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ISOLATION AND CHARACTERIZATION OF ANTIVIRAL SUBSTANCE AGAINST SALMONID VIRUSES, 46NW-04A PRODUCED BY AN AQUATIC BACTERIUM, *PSEUDOMONAS FLUORESCENS* 46NW-04

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INTRODUCTION

The virus survival and its interaction with other microorganisms in aquatic environments are critical to public hygiene. Several previous reports have described the inactivation of viruses in natural water which at certain times seem to be related to microorganisms inherent in these environments (Fujioka et al., 1980; Toranzo et al., 1982, 1983; Ward et al., 1986), showing the possibility of bacterial proteolytic enzyme action (Cliver and Herrmann, 1972; Toranzo et al., 1982, 1983; Ward et al., 1986). However, these reports did not include isolation and characterization of the proteolytic enzymes from the bacteria.

We also have reported inactivation of salmonid viruses in the waters collected from aquatic environments (Kamei et al., 1987b; 1987c; 1988a; 1988b). In those papers, we proposed that the inactivation might be caused by the indigenous bacteria present in the natural water (Kamei et al., 1987b; 1988a) and this possibility was proved by screening of bacteria with antiviral activity from the virus-inactivated water samples (Kamei et al., 1987c; 1988b). One of these bacteria, *Pseudomonas* sp. 46NW-04 was found to have potent antiviral activity against infectious hematopoietic necrosis virus (IHNV) and identified as *Pseudomonas fluorescens* biovar I (Kamei et al., 1988c). In our recent report, we already have described the successful isolation of a low molecular weight antiviral agent 46NW-04A produced by this bacterium (Kimura et al, 1990).

We report in this paper more detailed characteristics of 46NW-04A and its chemical structure. Also, the antiviral mechanism is discussed.

MATERIALS AND METHODS

Viruses and cell culture

Infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), and *Oncorhynchus masou* virus (OMV) (Kamei et al., 1987a) were used in this study. Rainbow trout (*Oncorhynchus mykiss*) gonad cells, RTG-2 (Wolf and Quimby, 1962) were used for propagation of viruses and chinook salmon (*Oncorhynchus tshawycha*) embryo cells, CHSE-214 (Fryer et al., 1965) were used for plaque assay. Cells were grown at 15 ℃ in MEM10-Tris medium (Kamei et al., 1987a). The 1-day-old confluent cell monolayers were subjected to plaque assay.

Plaque assay

The antiviral substance 46NW-04A was first dissolved in dimethyl sulfoxide (DMSO) and diluted with Hanks' balanced salt solution (Hanks' BSS) to make a stock solution of 1 mg/ml. The stock solution was diluted to desired concentrations by Hanks' BSS containing 1% DMSO. A 0.2 ml aliquot of diluted substance was mixed with an equal volume of IHNV suspension (approximately 150 PFU/0.1 ml) and left at 15 ℃ for 1 hour. Plaquing procedures were then conducted as described previously (Kamei et al., 1988b). One % DMSO in Hanks' BSS was used as a control.

Isolation and purification of 46NW-04A

One loopful of *Pseudomonas fluorescens* 46NW-04 stock culture was inoculated to 100 ml CYG broth medium (Kamei et al., 1988b) in 500 ml-Sakaguchi flask and incubated with agitation at 160 rpm at 25 ℃ for 2 days. Four 2.5 L CYG broths in 5L-Ehrenmeyer's flasks were inoculated with 25 ml each of broth culture and incubated with agitation at 25 ℃ for 3 days. The broth culture was centrifuged at 6,600 x g at 4 ℃ for 20 min. the supernatant was extracted twice with an equal volume of ethyl acetate and the ethyl acetate layer was concentrated to 2 L under reducing pressure at 25 ℃. The concentrated solvent was dehydrated with Na_2SO_4 (anhydrous) and concentrated to dryness. This extraction was conducted three times to obtain extracts from 30 L culture fluids. Detection of 46NW-04A was carried out by anisaldehyde reagent (Stahl and Kaltenbach, 1961). 46NW-04A was partially purified by column chromatography and TLC on silica gel. Then, it was finally purified by HPLC on C_{18}-phenyl gel to determine its physicochemical properties and chemical structure. Isolation and purification procedures are described in detail elsewhere (Kimura et al., 1990).

