Establishment of an Antiviral Activity Assay and the Identification and Partial Purification of Interferon-like Protein from Rainbow Trout Gonadal Cells (RTG-2)

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Shih-Bin Lin, Chih-Hsin Lin and Ya-Li Hsu (2001) Establishment of an antiviral activity assay and the identification and partial purification of interferon-like protein from rainbow trout gonadal cells (RTG-2). *Zoological Studies* **40** (3): 240-246. In this study, a rapid (performed within 24 h) and effective bioassay method free from overestimation or false positives for antiviral activity due to virus residue (within an MOI range of 6.4×10^{-4} to 0.4) was established. Antiviral proteins, generated by infectious pancreatic necrosis virus (IPNV) induction of rainbow trout gonadal cells (RTG-2), were partially purified by HPLC size exclusion chromatography and native polyacrylamide gel electrophoresis (PAGE); the chromatography demonstrated 2 possible antiviral proteins with molecular weights of 100 and 18 kDa, respectively. Electrophoresis produced 1 antiviral protein with a molecular weight of about 100 kDa identified through Western blotting with polyclonal anti-human IFN-γ. The yield of antiviral protein obtained from 10-d-old cells was 1.6-3.2 times higher than that from 6-d-old cells. http://www.sinica.edu.tw/zool/zoolstud/40.3/240.pdf

Key words: Interferon-like protein, Antiviral protein, RTG-2, IPNV.

Interferon, which has been well studied in mammalian species, is a term that generally describes those proteins with antiviral activity (De Maeyer and De Maeyer-Guignard 1988). In addition, interferon also possesses immunomodulatory and antiproliferative functions (Stewart 1979), hence it has been considered for use in the prevention and therapy of infectious diseases. Based on different biological functions and physical-chemical properties, interferons are classified into 2 types: type 1 (IFN-α and -β) and type 2 (INF-γ) (Stewart 1980, Hosoi et al. 1988, Sano et al. 1988).

Aquaculture is a major industry in many countries around the world. However, very often, fish grown in intensive hatcheries are susceptible to the threat of infectious diseases, especially viral diseases. So far, no effective way to control viral diseases has been found. Interferon, having been used in clinical applications in mammalian systems, may have potential for use in the therapy or prevention of virus diseases in fish. A fish interferon or interferon-like protein has been identified in several fish species (Beasley and Sigel 1967, Oie and Loh 1971, De Sena and Rio 1975, Tamai et al. 1993a b); however, fish antiviral protein has been purified and cloned only from oncogene-immortalized flatfish lymphocytes and has shown very limited homology to mammalian interferon (Tamai et al. 1993a).

To characterize fish antiviral protein, an interferon-like protein virally induced in a rainbow trout gonad cell line (RTG-2) was identified and partially purified in this study. In addition, in order to evaluate the antiviral protein rapidly and effectively, a modified method for assaying antiviral activity was established.

MATERIALS AND METHODS

Cell cultures and virus strain

All cell cultures were grown in Eagle's minimal

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essential medium (MEM-10) (Gibco, Gaithersburg, MD) at 20 °C, to which penicillin (100 U/mL), streptomycin (100 mg/mL), fungizone (2.5 mg/mL), gentamycin (25 mg/mL), and 10% fetal calf serum (FCS) (Gibco) was added. The maintenance medium (MEM-0) contained the same material as the growth medium, but lacked FCS. A rainbow trout gonad cell line (RTG-2) was used for producing IFN-like protein and for bioassaying antiviral activity. The virus used for induction of the IFN-like protein was infectious pancreatic necrosis virus (IPNV), strain T42G, that was propagated in chinook salmon embryo cells (CHSE-214). Virus titer was determined by the $TCID_{50}$ assay of confluent monolayers of CHSE-214 cells in a 96-well microtiter plate and was interpolated as described by Reed and Muench (1938).

Preparation of IFN-like protein samples

The capacities of 2 different ages of cells, 6 and 10-d-old, producing IFN-like protein induced by virus at different MOIs (of 0.1, 0.25, or 1) or by UV-inactivated virus at a MOI of 1 were compared. For induction, adherent cells cultured in 175-T flasks were washed once with PBS. Two milliliters of virus at the desired MOI in MEM-0 was then added. After adsorption for 1 h, the induction medium was aspirated, and cells were washed 3 times with PBS; MEM-0 was subsequently added for collecting the secreted antiviral protein. Production medium, at about 1 l/batch, was collected after a 24- or 48-h induction period. The collected medium was adjusted to pH 3.5 and stored for 24 h at 4 °C, after which the pH was adjusted to pH 7.0 before being concentrated and desalted by ultrafiltration processes through MiniPlate Bioconcentrators (3k cut off) (Amicon, Beverly, MA).

