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In vitro Antiviral Activities of Korean Marine Algae Extracts against Fish Pathogenic Infectious Hematopoietic Necrosis Virus and Infectious Pancreatic Necrosis Virus

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Abstract To investigate the antiviral activity of marine algae against fish pathogenic viruses, which are often the causes of viral disease in aquaculture, the 80% methanolic extracts of 21 species collected from the coast of Korea were screened for their *in vitro* antiviral activities on infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV), using a flounder spleen (FSP) cell-line. Among them, *Monostroma nitidum* (10 μg/mL) exhibited the strongest inactivation on IHNV, showing a 2 log reduced virus titre as compared to the control in the determination of direct virucidal activity. In addition, *Polysiphonia morrowii* (100 μg/mL) remarkably reduced the virus titres of treated cells by 2-2.5 log, for both IHNV and IPNV, in the determination of cellular protective activity, implying the existence of substances that may modulate innate host defense mechanisms against viral infections. These results reveal that some marine algae could be promising candidates as sources of antiviral agents or as health-promoting feeds for aquaculture.

Keywords: marine algae, antiviral, infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), *Polysiphonia morrowii*

Introduction

Marine algae are historically an exceptionally rich source of pharmacologically active metabolites, with antineoplastic, antimicrobial, and antiviral effects (1). Random screenings have been effective at finding marine algae with various biological activities, and many of these reports have been reviewed (2,3). Antiviral activity is one of the most important activities in marine drugs, and numerous algae and their constituents show potent antiviral activities. There are several reports concerning the antiviral activities of marine algae against human pathogenic viruses (3,4), but only a few have shown effects against fish pathogenic viruses (5,6). The cultivation of high value marine fish in Korea has grown rapidly since the 1990s. Concomitantly, there has been a high risk of viral disease occurring in these intensive marine aquaculture systems, and many viral diseases have been reported (7). Infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) in salmon and trout are important aetiological agents in aquaculture facilities. Outbreaks of infectious hematopoietic necrosis (IHN) result in losses approaching 100%, depending on the species and size of the fish, the virus strain, and environmental conditions (8). IPNV has been isolated from epizootics in cultured salmonids, and from a variety of aquatic animals in freshwater and sea water environments around the world (9-12). At the present time, vaccines are used as a preventive measure against several viral diseases; however, their efficacy is not complete. Furthermore, there are no effective antiviral agents to prevent and treat fish viral diseases, although several antiviral nucleotide analogues and other low molecular weight compounds are used to cure viral diseases in humans (6). Thus, the establishment of effective antiviral agents against fish viral diseases is urgently needed, either from known chemical compounds or from natural products such as marine algae. Potential antiviral substances that may be contained in marine algae would be likely candidates for applications in the control of fish viral diseases, and may be easily prepared as antiviral feeds for cultured fish. Therefore, in the present study, to obtain antiviral marine algae against fish pathogenic viruses, we investigated the antiviral activities of the 80% methanolic extracts of 21 species collected from the coast of Korea against 2 fish pathogenic viruses, IHNV and IPNV, that are often the cause of viral diseases in aquaculture, and are well-studied. To determine the marine algae extracts directly inactivating the viruses, as well as modulating innate host defense mechanisms against virus infection (13,14), screening was performed using dual assay paradigms: i) a direct virucidal activity test and ii) a cellular protective activity test.

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Materials and Methods

Algae The marine algae were collected from the Wando and Yeosu coasts in the Republic of Korea from November 2004 to April 2005. Voucher specimens have been deposited in our laboratory at Chonnam National University.

Preparation of algae extracts After cleaning the surface of the thalli to remove visible epiphytes and dirt, the samples were freeze-dried and then ground to be used for extraction. We described the detailed processes for the extraction of the algae samples in a previous report (15).

Briefly, 15 g of freeze-dried algae was extracted with 1 L of 80%(v/v) aqueous methanol (MeOH) at 80°C 4 times, each of which took 1 hr for a total of 4 hr. The extracted solution was evaporated *in vacuo* and then freeze-dried.

Viruses and cell culture Two fish pathogenic viruses, IHNV (RtJe00) and IPNV (RtJe98), isolated from rainbow trout in Korea, were used in the present study. The viruses were propagated in plastic flasks infecting flounder spleen (FSP) cells at a multiplicity of infection of 0.01 plaque forming unit (PFU)/cell (16). After 1 week, when an extensive cytopathic effect (CPE) had occurred, the flasks were shaken to detach the cells. To release the virus, the infected cells were frozen and thawed twice and stored in aliquots at −80°C.

