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Purification of an Antiviral Substance Produced by *Alteromonas* sp. and Its Virucidal Activity against Fish Viruses

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An antiviral substance of a high molecular weight, low cytotoxity and potent virucidal activity was purified from the culture supernatant of a marine *Alteromonas* sp. 48HS-27. Maximum production of this antiviral substance by the strain in MCYG broth was attained by 72 h-incubation at 25°C. By the purification procedure involving ultrafiltration, precipitation with ammonium sulfate and acetone, gel filtration and native-polyacrylamide gel electrophoresis (PAGE), a polypeptide (48HS-27A) with antiviral activity was obtained at a 270-fold purification with 6.20% yield from the culture supernatant. Molecular weight of the purified 48HS-27A was estimated as approximately 52 kDa by both native and sodium dodecyl sulfate (SDS) PAGE. The 50% infection inhibitory concentrations of this substance were from 0.09 to 2.51 μg/ml against one herpesvirus and five rhabdoviruses, whereas the minimal cytotoxic concentration of the substance was 144 μg/ml against FHM and CHSE-214 cells. The purified 48HS-27A had proteolytic activity against casein and bovine serum albumin.

Key words: marine bacteria, *Alteromonas*, antiviral substance, polypeptide

For the prevention of fish viral diseases, it is important to know the survival of viruses in natural water because waterborne transmission is one of the most common routes for infectious diseases of aquatic organisms.

Many investigators have reported that the survival of viruses in natural water is influenced by extracellular compounds released from the ambient microorganisms (Fujioka *et al.*, 1980; Toranzo *et al.*, 1983; Yoshimizu *et al.*, 1986; Kamei *et al.*, 1987b, 1987c), and several compounds were reported to be responsible for the viral inactivation process in aquatic environments. Ward and Ashley (1977) have reported ammonia as a low molecular weight and heat-stable virucidal agent in active sludge. Kimura *et al.* (1990) have purified a low molecular weight peptide with antiviral activity produced by bacteria and have reported that this peptide, namely 46NW-04A, was composed of nine amino acids bound at its *N*-terminal with 3-hydroxy decanoic acid. However, most other investigators suggest that viral inactivation is caused by high molecular weight and heat-labile substances produced by bacteria, such as proteolytic enzyme or ribonuclease (Cliver and Herrmann, 1972; Ward and Ashley, 1976; O'Brien and Newman, 1977; Toranzo *et al.*, 1983). Although some changes in viral integrity during the enzymatic inactivation process have been recognized (Ward and Ashley, 1976; O'Brient and Newman, 1977; Toranzo *et al.*, 1983), these reports did not include isolation and characterization of these substances.

In our previous report, we described a simple classification method for antiviral substance produced by 63 bacterial strains which had been isolated from various environmental water and had the antiviral activity against infectious hematopoietic necrosis virus (IHNV) (Myouga *et al.*, 1993). The antiviral substances produced by the four selected strains were partially characterized and one of the strains, *Alteromonas* sp. 48HS-27, produced a high molecular weight and heat-labile substance. In the present study, we first purified the antiviral substance produced by *Alteromonas* sp. 48HS-27, and secondly evaluated the antiviral, cytotoxic, and enzymatic (proteolytic) activities of the purified substance.

Materials and Methods

Bacterium

Alteromonas sp. strain 48HS-27 was used for this

study. This bacterium was isolated from a sediment of Nanaehama coast near Hakodate, Japan in August, 1984 (Kamei *et al.*, 1987b), and was capable of producing an antiviral substance belonging to Type II, which causes direct inactivation of virus or inhibition of viral invasion into the cells (Myouga *et al.*, 1993). The strain has been stocked in TSB seawater medium (BBL) containing 20% glycerol at −80°C.

Viruses and cell lines

In this study, the following six viruses of fish Rhabdoviridae were used: genus *Lissavirus*; infectious hematopoietic necrosis virus (IHNV strain ChAb) and *Rhabdovirus olivaceus* (HRV strain 8401-H), and genus *Vesiculovirus*; spring viremia of carp virus (SVCV), eel virus Europe X (EVEX), eel virus of America (EVA), and pike fry rhabdovirus (PFR). SVCV were kindly provided by Dr. B. J. Hill (Fish Disease Laboratory, Ministry of Agriculture, Fisheries and Food, Weymouth, UK), EVEX and EVA by Dr. T. Sano (Laboratory of Fish Pathology, Tokyo University of Fisheries, Japan), and PFR by Dr. W. Anne (Institute of Zoology and Hydrobiology, University of Münich, Germany). Other three kinds of fish viruses were also used: a herpesvirus; *Oncorhynchus masou* virus (OMV strain 00-7812), a birnavirus; infectious pancreatic necrosis virus (IPNV strain ATCC VR299), and a virus isolated from brain of abnormally swimming salmonid (strain BrCo-9221, Oh *et al.*, 1995).