Antiviral activity assay

Two-fold serially diluted samples were assayed in triplicate according to the method of Rubinstein et al. (1981) with some modifications: the established RTG-2 cells, cultured in 96-well microtiter plates to a confluent monolayer(approximately 4×10^4 cells/well), were incubated with prepared samples for 6 h followed by challenge with virus at an MOI of 100. After the CPE of the positive control group, which received no treatment but virus infection, was observed to be almost complete (> 90% in about 24 h), the sample-treated cells were stained with crystal violet, and the amount uptaken by cell was quantified by absorbance read at a wavelength of 595 nm. The antiviral activity titers of samples were expressed as dye uptake units (DU), determined as the reciprocal of the last dilution that gave more than 50% dye absorbance of negative control cells, which received no treatment.

Residual virus effects in the antiviral activity assay

In order to understand how the residual virus in induction medium could affect the antiviral activity assay, the following experiment was performed. Virus in different multiplicities of infection (MOI) (5-fold serial dilution of virus sample with MOIs from 2×10^{-7} to 2) was used to prime the established RTG-2 cells (in 96-well microtiter plates). After 6, 12, 18, or 24 h of priming infection periods, cells were challenged with virus at an MOI of 100. After the cytopathic effect (CPE) of positive was completed, cells were stained with crystal violet, and the amount uptaken by cells was quantified by reading the absorbance at a wavelength of 595 nm. Cell survival was determined by comparison with the negative control cells.
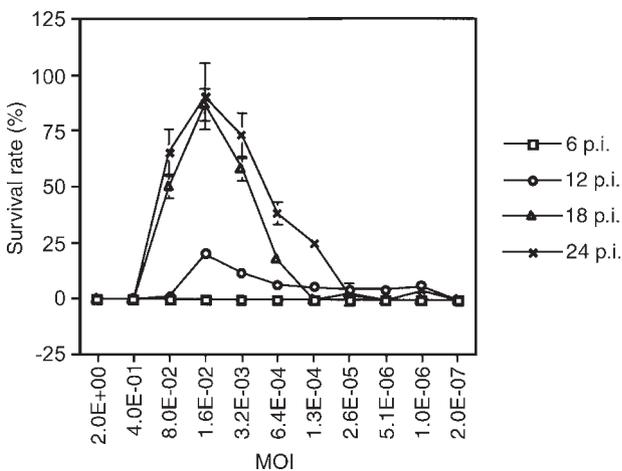
Fractionation of virus-induced samples

Two methods, high-performance size-exclusion chromatography (HPSEC) and native polyacrylamide gel electrophoresis (PAGE), were used to fractionate the concentrated samples. The HPSEC apparatus (Waters Associates, Milford, MA) was equipped with a Superose 6 HR 10/30 column (Pharmacia Fine Chemical, Uppsala, Sweden) and was operated at 25 °C with a UV detector at 280 nm. The eluent was PBS buffer (pH 7.2) at a flow rate of 0.5 ml/min, and fractions were collected at 0.5 ml/tube. The molecular weight markers (Sigma, St. Louis, MO) were α-amylase (*Mr* 200 kDa), alcohol dehydrogenase (*Mr* 150 kDa), BSA (*Mr* 67 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Fractions were concentrated and desalted by using a Centricon 3k prior to antiviral activity assay.

Native gels (10% resolving gels with 4% stacking gels) prepared as SDS-PAGE (Laemmli 1970), except containing no SDS or reducing agent, were used to fractionate the concentrated samples with Bio-Rad Mini-Protean II electrophoresis system (Hercules, CA). After electrophoresis, the gels were excised into 15 pieces at a width of about 3 mm and were denoted as fractions 1 to 15 from top to bottom. The sliced gels were crushed, and the protein was eluted with 0.2 ml distilled water twice for the antiviral activity assay. Just prior to the assay, the eluents were concentrated and desalted with a Centricon 3k against PBS buffer.

SDS-PAGE, containing the broad-range protein markers (2 to 212 kDa) purchased from New England Biolab (Beverly, MA), and Western blotting were performed to analyze the antiviral activity-containing fractions.

