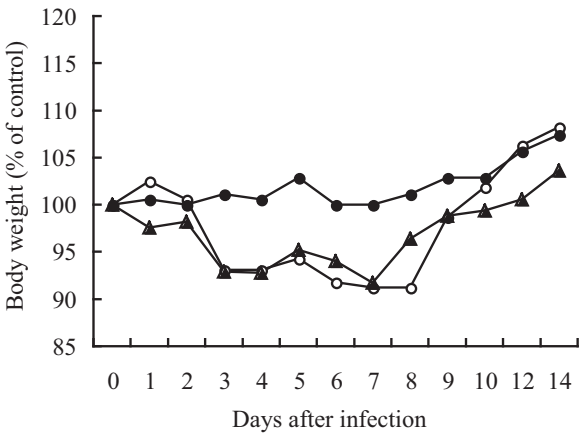

Fig. 3. Effects of oral administration of BRP-4 on body weight of mice infected with IFV. Mice were intranasally inoculated with IFV at day 0. BRP-4 and oseltamivir were given by oral administration twice a day from 7 days before virus inoculation to 7 days after virus inoculation. Control mice were treated orally with distilled water. ○, no drug control; ●, oseltamivir (0.2 mg/day); and ▲, BRP-4 (5 mg/day).

adsorption to the receptor on host cell membranes. Moreover, the moderate inhibitory effect on virus penetration was also involved in the antiviral target of both BRP-2 and BRP-4.

3.5. *In vivo* anti-HSV-2 efficacy of BRP-4

Since HSV-2 invades through and causes disease at the vaginal tract mucosa, which is the first line against HSV-2, the intravaginal infection of mice was chosen as the appropriate infection model for *in vivo* anti-HSV-2 assay. Mucosal immune response makes the local administration of polysaccharides possible. Therefore, whether or not the most abundant pectin-type polysaccharide (BRP-4) obtained from *B. rubra* could exert protection against viral infection at the site of viral entry led us perform the animal test of BRP-4.

The effect of BRP-4 at a dose of 1 mg per administration on intravaginal infection of mice with HSV-2 was evaluated. Two daily intravaginal administration of BRP-4 for 7 days delayed significantly the appearance of vaginal lesions compared with PBS-treated control group (Fig. 2). As shown in Table 4, the mean titers of virus shed on day 3 were reduced from 120×10^2 PFU/100 μL in PBS-treated control mice to 0.14×10^2 PFU/100 μL in mice treated with BRP-4 ($P < 0.01$). As predicted from viral shedding and severity of infection, four-fifths of ACV-treated and two-fifths of BRP-4-treated mice survived the virus challenge, respectively, while no survival was observed in PBS-treated control group by day 10 after infection.

Taken together, BRP-4 showed higher therapeutic efficacy based on titers of viral shedding, infectious severity and survival rate in this intravaginal model compared with those of the control group. The inhibition of viral binding and penetration at vaginal mucosa might contribute to the *in vivo* efficacy of this compound, as judging from its *in vitro* anti-HSV-2 targets. On the other hand, when viruses invade the mucosal surface, they are countered by host immune response. Several studies showed that activation of mucosal innate and systemic immune responses were responsible for host protection against herpetic viral infection (Han et al., 2007; Kwant-Mitchell, Ashkar, & Rosenthal, 2009; Peng et al., 2009). Therefore, it is also possible that BRP-4 may exert *in vivo* anti-HSV-2 effect by modulating the mucosal immune responses.

3.6. *In vivo* anti-IFV efficacy of BRP-4

The effect of orally administered BRP-4 against IFV infection was evaluated on the basis of the weight loss, virus production and virus-specific antibody titers in IFV-infected mice. Control mice treated with water showed moderate reduction in body weight compared with OSV-treated mice (Fig. 3). The body weight of BRP-4-treated mice showed a similar loss of body weight to that seen in control mice. As shown in Fig. 4(a) and (b), BRP-4 significantly decreased the virus titers of both BALF and the lung at 3 days post-inoculation compared to control group.

It has been reported that resistance to influenza is mediated through neutralizing antibodies secreted locally and systemically (Clements, Betts, Tierney, & Murphy, 1986; Topham, Tripp, & Doherty, 1997). Therefore, we determined the neutralizing antibody titers in sera and BALFs, and mucosal IgA antibody titers of mice at 14 days post-inoculation. Fig. 4(c) shows the effects of oral administration of BRP-4 (5 mg/day) on the neutralizing antibody response to IFV. In the BRP-4-treated group, the antibody titers were higher in both sera and BALF than those of control group without significant differences. Furthermore, when the effects on the secretion of mucosal IgA antibody in BALF and the intestine were estimated by ELISA system, BRP-4 increased IgA levels of both BALFs ($P < 0.05$) and the intestine, whereas OSV significantly reduced the IgA levels compared with those in control group (Fig. 4(d)). These results suggested that the effect of BRP-4 on the enhancement of secretory IgA levels might be at least a part of *in vivo* anti-IFV efficacy.

Table 4
Effect of BRP-4 treatment on survival of mice infected with HSV-2.

Treatment	Dose	Survivor/total	Mortality (%)	Virus titer ($\times 10^2$ PFU/100 μL)[a]
Control	–	0/5	100	120 ± 73
Acyclovir	0.2 mg/20 μL	4/5	20	0.06 ± 0.13**
BRP-4	1 mg/20 μL	2/5	60	0.14 ± 0.26**

[a] Virus titers were determined at 3 days after virus inoculation.
** $P < 0.01$ *vs.* control (PBS).