Stocks of viruses were prepared by RTG-2 (Wolf and Quimby, 1962), CHSE-214 (Lannan *et al.*, 1984) or FHM cells (Gravell and Malsberger, 1965). Antiviral activity was evaluated using either CHSE-214 or FHM cells, while cytotoxicity was determined for both cells.

Assay of antiviral activity and cytotoxicity

The bacterial culture fluid or the purified substance was reacted with viruses at 15°C for 2 h, and the antiviral activity was determined according to the plaque assay method described by Kamei *et al.* (1987a). A serial two-fold dilution of the sample was prepared by Hanks' balanced salt solution (Hanks' BSS, Gibco) and the 50% inhibitory dilution rate (IHD_{50}) or concentration (IC_{50}) of each sample was calculated from the dilution rate or dose response curves according to Hayden *et al.* (1980). To compare our results with the data of known antiviral agents against fish viruses, the minimal inhibitory concentration (MIC) required to completely inhibit the 100 PFU virus was examined.

Cytotoxic effect was observed for 7 days after addition of the purified antiviral substance to the culture medium of cells.

Culture conditions for production of antiviral substance

Alteromonas sp. 48HS-27 was precultured on SA medium (Yamamoto *et al.*, 1982) at 25°C for 48 h. Then a loopful of the precultured cells was suspended into 10 m*l* of 50% Herbst's artificial seawater. In order to determine the optimal incubation temperature to produce the antiviral substance, 10 m*l* of MCYG broth (0.5% casamino acids, Difco; 0.05% yeast extract, Difco; 0.1% glucose in 50% Herbst's artificial seawater; pH 7.8: Kamei *et al.*, 1987c) in a L-form test tube (length 18 cm, diameter 1.7 cm) was inoculated with 100 μl of the bacterial suspension, and was incubated for 48 h at 15–30°C with shaking by temperature gradient bio-photorecorder (TN-112D, Toyo Kagaku Sangyo Co., Lit.). For the optimal incubation time, 10 m*l* of the bacterial suspension was inoculated into 1 *l* of MCYG broth in a 2 *l* Erlenmeyer flask, and then the culture was incubated at 25°C with shaking and a 10 m*l* aliquot of the culture was periodically removed. Of the 10 m*l* bacterial culture fluid samples, 8 m*l* aliquots were used for determination of pH and the optical density at 620 nm, and the other 2 m*l* aliquots were employed for the plauqe assay after filtered through a 0.45 μm-pore size filter (Millex-HA, Millipore).

Isolation and purification of antiviral substance

One loopful of the stock culture of *Alteromonas* sp. 48HS-27 was inoculated into 50 m*l* of MCYG broth in an Erlenmeyer flask and incubated at 25°C for 48 h with shaking. Four Erlenmeyer flasks each containing 2.5 *l* of MCYG broth were inoculated with 10 m*l* of the culture. After incubation at 25°C for 72 h with shaking, the culture was centrifuged at 13,000 $\times g$ at 1°C for 20 min and the supernatant was used as the starting material for purification of antiviral substance. All operations for isolation and purification were carried out at 0–1°C.

The culture supernatant of about 10 *l* was concentrated to 500 m*l* by tangential flow ultrafiltration using a Millipore Pellicon cassette system with polysulfone membrane (filter type; PT) which allows for