Western blotting

Western blotting was performed according to the method of Gershoni and Palade (1983) with some modifications. Briefly after electrophoresis, the protein in gel was transferred onto a PVDF membrane (Applied Biosystems, Foster City, CA) using a Semi Dry Electroblotter (Sartorius North America, Long Island, NY) and transfer buffer containing 20% methanol, 39 mM glycine, 48 mM Tris, and 0.0375% SDS at a constant current of 3 mA/cm^2. The blot was blocked with 4% skim milk in PBS containing 0.1% Tween 20 (PBST) overnight at 4 °C followed by incubation with primary antibody (1:2500 dilution in PBST) and polyclonal rabbit anti-human IFN-α or rabbit anti-human IFN-γ (Pepro Tech EC, London, UK) for 2 h. After washing with PBST, HRP-conjugated goat anti-rabbit IgG (Bio-Rad) was added, and the sample was incubated for an additional hour at room temperature. The ECL kit purchased from Amersham (Buckinghamshire, UK) was used for blotting.

RESULTS

Modification of the antiviral activity assay

The conventional antiviral activity assay (Rubinstein et al. 1981) suffers from the possible overestimation caused by residual virus in the induction medium. According to figure 1, cells primed with virus infection at MOIs between 8×10^{-2} and 1.3×10^{-4} for incubation times longer than 12 h survive better following viral challenge. The results clearly show that minute amounts of virus for an appropriate incubation time can confer strong protection against virus challenge. This suggests that even a minute amount of virus existing in a test sample will cause significant overestimation of antiviral activity. The potential effect of virus residue can be eliminated with a priming period shorter than 6 h. Therefore, the proper incubation time to exclude the effect of virus residue in the assay was determined to be 6 h, a time that was used for all subsequent assays of antiviral activity. It was also found that the highest cell survival could be obtained when cells were primed with virus infection at an MOI of 1.6×10^{-2} and with an incubation period longer than 18 h; these conditions were determined to be optimum for inducing antiviral proteins.

Effects of incubation time and virus residue on antiviral activity assay

Since a minute amount of virus, as shown in figure 1, can confer strong antiviral activity to RTG-2 cells, during the assay period, the antiviral activity of tested samples resulting from unavoidable viral residue might lead to overestimation of antiviral activity. For this reason, two different batches of induction medium, S18 and S19, with virus residues of $TCID_{50}$/ml of 10^5 and 10^6, respectively, were evaluated for their antiviral activity at 2 different incubation times (6 and 24 h) (Table 1). There was no difference in the antiviral activities of the 2 batches when the assay

Fig. 1. RTG-2 cells infected with a broad range of tested IPNV MOIs (2×10^{-7} to 2.0), and, after the indicated postinfection (p.i.) incubation times (6 [□], 12 [○], 18 [Δ], and 24 h [×]), cells were challenged with an IPNV MOI of 100. Antiviral activity determined as survival rate (%) was plotted against the tested virus MOI at 4 different postinfection incubation times. MOI expressed as "4.0E-01" stands for 4.0×10^{-1}. Vertical bars represent the standard error of the mean.

Table 1. Effects of incubation time and viral residue in the inducing medium on the antiviral activity assay

Batch number	S18	S19
Viral residue in induction medium ($TCID_{50}$/ml)	10^5	10^6
Antiviral activity (DU/100 ml) determined at 6 h of incubation	512	512
Antiviral activity (DU/100 ml) determined at 24 h of incubation	8192	32768

was done with an incubation time of 6 h; however, antiviral activity increased as the virus residue increased when the assay was done with an incubation time of 24 h (Table 1). According to the result obtained from figure 1, overestimation of activity was probable at an incubation time of 24 h. The results obtained at an incubation time of 6 h may have been underestimated.

Parameters affecting the induction of antiviral activity

Both virus MOI and cell age were evaluated for their effects on the induction of antiviral activity. Samples were prepared by inducing RTG-2 cells (at 6- or 10-d-old) with UV-inactivated virus (MOI = 1) and native virus with MOIs of 0.01, 0.1, or 1.0, respectively. According to table 2, it was found that the UV-inactivated virus (S17) appeared to be less efficient at inducing antiviral activity than was the native virus (S18). Also, it appeared that virus MOIs at between 0.01 to 1.0 (S18-S20) showed no difference in their ability to induce antiviral activity. Moreover, more antiviral activity could be induced in 10-d-old cells than in 6-d-old cells (S20 vs. T20) (Table 2).

Partial purification and identification of antiviral proteins

Concentrated samples were partially purified. An HPSEC protein profile with corresponding antiviral activity profile of the concentrated sample is shown in figure 2, in which antiviral activity suffered from low resolution and was broadly distributed. The 2 highest antiviral activity peaks are located at molecular weights of about 110 and 18 kDa, respectively.