Chemical analysis

Solubility and color reaction of 46NW-04A were tested with several solvents and reagents (Kimura et al., 1990). Melting point was measured on a Micro Melting Point Apparatus (Yanako). Optical rotation was performed on an AA-5 spectrometer (Optical Activity LTD.). Ultraviolet spectrum was recorded on a 150-20 spectrophotometer (Hitachi). Infrared spectrum was recorded on an A-100 spectrophotometer (Japan Spectrascopic CO., LTD.). FAB-MS, GC-MS, SI-MS, NMR, and amino acid analyses were conducted and the chemical structure of 46NW-04A was determined by Pharmaceutical Research Laboratories of Sapporo Breweries LTD., Japan.

Antiviral activity of 46NW-04A against salmonid viruses

Antiviral activity of 46NW-04A was examined by using RNA viruses, IHNV and IPNV, and also the DNA virus, OMV. Serial two-fold dilutions of the substance were made 1% DMSO in Hanks' BSS to give the concentrations of 100, 50, 25, 12.5, and 6 μg/ml. Each sample was assayed for comparative antiviral activities against these viruses by plaque reduction described above.

Antiviral mechanism of 46NW-04A

To examine the antiviral effect of 46NW-04A prior to virus inoculation, 46NW-04A in Hanks' BSS with 1 % DMSO was added to 1-day-old confluent CHSE-214 cell cultures in 24-well plate to give a final concentration of 10 μg/ml. After incubation at 15 ℃ for 12 or 24 hours, the cell culture medium was removed and 150 PFU/0.1 ml of IHNV was inoculated. To examine this antiviral activity during virus adsorption, 50 μg/ml of 46NW-04A was added to the cells at 0 or 15 min after the virus was inoculated. Furthermore, to measure the antiviral activity after virus adsorption, 46NW-04A was added to the cell culture to give a final concentration of 10 μg/ml after the virus-infected cells were incubated for 1 or 12 hours at 15 ℃. Then, comparative plaque reduction assay was done as described above.

Direct effect of 46NW-04A on IHNV particles was measured for the plaque reduction on the time course of IHNV treatment with 46NW-04A. A 2.0 ml of 46NW-04A was added to the equal volume of IHNV suspention (200 PFU/0.1ml) to give 25 μg/ml and mixed thoroughly. After 0, 5, 10, 15, 20, 30, 40, 50, and 60 min incubation at 15 ℃, a 0.4 ml-aliquot was withdrawn and used to measure the comparative virus infectivity by plaque assay. Each assay was done in duplicate (0.2 ml/well) in 24-well plate. In this case, the cells were rinsed twice with Hanks' BSS after virus was adsorbed for 1 hour to remove 46NW-04A from the cell cultures. The control sample was reacted with Hanks' BSS with 1 % DMSO for 60 min.

RESULTS

Characteristics and chemical structure of 46NW-04A

A 709 mg of antiviral agent 46NW-04A was isolated from 30 L of culture fluid of *Pseudomonas fluorescens* 46NW-04 by ethyl acetate extraction and partially purified by both column and thin layer chromatography on silica gel. Finally, 46NW-0A was purified by HPLC on C_{18}-phenyl gel to determine its physicochemical characteristics and chemical structure. Molecular weight of 46NW-04A was edetermined to be 1,125 by FAB-MS (previously we had reported the molecular weight to be 1,126: Kimura et al., 1990). The molecular formula was determined to be $C_{54}H_{95}N_9O_{16}$ by both ^{13}C-NMR and calculation from elemental analysis (C:H:N=54:94.8:8.8). Ultraviolet and infrared spectra suggested that 46NW-0A might be peptide (probably it is composed in cyclic form; because ninhydrin color