The FSP cell line was used as the host cells for the viruses, and cultured using Eagle's minimum essential medium (EMEM, Invitrogen Co., Carlsbad, CA, USA), which was supplemented with fetal bovine serum (FBS, Invitrogen Co.) at 2% for virus production and 10% for routine cell culture. For routine cell propagation, the FSP cells were incubated at 20°C under normal atmospheric conditions.

Cytotoxicity assay The cytotoxicities of the algae extracts were evaluated by 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described by Mosmann (17) with slight modification, using the FSP cell line. Briefly, the FSP cells were seeded into a 96-well plate to be approximately 70% confluent, and were then incubated overnight at 15°C. After the algae extract was added to the cell culture to give final concentrations from 200.0 to 3.1 μg/mL in a series of 2-fold dilutions, the cells were incubated at 15°C for 48 hr. MTT(Sigma-Aldrich Chemical Co, St. Louis, MO, USA) solution was added to the cell culture medium and incubated for 4 hr. The absorbance of formed formazan was measured at 570 nm by a microplate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA, USA), and the growth rate as an index of cytotoxicity was calculated by dividing the test cell's absorbance by the absorbance of the corresponding control cells. The antiviral activities of all the algae extracts were tested at non-cytotoxic concentrations.

Assessment of antiviral activity Stock solutions of the algae extracts for the antiviral assay were prepared in dimethyl sulfoxide (DMSO) and kept at −20°C, until appropriate dilutions of the solutions were used in each assay (final concentration of DMSO: 0.1%). Two assay methods were used to determine the antiviral activity. The direct virucidal activity of the algae extracts was measured to determine the direct inactivation of IHNV or IPNV ($10^{7.5}$ 50% tissue cultured infectious dose, $TCID_{50}$/mL), a 10-fold serial dilution of the viruses was treated with an equal volume of algae extract. After 1 hr, an aliquot of the mixture was added to the FSP cell monolayer in 96-well culture plates at 20°C. Then, to observe the cytopathic effects (CPE), the cells were stained with 2% crystal violet for 10 min. The $TCID_{50}$ value was calculated by the method of Burleson *et al.* (18). The cellular protective activity of the algae extracts was measured to determine the host defense mechanisms of the FSP cells against IHNV or IPNV, the FSP cells were seeded into 96-well culture plates. After the cells attached to the plates, each algae extract was added into the 96-well culture plates and then incubated for 24 hr at 20°C. After the cells were washed out twice by Hank's balanced salt solution (HBSS), a 10-fold serial dilution of the viruses ($10^{7.5}$ $TCID_{50}$/mL) was infected to the pretreated FSP cell monolayer. The procedures followed were the same as those for the direct virucidal activity.

Results and Discussion

Antiviral activities of 21 species of Korean marine algae extract against IHNV and IPNV In the present study, to obtain antiviral marine algae against the fish pathogenic viruses, IHNV and IPNV, 21 species of Korean marine algae were screened. They consisted of 3 Chlorophyta, 5 Phaeophyta, and 13 Rhodophyta. The results of the screenings are summarized in Table 1. It can be seen that 11 species of the algae extracts exhibited significant antiviral activities on IHNV and/or IPNV, showing over 1 log reduced virus titres as compared to the control virus titre ($10^{7.5}$ $TCID_{50}$/mL). *Monostroma nitidum*, *Nemacystus decipiens*, *Sargassum thunbergii*, *Undaria pinnatifida*, *Gracilariopsis chorda,* and *Grateloupia filicina* showed effective direct virucidal activity for only IHNV; while *Ulva pertusa*, *Corallina officinalis Gracilaria textorii*, *Lomentaria catenata,* and *Polysiphonia morrowii* were effective on both IHNV and IPNV. Particularly, *M. nitidum* exhibited the strongest direct virucidal activity, showing a 2 log reduced virus titre ($10^{5.5}$ $TCID_{50}$/mL) in the treated cells as compared to the control ($10^{7.5}$ $TCID_{50}$/mL) for IHNV, followed by *N. decipiens* ($10^{6.0}$ $TCID_{50}$/mL) and *U. pinnatifida* ($10^{6.0}$ $TCID_{50}$/mL). Most of the 11 antiviral marine algae extracts showed efficacies in the direct virucidal activity test. In contrast, *P. morrowii* remarkably inhibited the replication of both IHNV and IPNV, reducing virus titres to $10^{5.0}$ $TCID_{50}$/mL on IHNV and $10^{5.5}$ $TCID_{50}$/mL on IPNV, when it was pretreated on the cells (cellular protective activity test); while it showed moderate antiviral activity for virus inactivation with virus titres of $10^{6.8}$ $TCID_{50}$/mL and $10^{6.5}$ $TCID_{50}$/mL on IHNV and IPNV, respectively.