In addition to HPSEC, antiviral protein was also partially purified by native PAGE as described above; among the fractions eluted from native PAGE slices,
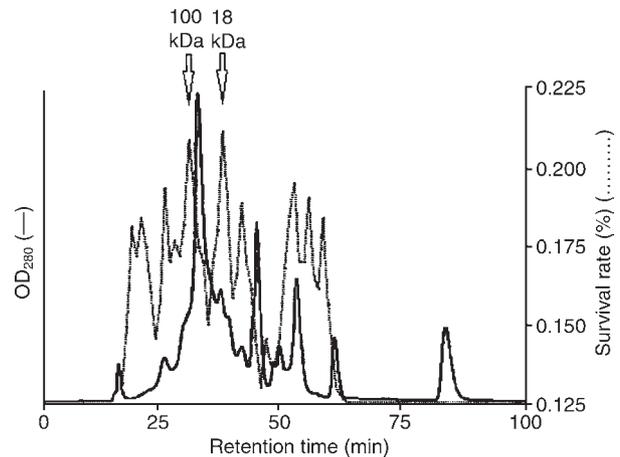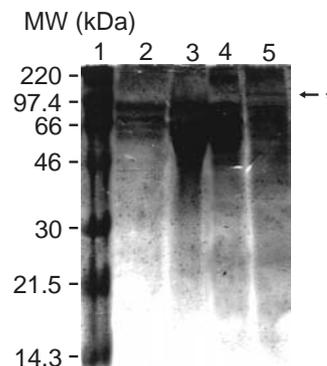

Fig. 2. HPSEC profile (——) and antiviral activity profile (·········) of a concentrated antiviral protein-containing sample.

antiviral activities were detected in fractions 2 to 4. These fractions comprised more than 80% of the initial activity and were further concentrated and analyzed by both SDS-PAGE and Western blotting. The former stained with silver staining (Fig. 3) demonstrating a differential band in the induced sample, which was not seen in the non-induced sample; subsequent Western blotting also demonstrated a band detected by polyclonal IFN-γ antibody at the same position (about 100 kDa) (Fig. 4). However, polyclonal IFN-α antibody detected no protein.

DISCUSSION

Identifying the antiviral activity of interferon, especially during IFN purification requires a reliable,

Table 2. Comparison of antiviral activity of samples from different batches prepared by varying the induction conditions

Batch number	*S17	S18	S19	S20	$T20
MOI (IPNV/RTG-2 cells) for induction	#1	1	0.1	0.01	0.01
Antiviral activity (DU/100 ml) determined at 6 h of incubation	256	512	512	512	1200

*S, cells were 6-d-old.
$T, cells were 10-d-old.
#Virus used was UV inactivated.

Fig. 3. Silver staining of 12.5% SDS-PAGE analysis of antiviral protein-containing fractions (2, 3, and 4) eluted from native gel. Lane 1, standard marker; lane 2, negative control; lanes 3, 4, and 5 are fractions 4, 3, and 2, respectively. *, suspected antiviral protein. For details see "Materials and Methods".

reproducible, and rapid assaying method. Several methods for interferon assay have been developed for mammalian interferon, including antiviral activity assays for type I interferon (Wheelock 1966, Pestka et al. 1983) and antiproliferative assays for type II interferon (Aebersold et al. 1986, Tyring et al. 1986). These assay systems established for mammalian IFN have been adopted or modified for identifying the IFNs of other species.

Antiviral bioassays are regularly performed by incubating samples with targeted cells for at least 24 h before viral challenge. However, as shown in figure 1, even traces of viral contamination (a MOI of as little as about 1.3×10^{-4}) can cause false positive results or overestimation of antiviral activity if the incubation time is longer than 12 h; but no antiviral activity could be detected at an incubation of 6 h. According to these results, in a regular antiviral bioassay (i.e., more than a 24-h incubation time), antiviral activity of a virally induced sample might be significantly overestimated from residual virus in the sample. It was found that viral interference could be avoided by acid treatment (Cantell et al. 1971); however, it could not completely inactivate IPNV (data not shown), and the residual virus could still exert its effect. As a result, a compromise incubation time of 6 h, although it might underestimate antiviral activity, was used throughout this study. The antiviral activity of the INF-like molecule obtained from RTG-2 cells by De Sena and Rio (1975) (determined by using the regular bioassay method) had a much higher titer than did ours, which might be due to overestimation.

Aging of cell cultures after confluency increases the interferon yield to some extent (Havell and Vilcek 1972, Van Damme and Billiau 1981). This phenomenon was also seen in our study. There was about

2.3-fold increased antiviral activity when cells were aged for an additional 4 d (i.e., 4 d after confluency) before induction (Table 1).