the retention of substances larger than 30,000 molecular weight, and then 500 m*l* of ice-cold distilled water was added. After the addition of 714.5 g of solid ammonium sulfate to give 95% saturation, the culture was allowed to stand for 12 h. The precipitate obtained by centrifugation at 16,000×*g* for 20 min was dissolved into 20 m*l* of a 16 mM Tris-HCl buffer (pH 8.0). Forty m*l* of ice-cold acetone was added to the 20 m*l* solution and the mixture was left to stand for 2 h. The precipitate was withdrawn by centrifugation at 9,500×*g* for 20 min. This procedure of dissolution and precipitation was repeated three times. The finally obtained precipitate was dissolved in 20 m*l* of the 16 mM Tris-HCl buffer (pH 8.0). The solution was subjected to ascending gel filtration chromatography (5 m*l*×4 times) by Sephacryl S-500 (Pharmacia) column (2.6 cm×40 cm) in 16 mM Tris-HCl buffer (pH 8.0) as an eluant (flow rate; 30 m*l*/h). Four fractions (each 5 m*l*) showing anti-IHNV activity were pooled (80 m*l* in total) and concentrated again to 20 m*l* by ultrafiltration using 20,000 molecular weight cut-off filter (Centricut U-20, Kurabou). The partially purified substance was further purified two times by native-polyacrylamide gel electrophoresis (PAGE) (Davis, 1964) using vertical slab gels (9×6.7×0.1 cm) prepared with different gel concentrations (12.5% and ensuing 7.0%). Electrophoresis was performed at a constant current of 15 mA per gel, and the gel was stained with coomassie brilliant blue R-250 or silver nitrate. Periodic acid-Schiff (PAS) staining was also conducted to examine the presence of glycoproteins. After electrophoresis, a portion of the gel with antiviral activity was cut out from the gel, homogenized in 2 m*l* of 16 mM Tris-HCl buffer (pH 8.0) and eluted into the buffer by incubation for 12 h. Homogeneity of antiviral substance was confirmed by native-PAGE using 12.5% gel. The protein concentration was measured by the improved Lowry's method (Bearden, 1978), with bovine serum albumin (Wako) as a standard.

Estimation of molecular weight

Molecular weight of the purified antiviral substance was estimated by both native- and sodium dodecyl sulfate (SDS)-PAGE.

The molecular weight by native-PAGE was determined according to the method of Bryan (1977) with the undenatured protein molecular weight marker kit (MW-ND-500 Kit, Sigma) as standard. Retardation coefficients (K_R) of antiviral substance and each marker protein were calculated from the negative slopes of Ferguson plot (Ferguson, 1964). Regression line of the plot was drawn by the least square method.

SDS-PAGE was performed as described by Laemmli (1970). The molecular weight in this method was determined according to Weber and Osborn (1969) with the SDS molecular weight marker kit (MD-SDS-70L, Sigma) as standard.

Proteolytic activity of purified antiviral substance

Proteolytic activity was determined according to Hagihara *et al.* (1956). One m*l* of the assay mixture contained 1% proteinaceous substrate and 1.1 μg of purified antiviral substance in 16 mM Tris-HCl buffer (pH 8.0). After incubation for 120 min at 15°C, the reaction was stopped by addition of 1 m*l* 0.4 M trichloroacetic acid (TCA). Then, 2.5 m*l* of 0.4 M Na_2CO_3 and 0.5 m*l* of 1 N phenol reagent (Wako) were added to 0.5 m*l* of supernatant of the mixture. After incubation for 20 min at 30°C, absorbance was measured at 660 nm. Blank was prepared by adding the substance after addition of TCA solution. An increase in 0.001 absorbance per m*l* in the present experimental conditions was defined as one unit of proteolytic activity.

Results

Optimal culture conditions for antiviral substance

Effect of incubation temperature on production of the antiviral substance was tested at 15 to 30°C for 48 h (Fig. 1). The bacterium grew well between 20 and 30°C, and maximal growth was observed at 28°C. Anti-IHNV activity was detected from the culture at 20–30°C, and the highest activity (4.6 IHD_{50}) was observed in the culture at 25°C. After the 48-incubation at 20–30°C, pHs of the culture were between 7.1 and 8.2. Subsequently, the production of antiviral substance was tested at 25°C for 180 h (Fig. 2). Bacterial growth reached a maximum at 72 h incubation. Obvious antiviral activity was observed from the late log phase to the death phase of bacterial growth and the maximal activity was observed at 72 and 96 h incubations.

Purification of antiviral substance

The results of purification of antiviral substance were summarized in Table 1. For the first step of

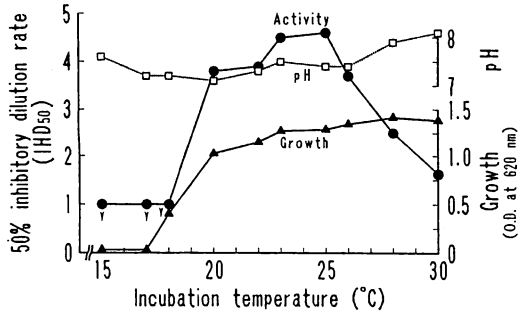


Fig. 1. Effect of incubation temperature on growth of *Alteromonas* sp. 48HS-27 and production of anti-IHNV substance (48 h incubation).
Y: less than detectable limit, O.D. = optical density