Table 1. Physicochemical properties of antiviral substance 46NW-04A

Appearance	white powder
Melting point	232 ~ 235 ℃
Optical rotation ($[\alpha]_D^{25}$)	−48° (c 2.0, MeOH)
Molecular weight (by mass spectrometry)	1,125
Elemental analysis (found)	C, 54.27 %; H, 7.94 %; N, 10.37 %
Molecular formula	$C_{54}H_{95}N_9O_{16}$
UV λ_{max}^{MeOH} nm (ε)	204 (18200)
IR (KBr) cm^{-1}	3,300, 2,950, 2,920, 2,850, 1,730, 1,650, 1,520, 1,460, 1,370, 1,270, 1,060
Stability	stable at pH 3 ~ 10
Solubility: soluble	methanol, ethanol, ethyl acetate, acetone, benzene, chloroform, diethyl ether, dimethyl sulfoxide, pyridine
insoluble	*n*-hexane, petroleum ether, water
Color reaction: positive	anisaldehyde, bromocresol green, dinitrophenylhydrazine, dragendorff, 8-hydroxyquinoline-NH_3
negative	potassium permanganate, anthron, Barton, cupper acetate, Legal, Molish, ninhydrin, Rhodamine

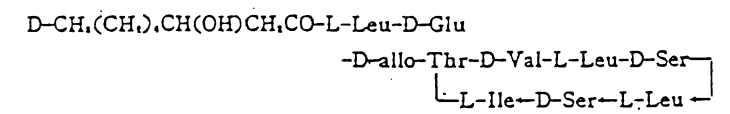

Fig. 1. Chemical structure of antiviral substance, viscosin (Hiramoto et al., 1970) similar to 46NW-04A.

reaction was negative) (Table 1). Furthermore, both ^{1}H and ^{13}C-NMR spectra also revealed a typical peptide patern, proving that 46NW-04A is a peptide (data not shown). Finally, amino acid analysis proved that 46NW-04A has nine amino acids (three leucines, two serines, glutamic acid, valine, isoleucine, and threonine). Since 46NW-04A was negative to ninhydrine reaction, as this substance was completely hydrolyzed to determine unknown substance which might bind to N-terminal of leucine by GC-MS, the unknown substance was shown to be 3-hydroxy decanoic acid (HDA). Furthermore, amino acid sequencing analysis by FAB-MS showed that 46NW-04A is composed of the amino acids in the following order (: HDA ← Leu ← Glu ← Thr ← Val ← Leu ← Ser ← Leu ← Ile). These data indicated that 46NW-04A was very similar to the known antiviral substance Viscosin (Hiramoto et al., 1970). The structure of Viscosin reported by Hiramoto et al. is shown in Fig. 1. However, the data of melting point and optical rotation of 46NW-04A were different from those of Viscosin, indicating some optical isomers probably may be present in 46NW-04A composed of the amino acids or HDA.

Antiviral activity against salmonid viruses

Antiviral activity of 46NW-04A was examined using both DNA (OMV) and RNA viruses (IHNV and IPNV). The antiviral activity against salmonid viruses at tested concentrations ranging from 3 to 50 μg/ml is present in Table 2. Plaque

Table 2. Antiviral activity of 46NW-04A against salmonid viruses

Concentration of 46NW-04A	Plaque reduction		
	OMV	IHNV	IPNV
50 μg/ml	100%	100%	0%
25 μg/ml	100%	100%	0%
12.5 μg/ml	100%	94%	0%
6 μg/ml	60%	58%	0%
3 μg/ml	25%	37%	0%

formation of OMV was completely inhibited at a concentration of 12.5 μg/ml of this agent. Even at 6μg/ml, 60 % plaque reduction of the virus was observed, but little or no antiviral activity was noted at 3 μg/ml. Similar results were obtained with IHNV, virus plaque reduction was 94 % at 12.5 μg/ml and 37 % at 3 μg/ml, suggesting that 46NW-04 is probably an antiviral substance which specifically effects enveloped viruses, regardless of whether they are DNA or RNA viruses.