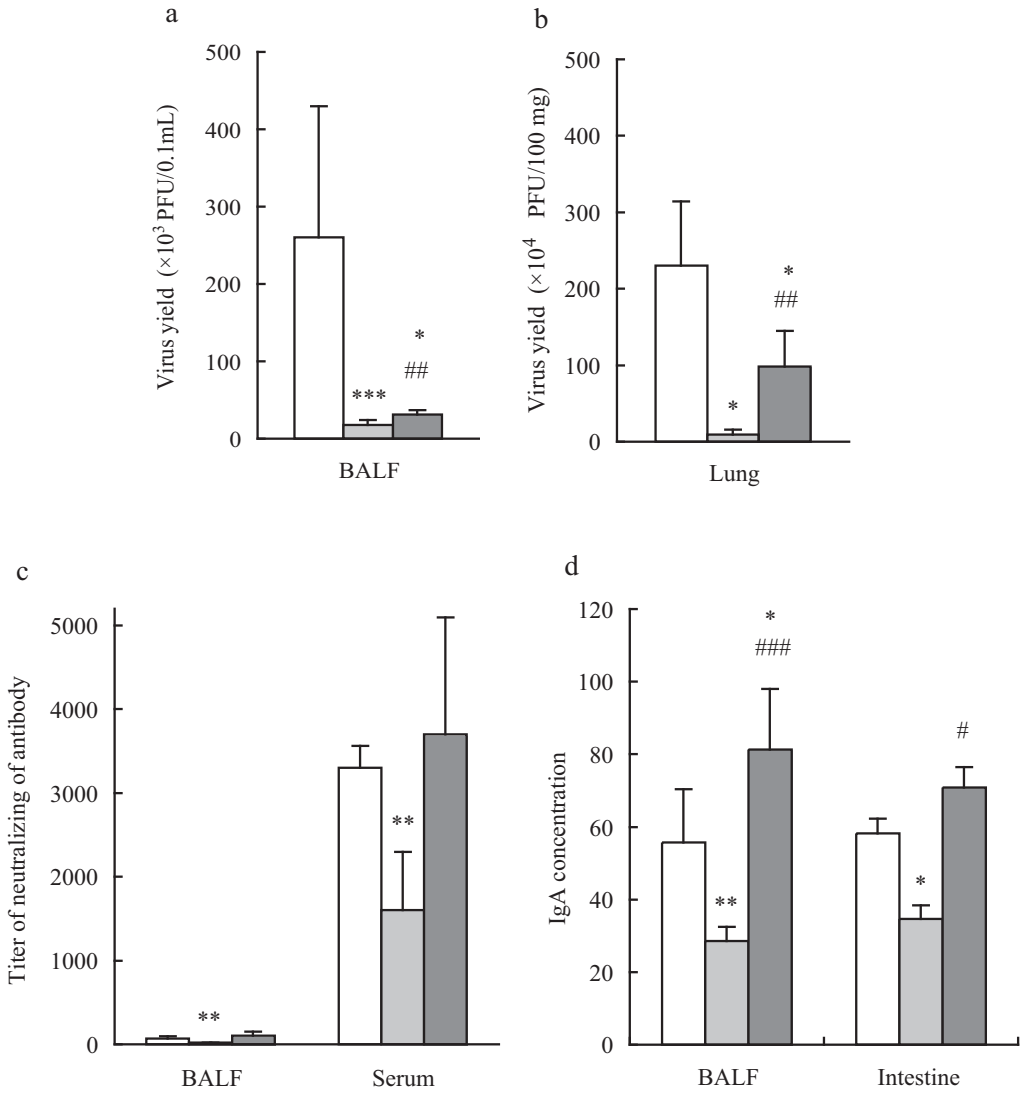

Fig. 4. Effect of BRP-4 on the virus replication, the production of virus-specific neutralizing antibody and mucosal IgA in the mice infected with IFV. Mice were treated as described in Fig. 3. Virus titers in BALF (a) and lung (b) samples were determined at 3 days after virus infection. Neutralizing antibody titers (c) were determined using by plaque assay at 14 days after intranasal infection with IFV. IFV-specific IgA levels (d) in BALFs (ng/mL) and in the intestine (ng/100 mg) were determined by ELISA method. Data are shown as mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* oseltamivir. □, control; ▒, oseltamivir (0.2 mg/day); and ■, BRP-4 (5 mg/day).

On the basis of enhancement or maintenance of the production of systematic and local IFV-specific antibodies by oral administration of BRP-4, it was suggested that BRP-4 might be a promising candidate against IFV infection. Notably, in accordance with recent research (Takahashi et al., 2010), the results of OSV suppressing the humoral and mucosal immunity was also observed in present study. This could increase the risk of re-infection in patients following OSV treatment.

In conclusion, two pectin-type polysaccharides BRP-2 and BRP-4 obtained from *B. rubra* were found to exert potential anti-HSV-2 effects *in vitro* mainly by interfering with the absorption of virus to host cells. Furthermore, the most abundant pectin-type polysaccharide BRP-4 showed a high therapeutic efficacy in the mouse model intravaginally infected with HSV-2 as judged from the severity of herpetic lesions, the survival rate of mice and virus shedding. On the other hand, orally administered BRP-4 resulted in moderate therapeutic efficacy against IFV based on virus yields in the mice. Moreover, BRP-4 stimulated the production of neutralizing antibody and the secretion of mucosal IgA in IFV-infected mice in spite of less antigens (viruses), implicating an attribution to the protective effect of oral administration of BRP-4 on IFV. This is the first report of antiviral polysaccharides isolated from *B. rubra*.

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