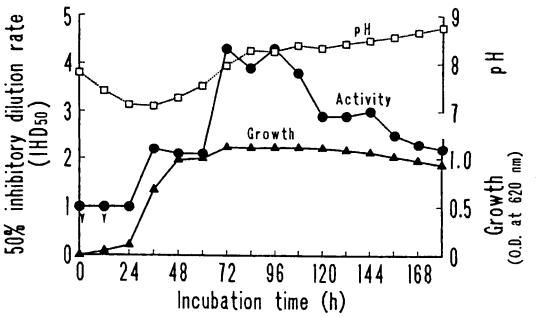


Fig. 2. Time course of growth of *Alteromonas* sp. 48HS-27 and production of anti-IHNV substance at 25°C.
Y: less than detectable limit, O.D. = optical density

Table 1. Purification of antiviral substance from the culture supernatant of *Alteromonas* sp. 48HS-27

Purification step	Volume (ml)	Protein (mg)	Total activity (IHD_{50}*$\times 10^2$)	Specific activity (IHD_{50}*/mg)	Purification factor (-fold)	Yield (%)
Culture supernatant	10,000	6,530	2,160	33.1	1.00	100
Ultrafiltration (MW 30,000>cut off)	1,000	2,590	2,080	80.3	2.43	96.3
$(NH_4)_2SO_4$ precipitation (95%)	40	1,700	1,920	113	3.41	88.9
Acetone precipitation (3 times)	20	188	493	262	7.92	22.8
Gel filtration (Sephacryl S-500)	20	7.71	210	2,720	82.2	9.72
Native-PAGE (12.5 & 7.0% gels)	20	1.50	134	8,930	270	6.20

*50% inhibitory dilution rate for IHNV (ChAb).

purification, 10*l* of the culture supernatant was concentrated by ultrafiltration, amonium sulfate precipitation and precipitation with acetone. The combination of ultrafiltration and ammonium sulfate precipitation was efficient to concentrate the anti-IHNV substance by 3.41-fold with a 88.9% yield. Repeated precipitation with acetone removed solvent-soluble substances from the sample and resulted in a 7.92-fold purification with a 22.8% yield. Further purification by Sephacryl S-500 gel filtration (fraction No. 33–36) and concentration by ultrafiltration resulted in 82.2-fold purification with a 9.72% yield. However, at this point the fractions contained three substances other than antiviral substance that appeared at 0.07 of relative mobility (Rf) in native-PAGE using 12.5% gel (Fig. 3A). One (Rf 0–0.08) was detected by periodic acid-Schiff staining (PAS) as a tailed band and the other two (Rf 0.12 and 0.17) by both coomassie brilliant blue R-250 (CBB) and silver nitrate (SN) staining (the band of Rf 0.17 was

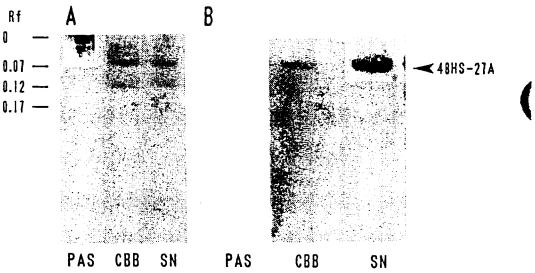


Fig. 3. Native-polyacrylamide gel electrophoresis of partially purified (after gel filtration; A) and purified (after electrophoresis with 12.5 & 7.0% gels; B) antiviral substance (48HS-27A). After electrophoresis with 12.5% gels, the gels were stained with periodic acid-Schiff (PAS), coomassie brilliant blue (CCB), and silver nitrate (SN).