Interferon is known to exhibit strong hydrophobicity and to tend to aggregate together (Rubinstein et al. 1979); therefore, only under denaturing conditions (e.g., with 4 M urea) can interferon be well resolved by a gel filtration column. This property of aggregation was also seen in our result, where the antiviral activity profile resolved by HPSEC ranged from molecular weights of below 200 Da to as high as 2000 kDa. Such a broad distribution could be caused by the following two conditions: (1) a delay in retention time by interaction between the matrix and proteins, or (2) formation of the polymeric form of antiviral proteins. The possible molecular weight of native-formed antiviral protein was discerned at 2 peaks: 100 and 18 kDa.

We also used native PAGE to partially purified the antiviral protein. The antiviral activity-containing fractions were further differentiated by a subsequent SDS-PAGE (Fig. 3) and Western blotting (Fig. 4). We found a single band at about 100 kDa using the polyclonal anti-human IFN-γ antibody. The molecular weight of this band was similar to that of 94 kDa reported by De Sena and Rio (1975), and could be the polymeric form of fish interferon. Fish INF-γ-like proteins have been reported by Graham et al. (1990) from rainbow trout leukocytes. These proteins had molecular weights of between 19 and 32 kDa, and they may represent the dissociating form of fish interferon.

It has been postulated that INF-γ exerts its function in the polymeric form (e.g., as a dimer [Yip et al. 1981] or a tetramer [Pestka et al. 1983]). Moreover, a new class of INF-γ (N-INF-γ) purified from rat neuron tissue displayed a similar biological function to lymphocyte-derived recombinant INF-γ with 3 molecular weights of 54, 62, and 66 kDa on PAGE (Olsson et al. 1994), which implies a conformational similarity between them. In addition, invertebrate species, such as tunicates, have been shown to possess cytokine-like molecules (e.g., interleukin 1-like molecules) and had much bigger molecular weights than did interleukin 1-like molecular identified in mammalian species (Beck et al. 1986 1989, Beck and Habicht 1991). These results raise the possibility that the larger proteins are the ancestral form of interferons.

In the present study, we pointed out the possible overestimation of antiviral activity obtained by using regular assay methods. The implication of this observation is that RTG-2 cells produce a very low amount of antiviral protein. The positive signal pro-
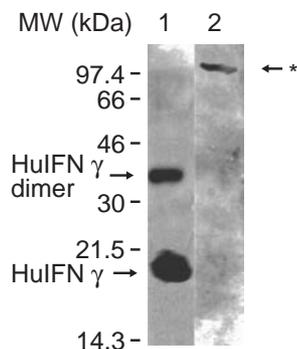
Fig. 4. Western blot analysis with rabbit-anti-hu-IFN-γ. Lane 1 is recombinant human IFN-γ. Lane 2 represents fraction 1 eluted from native gel. *, suspected antiviral protein. For details see "Materials and Methods".

duced by the polyclonal anti-human INF-γ antibody only provides indirect evidence for the existence of fish interferon. Therefore, it is key to obtain sufficient pure antiviral protein before more substantial results can be produced. To achieve this goal, we plan to overproduce this protein by cell selection and cloning.

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抗病毒活性檢測法之建立與虹鱒生殖腺細胞株(RTG-2)之抗病毒蛋白質
的辨識與部份純化

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　　本實驗建立了一套快速(24小時內)有效的抗病毒活性生物檢測法。低濃度感染性胰臟壞死病毒(IPNV)(MOI介於0.00064至0.4)在虹鱒生殖腺細胞株(RTG-2)內培養超過12小時後，可明顯提高RTG-2細胞在後續的高濃度(MOI=100)IPNV病毒攻毒下的存活率。 IPNV病毒可誘發RTG-2產生抗病毒蛋白質。實驗亦發現誘發液中殘留的病毒可造成受測樣本之抗病毒活性高估或假顯性的現象。若經濃縮之抗病毒蛋白質粗誘發培養液，分別利用HPLC分子篩色層分析管柱及nativePAGE作部份純化。前者的分析圖譜中發現兩處(100及18 kDa)有較高的抗病毒活性，惟不明顯。而後者(native PAGE)則在約100 kDa處發現有明顯抗病毒活性，且經西方墨點法之抗人類干擾素γ多株抗體處理後，發現該處有訊號產生。本實驗亦發現經由十日齡細胞所誘發出來的抗病毒活性，高於由六日齡細胞所誘發出來者約1.6至3.2倍。

關鍵詞：類干擾素，抗病毒蛋白質，虹彩鱒魚生殖腺細胞株(RTG-2)，感染性肝胰臟壞死病毒(IPNV)。

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