Antiviral mechanism

In order to elucidate the antiviral mechanism of 46NW-04A against IHNV, 46NW-04A was added to cell cultures before or after inoculation of IHNV and the

antiviral activity was measured. When 46NW-04A was added to cells at 24 or 12 hours prior to IHNV inoculation, no antiviral activity was observed (Table 3).

Table 3. Effect of addition time of 46NW-04A to IHNV-infected cells on anti-IHNV activity

Addition time	Concentration	Plaque reduction
Pre-infection		
- 24 h	10 μg/ml	0%
- 12 h	10 μg/ml	0%
Post-infection		
0	50 μg/ml	100%
+ 15 min	50 μg/ml	98%
+ 1 h	10 μg/ml	33%
+ 12 h	10 μg/ml	0%

However, when this antiviral substance was added just after IHNV was inoculated to the cells, strong antiviral activity was observed, showing 100 % plaque reduction. This strong antiviral activity was still observed even at 15 min after the virus was inoculated. However, when 46NW-04A was added to the cells 1 hour after virus inoculation, the antiviral activity was reduced to 33 % plaque reduction, 12 hours after the virus was inoculated, no antiviral activity was observed. From these results, the antiviral effect of 46NW-04A seemed to be acting on the begining stage of virus adsorption to the cells, probably acting directly on the virus particles.

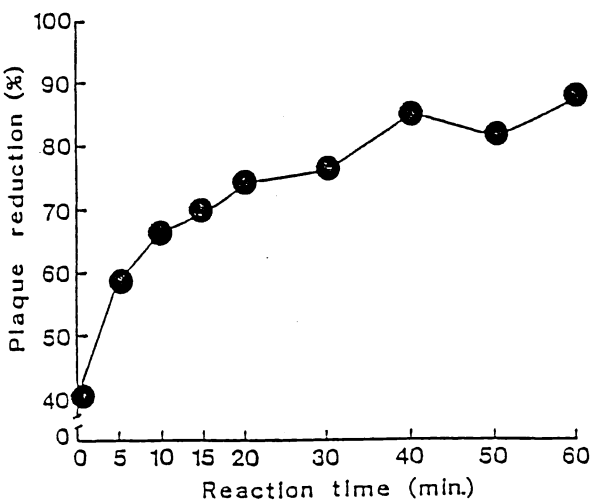

Fig. 2. Effect of reaction time of antiviral substance 46NW-04A with IHNV particles on the infectivity of IHNV.

To examine this mechanism, 46NW-04A was preincubated with IHNV before conducting the plaque assay. When IHNV and 46NW-04 was mixed and immediately assayed, a 40 % plaque reduction was observed (Fig. 2). However, when IHNV and 46NW-04A were preincubated 5 min before the assay, a 59 % plaque reduction was

observed. This antiviral effect was stronger, when the reaction time was longer, indicating 76 % plaque reduction after 30 min and 87 % plaque reduction after 60 min. These results strongly reinforced the hypothesis that 46NW-04A directly affects the virus particles. The mechanism of antiviral action of 46NW-04A is to block the attachment sites of IHNV, probably spikes on the envelopes.

DISCUSSION

Previously, we reported that fish pathogenic viruses were inactivated in all the water samples including fresh, brackish, and sea waters, and that the inactivation is possibly caused by some bacterial metabolites released in the water (Kamei et al., 1987b, 1988a; Yoshimizu et al., 1986). This possibility was proved by screening bacteria with antiviral activity from 1,453 bacterial isolates from the water and sediments collected from aquatic environments (Kamei et al., 1987c, 1988b). In our next step of this research series, we succeeded in isolating the antiviral substance from the culture fluid of *Pseudomonas fluorescens* biovar I 46NW-04 by extraction with ethyl acetate and purification with column chromatography and TLC on silica gel. Eventually, 709 mg of antiviral substance 46NW-04A was isolated and purified from 30 L of the culture fluid.