Constituents responsible for the antiviral activities against IHNV and IPNV from algae Another observation worth mentioning is that in addition to these 11 algae, slight to moderate antiviral activities were observed in several other algae. Our screening results show that most of the screened marine algae extracts had antiviral activities against IHNV or IPNV. These universal antiviral activities that were revealed in the marine algae extracts may be attributed to the broad extraction capacity of the 80% MeOH used as the extraction solvent. Polar organic solvents such as MeOH or ethanol are often used in the extraction of water-soluble substances from natural products rather than water, since these substances are mostly stored in protected states such as bound to membranes, compartmentalized, protected by lipophilic materials, etc (19,20). Therefore, the extraction of marine algae using 80% MeOH, as in the present study, may provide substances in the range of small lipophilic to water-soluble

Table 1. Antiviral activities of marine algae extracts against IHNV and IPNV[1)]

Species (concentration: μg/mL)	IHNV		IPNV	
	Direct virucidal activity[2)] ($TCID_{50}$/mL)	Cellular protective activity[3)] ($TCID_{50}$/mL)	Direct virucidal activity ($TCID_{50}$/mL)	Cellular protective activity ($TCID_{50}$/mL)
Chlorophyta				
Enteromorpha prolifera (100)	$10^{6.8}$	$10^{7.0}$	$10^{6.5}$	$10^{7.5}$
Monostroma nitidum (10)[4)]	**$10^{5.5}$**	$10^{6.5}$	$10^{7.0}$	$10^{7.5}$
Ulva pertusa (100)	**$10^{6.3}$**	$10^{6.5}$	**$10^{6.4}$**	$10^{7.5}$
Phaeophyta				
Agarum clathratum (100)	$10^{7.5}$	$10^{7.5}$	$10^{6.8}$	$10^{7.5}$
Nemacystus decipiens (100)	**$10^{6.0}$**	$10^{7.0}$	$10^{7.0}$	$10^{7.5}$
Sargassum horneri (100)	$10^{6.5}$	$10^{7.1}$	$10^{7.3}$	$10^{7.5}$
Sargassum thunbergii (10)[4)]	**$10^{6.3}$**	$10^{7.3}$	$10^{6.5}$	$10^{7.5}$
Undaria pinnatifida (100)	**$10^{6.0}$**	$10^{6.9}$	$10^{7.1}$	$10^{7.5}$
Rhodophyta				
Campylaephora crassa (100)	$10^{6.7}$	$10^{7.5}$	$10^{7.3}$	$10^{7.5}$
Chondrus ocellatus (100)	$10^{7.5}$	$10^{7.0}$	$10^{7.5}$	$10^{7.5}$
Corallina officinalis (100)	$10^{6.5}$	$10^{7.3}$	**$10^{6.3}$**	**$10^{6.3}$**
Gelidium amansii (100)	$10^{7.5}$	$10^{7.5}$	$10^{6.5}$	$10^{7.5}$
Gloiopeltis furcata (100)	$10^{6.7}$	$10^{7.0}$	$10^{6.5}$	$10^{7.5}$
Gracilaria textorii (100)	**$10^{6.3}$**	$10^{6.9}$	**$10^{6.2}$**	$10^{7.4}$
Gracilariopsis chorda (100)	**$10^{6.3}$**	$10^{6.7}$	$10^{7.5}$	$10^{7.3}$
Grateloupia filicina (100)	**$10^{6.4}$**	$10^{7.3}$	$10^{7.0}$	$10^{7.0}$
Grateloupia lanceolata (100)	$10^{6.7}$	$10^{6.7}$	$10^{6.5}$	$10^{7.5}$
Lomentaria catenata (100)	$10^{6.5}$	$10^{7.5}$	$10^{6.4}$	$10^{7.5}$
Polysiphonia morrowii (100)	$10^{6.8}$	**$10^{5.0}$**	$10^{6.5}$	**$10^{5.5}$**
Pterocladiella capillacea (100)	$10^{7.3}$	$10^{7.5}$	$10^{7.5}$	$10^{7.5}$
Symphyocladia latiuscula (10)[4)]	$10^{6.8}$	$10^{7.0}$	$10^{7.5}$	$10^{7.5}$

[1)]Virus titre of IHNV inocula was $10^{7.5}$ $TCID_{50}$/mL; virus titre of IPNV inocula was $10^{7.5}$ $TCID_{50}$/mL.
[2)]A 10-fold serial dilution of the viruses was incubated with an equal volume of algae extract. After 1 hr, an aliquot of the mixture was added to the cell monolayer in 96-well culture plates at 20°C.
[3)]Each algae extract was added into the 96-well culture plates and then incubated for 24 hr at 20°C. After the cells were washed out, a 10-fold serial dilution of the viruses ($10^{7.5}$ $TCID_{50}$/mL) was added to the pretreated cell monolayer.
[4)]Due to cytotoxicity, these marine algae extracts were treated at 10 μg/mL.

substances such as oligo- or poly-saccharides.