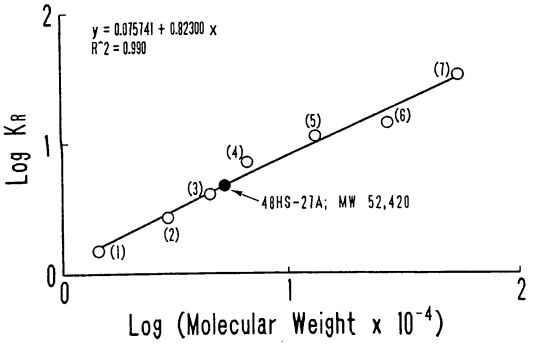


Fig. 4. Molecular weight estimation of purified antiviral substance (48HS-27A) by native-polyacrylamide gel electrophoresis. Open circles indicate standard proteins.
(1) α-lactalbumin from bovine milk (MW 14,200), (2) carbonic anhydrase from bovine erythrocytes (MW 29,000), (3) albumin from chicken egg (MW 45,000), (4) albumin monomer from bovine serum (MW 66,000), (5) albumin dimer from bovine serum (MW 132,000), (6) urease trimer from Jack bean (MW 272,000), (7) urease hexamer from Jack bean (MW 545,000)
K_R: − slope calculated by Ferguson plots (retardation coefficients)

unclear). These impurities were completely excluded by following native-PAGE using 7.0% gel, and a single band with high antiviral activity (Rf 0.07) was observed in 12.5% gel of native-PAGE (Fig. 3B). The final yield and purification factor of the purified antiviral substance were 6.20% and 270-fold, respectively.

Molecular weight of purified antiviral substance

Molecular weight of the purified antiviral substance estimated by native- and SDS-PAGE was approximately 52 kDa. The result obtained by native-PAGE is shown in Fig. 4 as a representative.

Cytotoxic effect of purified antiviral substance against fish cells

Cytotoxic effects of the purified antiviral substance against FHM and CHSE-214 cells were observed at concentrations higher than 144 μg/ml (Table 2).

Antiviral activity of purified substance against fish pathogenic viruses

Antiviral activity of the purified substance was evaluated by susceptibility test for nine fish viruses including IHNV (Table 3). The most susceptible virus to the substance was OMV belonging to Herpesviridae and the 50% inhibitory concentration (IC_{50}) of the substance was 0.09 μg/ml. Five rhabdoviruses except for EVEX were also susceptible to this substance and the IC_{50} were 0.27, 0.56, 1.10, 1.16 and 2.51 μg/ml for HRV, IHNV, SVCV, PFR and EVA, respectively. The susceptibility of EVEX to the substance was lower than other five rhabdoviruses and the IC_{50}s was 11.4 μg/ml. No antiviral activity of this substance was observed even at the concentration of 72 μg/ml against IPNV and virus from brain of salmonid, which belong to Birnaviridae and retro-like virus, respectively. As shown in Table 3, the

Table 2. Cytotoxic effect of the purified antiviral substance against fish cell lines

Cell line	Concentration (μg/ml)					
	228	144	72	36	18	9
FHM	+	+	−	−	−	−
CHSE-214	+	+	−	−	−	−

+: positive, −: negative.

Table 3. Antiviral activity of the purified antiviral substance against fish pathogenic viruses

Virus	IC_{50}* (μg/ml)	MIC** (μg/ml)
HRV	0.27	1.26
IHNV	0.56	4.50
SVCV	1.10	5.04
PFR	1.16	10.1
EVA	2.51	20.5
EVEX	11.4	>40.3
OMV	0.09	0.28
IPNV	>72.0	>72.0
BrCo 9221	>72.0	>72.0

*50% inhibitory concentration.
**minimal inhibitory concentration; see Materials and Methods.

Table 4. Proteolytic activity of the purified antiviral substance

Substrate	Activity* (units/ml)
Casein	686
Bovine serum albumin	66
Gelatin	0

*see Materials and Methods.

minimal inhibitory concentrations (MIC) of the purified substance were determined as 0.28, 4.50 and >72.0 μg/ml against OMV, IHNV and IPNV, respectively.

Proteolytic activity of purified antiviral substance

Purified antiviral substance showed proteolytic activity against casein and bovine serum albumin with 686 and 66 units/ml, while no activity was observed against gelatin (Table 4).

Discussion

In this paper, we attempted to purify the antiviral substance produced by *Alteromonas* sp. 48HS-27. The antiviral substance was successfully purified from the culture supernatant by a procedure involving ultrafiltration, ammonium sulfate precipitation, precipitation with acetone, gel filtration and native-PAGE. The final yield and purification factor of the purified antiviral substance, designated as 48HS-27A, were 6.20% and 270-fold, respectively (Table 1). A sigle band of 48HS-27A retaining intense antiviral activity was detectable in the gel of native-PAGE by coomassie brilliant blue R-250 or silver nitrate staining but not detectable by periodic acid-Schiff staining (Fig. 3B). The molecular weight of 48HS-27A was estimated to be approximately 52 kDa by both native- and SDS-PAGE (Fig. 4). These results suggest that 48HS-27A is a polypeptide and includes no sugar in its molecular structure. No antiviral activity was observed in any part of the gel after SDS-PAGE, indicating that 48HS-27A lost its antiviral activity after a sodium dodecyl sulfate treatment.