Several investigators have reported that the lack of viral survival in natural water is caused by microorganisms and some extracellular coumpounds released from the microorganisms (Toranzo et al., 1982, 1983; Ward et al., 1986). However, there have been no reports on antiviral substances actually isolated and characterized from the microorganisms native to aquatic environments, although several investigators suggested the causative agent of viral inactivation was proteolytic bacterial enzymes (Toranzo et al., 1982, 1983; Ward et al., 1986). However, since we first succeeded in the isolation and characterization of a low molecular weight antiviral substance (molecular weight: 1,125) from an aquatic bacterium, we propose that the virus inactivation in natural water is also likely caused by low molecular weight compounds released by aquatic bacteria. The mechanism of this antiviral substance is probably to block attachment sites on the virus surface.

So far, we have several reports about the antiviral antibiotics, some are blocking of cell receptors (Deig et al., 1974; Ehresmann et al., 1977; Richards et al., 1978), and some are inhibiting of virus adsorption (Davie et al., 1962), transcription (Awaya et al., 1979; Aswell et al., 1977; Ch'ien et al., 1973; Underwood, 1962), or translation (Taguchi et al., 1981). In this study, possibilty of blocking of cell receptors by 46NW-04A was tested by treating the cell surface before virus was added. When the cell surface was treated by 46NW-04A before and after IHNV was inoculated, the antiviral activity was observed just after the virus inoculation, indicating this antiviral mechanism is unlikely blocking of cell receptors and inhibition of transcription or translation. A more detailed study showed that 46NW-04A directly effected virus particles, because antiviral activity increased with prolonged reaction time with virus. From these results, the actual action of 46NW-04A against IHNV appeared to be blocking attachment sites of IHNV, probably spikes on the virus envelopes. This antiviral mechanism was elucidated by the fact that herpes virus, OMV was also inactivated, but not non-enveloped virus, IPNV by 46NW-04A. By these antiviral mechanisms, these antiviral substance-producing bacteria may inactivate viruses and play a very important role on control of outbreaks of fish viral diseases in nature.

SUMMARY

The antiviral agent 46NW-04A, effective against salmonid viruses, was isolated and partially purified from the culture fluid of *Pseudomonas fluorescens*

46NW-04 by ethyl acetate extraction and TLC on silica gel. It was finally purified by HPLC on C_{18}-phenyl gel and the physicochemical characteristics and chemical structure were determined. MS, NMR, and amino acid analyses proved that 46NW-04A is a cyclic peptide composed of three leucines, two serines, threonine, glutamic acid, valine, isoleucine, and 3-hydroxy decanoic acid. Its antiviral activity measured as 100% plaque reduction was observed at 25 μg/ml against *Oncorhynchus masou* virus (OMV) and infectious hematopoietic necrosis virus (IHNV). However, no antiviral activity was observed against infectious pancreatic necrosis virus (IPNV) at the concentrations tested. 46NW-04A appeared to specifically effect the enveloped viruses. This antiviral mechanism may act by blocking attachment sites of the viruses, probably spikes.

REFERENCES

Aswell J F, Allen G P, Jamieson A T, Campbell D E, Gentry G A (1977) Antiviral activity of arabinosylthymine in herpesviral replication: Mechanisms of action in vivo and vitro. Antimicrob Agents chemother 12: 243-25

Awaya J, Matsuyama K, Iwai Y, Omura S, Oiwa H, Hasegawa Y, Ikai T (1979) Production of 9-β-arabinosyladenine by a new species of *Streptomyces* and its herbicidal activity. J Antibiotics 32: 1050-1054

Ch' ien L T, Canon N J, Charamella J J, Dismukes W E, Whitley R J, Buchana R A, Alford C R (1973) Adenosine araboside treatment of severe herpesvirus hominis infectious in man. J Infect Dis 128: 658-663

Cliver D O, Herrmann J E (1972) Proteolytic and microbial inactivation of entroviruses. Water Res 6: 797-805

Davies W L, Grunert R R, Haff R F, MacGahen J W, Neumayer E M, Paulshock M, Watts J C, Wood T R, Hermann E C, Hoffmann C E (1962) Antiviral activity of 1-adamantanamine (amantadine). Science 144: 862-863

Deig E F, Ehresmann D W, Hatch M T, Riedlinger D J (1974) Inhibition of herpesvirus replication by marine algae extracts. Antimicrob Agents Chemother 6: 524-525