Polysaccharides from marine algae are well known to act as antiviral substances against human viruses. In particular, sulfated polysaccharides such as carrageenans and fucoidan are reported to inhibit a variety of viruses, including human immunodeficiency virus (HIV), herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), influenza A virus, and human cytomegalovirus (HCMV) (21). There are several reports on the antiviral activities of sulfated polysaccharides isolated from algae, which turned out to be active in the present study. Lee *et al.* (22) reported anti-HSV-1 activity for a sulfated polysaccharide isolated from *M. nitidum,* which showed the strongest direct inactivation of IHNV in the present study. Sulfated polysaccharides from *U. pinnatifida* and *G. filicina* showed anti-HSV and HIV-1 activities, respectively (23,24). Reports on the isolation of the famous antiviral sulfated polysaccharide, fucoidan, from *S. thunbergii,* also indirectly indicate its antiviral activity (25,26). Furthermore, ulvan, a sulfated polysaccharide isolated from *U. pertusa,* is reported to have antioxidant and antihyperlipidemic activities (27,28), which may suggest its potential antiviral activity, although it was not tested. To the best of our knowledge, there have been no reports to date on the inactivation of fish pathogenic viruses by sulfated polysaccharides isolated from algae. However, in an article by Fabregas *et al.* (5), the authors showed that extracts from marine microalgae exhibited replication inhibition on the viral hemorrhagic septicemia virus (VHSV), and suggested that this antiviral activity could be due to the sulfated polysaccharides contained in the microalgae. In line with this data, the antiviral activities of the 80% MeOH extracts of marine algae revealed in the present study are likely due to sulfated polysaccharides contained in the extracts. At this point, we cannot exclude the probability that small lipophilic substances contained within the 80% MeOH extracts also had antiviral action; because it has been reported that there are low molecular weight antiviral compounds such as terpenoids, bromophenols, and phlorotannins contained in marine algae (29-31). From *P. morrowii*, which showed the highest antiviral activity in the determination of cellular protective activity, 2 bromophenols were previously isolated from it, and have inhibitory activities on alpha glucosidase (32). Park *et al.* (31) reported that a bromophenol from the methanolic extract of *Symphyocladia latiuscula* showed anti-HSV-1

activity. Both *P. morrowii* and *S. latiuscula* belong to the family of Rhodomelaceae, which is famous for its characteristic bromophenols. In our study, the antiviral activity of *S. latiuscula* was shown to be moderate for IHNV, with a virus titre of $10^{6.8}$ $TCID_{50}$/mL. However, considering it was treated at the low concentration of 10 mg/mL due to its cytotoxicity, the existence of antiviral substances in *S. latiuscula* could be probable. Also, although their antiviral activities were not reported, the evidence suggests that the bromophenols isolated from *P. morrowii* could function as antiviral agents.

In addition, in contrast with most of the other algae extracts, *P. morrowii* remarkably inhibited the replication of both IHNV and IPNV when it was pretreated to cells, and also displayed moderate direct virucidal activitiy. This fact reflects that it can inactivate virus particles, as well as modulate or induce innate host cell defense mechanisms against virus infections. Recently, Falco *et al*. (14) reported the dual antiviral activity of human α-defensin-1 against VHSV. Human α-defensin-1 is a type of antimicrobial peptide (AMP) that controls viral replication and provides time for the generation of a more effective host adaptive immunity response. Researchers have revealed that human α-defensin-1 inactivates VHSV particles and inhibits VHSV replication in target cells by up-regulating genes related to the type I interferon (IFN) response, such as Mx proteins (13), and have suggested human α-defensin-1 as a model antiviral agent for fish.

At the present time, the application of vaccine-based immunization strategies is very limited, and the use of chemicals is restricted due to their potential harmful impact on the environment. Therefore, although further studies are needed on the identification of antiviral components, the *in vivo* toxicity, and the mechanisms involved in the antiviral activity of *P. morrowii,* the isolated components, or extracts from *P. morrowii,* could be candidates in the development of antiviral agents and health-promoting feed for fish.

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