Subsequently, the 50% inhibitory concentration (IC_{50}) and the minimal inhibitory concentration (MIC) of 48HS-27A were determined against fish pathogenic viruses (Table 3). The 48HS-27A had high virucidal activities against a fish herpesvirus (OMV) with 0.09 μg/ml (IC_{50}) and 0.28 μg/ml (MIC), and against five rhabdoviruses except for EVEX with 0.27–2.51 μg/ml (IC_{50}) and 1.26–20.5 μg/ml (MIC). As the plaque formation of these six viruses described above was inhibited by 50% at 57 to 1,600-fold lower concentrations of 48HS-27A than its mimimal cytotoxic concentration against fish cell lines (144 μg/ml, Table 2), the substance was regarded to be an effective antiviral agent against these six fish viruses. Information about known antiviral agents against fish viruses are limited and most of them are against Herpesviridae (Kimura *et al.*, 1983, 1988, 1990; Hasobe and Saneyoshi, 1985; Suzuki *et al.*, 1987). OMV was more susceptive to 48HS-27A than the following known antiviral agents: (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU, MIC; 12.5 μg/ml), 9-(2-hydroxyethoxymethyl)guanine (ACV, MIC; 12.5 μg/ml), 1-β-D-arabinofuranosylcytosine (AraC, MIC; 2.0 μg/ml), 46NW-04A (MIC, 12.5 μg/ml), 5-iodo-2′-deoxyuridine (IUdR, IC_{50}; 1.0–3.2 μg/ml) and guanine 7-*N*-oxide (IC_{50}; 10 μg/ml). However, 1-β-D-arabinofuranosyl-*E*-5-halogenovinyluracils reported by Suzuki *et al.* (1987) had higher activities than 48HS-27A against OMV (IC_{50}; 0.003–0.01 μg/ml). On the other hand, against fish Rhabdoviridae, there are few reports on effective antiviral agents, other than guanine 7-*N*-oxide (IC_{50}; 20 μg/ml) and 46NW-04A (MIC; 25 μg/ml) against IHNV reported by Hasobe and Saneyoshi (1985) and Kimura *et al.* (1990), respectively. The 48HS-27A was 6–36 fold more active than these agents against IHNV.

As mentioned earlier, there are many reports on high molecular and heat-labile extracellular substances from environmental bacteria which can reduce the infectivity of enteroviruses. These substances have been indicated to have enzymatic activities that cause degradation of viral proteins or nucleic acids (Cliver and Herrmann, 1972; Ward and Ashley, 1976), though no report on purification of the substances was noted. In a simulated study of digested sludge, poliovirus was treated with both 100 μg/ml of proteolytic enzyme (trypsin, chymotrypsin or pronase) and 20 μg/ml of ribonuclease, which were commercially available, at 4°C for 10 days as some substitutes for the bacterial enzymes related with viral inactivation in the environments, however no significant reduction was observed in its infectivity (Ward and Ashley, 1976). Other investigators have demonstrated that low molecular weight substances of less than 500, in addition to these enzymes, were involved in viral inactivation (Cliver and Herrmann, 1972). Therefore, Toranzo *et al.* (1983) concluded that the enzymatic degradation was not the first step in the viral inactivation process, although the enzymatic degradation of the virus was undoubtedly important. To know the significance of enzymatic activities in inactivation of viruses, purification and characterization of the enzymes having antiviral activity are required, because these enzymes

produced by bacteria may differ from the restrictively substituted enzymes used in the tentative experiment (Ward and Ashley, 1976) in their characters, e.g. substrate specificity, structure of active center, optimal conditions for activity and specific inhibitors, and the properties of enzymes may have an influence on the reduction of viral infectivity.

In this study, it was shown that 48HS-27A markedly reduced the infectivity of a fish herpesvirus and rhabdoviruses at the concentration of less than 2.51 μg/ml within 2 h-incubation at 15°C. The substance was a polypeptide of 52 kDa and as described previously, 77% of its anti-IHNV activity in culture supernatant was lost by heating at 60°C for 15 min (Myouga *et al.*, 1993). This substance had proteolytic activity against casein and bovine serum albumin (Table 4). Proteolytic activity of this substance against structural proteins of fish pathogenic viruses will be described in following reports.

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