Ehresmann D W, Deig E F, Hatch M T, DiSalvo L H, Vedros N A (1977) Antiviral substances from California marine algae. J Phycol 13: 37-40

Fryer J L, a. Yusha A, Pilcher K S (1965) The in vitro cultivatiion of tissue and cells of pacific salmon and steelhead trout. Ann N Y Acad Sci 126: 566-586

Fujioka R S, Loh P C, Lau L S (1980) Survival of human enteroviruses in the Hawaiian ocean environment: Evidence for virus-inactivating microorganisms. Appl Environ Microbiol 39: 1105-1110

Hiramoto M, Okada K, Nagai S (1970) The revised structure of viscosin, a peptide antibiotic. Tetrahedron Lett No 13: 1087-1090

Kamei Y, Yoshimizu M, Ezura Y, Kimura T (1987a) Plaque assay of *Oncorhynchus masou* virus (OMV). Fish Pathol 22: 147-152

Kamei Y, Yoshimizu M, Ezura Y, Kimura T (1987b) Effects of estuarine and marine waters on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). Bull Fac Fish Hokkaido Univ 38: 271-285

Kamei Y, Yoshimizu M, Ezura Y, Kimura T (1987c) Screening of bacteria with antiviral activity against infectious hematopoietic necrosis virus (IHNV) from estuarine and marine environments. Nippon Suisan Gakkaishi 53: 2179-2185

Kamei Y, Yoshimizu M, Ezura Y, Kimura T (1988a) Effects of environmental water on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). J Appl Ichthyol 4: 37-47

Kamei Y, Yoshimizu M, Ezura Y, Kimura T (1988b) Screening of bacteria with antiviral activity from fresh water salmonid hatcheries. Microbiol Immunol 32: 67-73

Kamei Y, Yoshimizu M, Ezura Y, Kimura T (1988c) Identification of *Pseudomonas* sp. 46NW-04 which produces antiviral agent against fish viruses. Bull Fac Fish Hokkaido Univ 39: 124-132

Kimura T, Yoshimizu M, Ezura Y, Kamei Y (1990) An antiviral agent (46NW-04A) produced by *Pseudomonas* sp. and its activity against fish viruses. J Aquat Animal Heal 2: 12-20

Richards J M, Kern E R, Glasgow L A, Overal J C, Deig E F, Hatch M T (1978) Antiviral activity of extracts from marine algae. Antimicrob Agents Chemother 14: 24-30

Stahl E, Kaltenbach U (1961) Dunnschichtchromatographie VI. Mitteilung. Spurenanalyse von Zuckergemischen auf Kieselgur-G Schichten. J Chromatog 5: 351-355.

Taguchi F, Imatani Y, Nagai D, Nakagawa A, Omura S (1981) Selective antiviral activity of the antibiotic 2'-amino-2'-deoxyribofuranosyl adenine. J Antibiotics 34: 313-316

Toranzo A E, Barja J L, Hetrick F M (1982) Antiviral activity of antibiotic-producing marine bacteria. Can J Microbiol 28: 231-238

Toranzo A E, Barja J L, Hetrick F M (1983) Mechanism of poliovirus inativation by cell-free filtrates of marine bacteria. Can J Microbiol 29: 1481-1486

Underwood G E (1962) Activity of 1-β-D-arabinofuranosylcytosine hydrochloride against herpes simplex keratitis. Pro Soc Exp Biol Med 111: 660-664

Ward R L, Knowlton D S, Winston P E (1986) Mechanism of inactivation of enteric viruses in fresh water. Appl Environ Microbiol 52: 450-459

Wolf K, Quimby M C (1962) Established eurythermic line of fish cells in vitro. Science 135: 1065-1066

Yoshimizu M, Takizawa H, Kamei Y, Kimura T (1986) Interaction between fish pathogenic viruses and microorganisms in fish rearing water: Survival and inactivation of infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus and *Oncorhynchus masou* virus in rearing water. Fish Pathol 21: 223-231.