Yellow-head virus: a rhabdovirus-like pathogen of penaeid shrimp

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ABSTRACT: Yellow-head virus (YHV), a highly virulent virus of cultured penaeid shrimp, was originally isolated from the black tiger shrimp *Penaeus monodon* in Thailand. It was initially described as a baculovirus, but was recently reported to be an RNA-containing virus. The present study reaffirms the genome of highly purified YHV to be an unsegmented single-stranded RNA with negative polarity and of approximately 22 kb size. When analysed by SDS-PAGE, the purified virus yielded at least 4 viral structural proteins of 170, 135, 67 and 22 kDa. The 135 kDa protein was determined to be glycosylated. YHV, as with VSV, a rhabdovirus, was found to agglutinate chicken red blood cells. The highly flexible enveloped bacilliform YHV particles measured 50–60 × 190–200 nm. Since the virus had a number of properties in common with rhabdoviruses, particularly plant rhabdoviruses, it was provisionally classified as a rhabdovirus.

KEY WORDS: Yellow-head virus · Rhabdovirus · Penaeid shrimp

INTRODUCTION

Yellow-head virus (YHV) has caused massive losses among shrimp farms in Thailand (Boonyaratpalin et al. 1993). The principal species affected is *Penaeus monodon* (black tiger shrimp), which is commonly cultured in Thailand and other Southeast Asian countries. YHV has also been shown to infect and cause disease in *Penaeus vannamei* and *P. stylirostris* (Lu et al. 1994), 2 species of shrimp commonly cultured in Hawaii and elsewhere in the western hemisphere.

The identification and classification of YHV has been a subject of controversy since it was first reported in 1993. Based on its general morphology and perinuclear site of accumulation, Boonyaratpalin et al. (1993) and Chantanachookin et al. (1993) reported that it was baculovirus-like. Baculoviruses have been previously reported to infect the penaeid shrimp species and, like most DNA-containing viruses, they were found to replicate and assemble in the nuclei of infected cells (Lightner 1993). By contrast, YHV was primarily localized in the cytoplasm of infected cells (Lu et al. 1994,

Wongteerasupaya et al. 1995). Furthermore, it was recently reported that YHV was an RNA-containing virus (Wongteerasupaya et al. 1995). The present study provides additional data to strongly suggest that YHV is most similar to the RNA-containing rhabdoviruses.

MATERIALS AND METHODS

Virus propagation. The virus was propagated in white shrimp (40 to 60 g *Penaeus vannamei*) by intramuscular injection of filtrates (0.2 ml of 5% w/v) from YHV-infected shrimp gill tissue. Three to four days after injection, hemolymph was harvested from moribund individuals by ablation of the telson and uropod, and stored at –80°C along with the shrimp carcasses until needed for virus purification.

Virus purification. To purify the virus from gill or head soft tissues, frozen infected shrimp were thawed and dissected. The tissues were suspended in TNE at 10% w/v (0.05 M Tris, 0.1 M NaCl, 1 mM EDTA) and homogenized [Brinkmann Polytron 3000 (Kinematica AG) with 90/Polytron PT-DA 3012/2TS]. Tissue debris was pelleted at 2500 × *g* for 30 min and the supernate further clarified by centrifugation at 11950 × *g* for

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30 min. Virus was pelleted at $62\,092 \times g$ for 1 h, resuspended in TNE, layered on top of a 30-40-50-60% (w/w) discontinuous sucrose gradient, and centrifuged at $92\,023 \times g$ for 2 h. The virus band was collected, diluted 5 times in TNE, and pelleted at $92\,023 \times g$ for 1 h. The virus pellet was resuspended in TNE, layered on top of a 35-55% (w/w) continuous sucrose gradient, and centrifuged at $192\,282 \times g$ for 16 to 20 h. The virus band was collected, diluted 5 times in TNE, and pelleted at $48\,070 \times g$ for 1 h. To purify the virus from hemolymph, a shorter version of the above procedure was employed, eliminating the homogenization step as well as the discontinuous sucrose gradient centrifugation.

Electron microscopy. Virus samples were negatively stained with 2% (w/v) uranyl acetate and examined under the Zeiss EM10/A electron microscope.

Silver staining. The structural proteins of the virus were analyzed by 5% and 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (1970). Samples were electrophoresed for 45 min at 200 V and the gels stained using the Silver Stain Plus kit (Biorad, CA, USA).

Preparation of anti-YHV antibodies. Polyclonal hyperimmune serum against YHV was generated in an adult New Zealand white rabbit using purified YHV as immunogen. Immunoglobulin G was purified from the antisera using recombinant bacterial protein-G columns (Gamma-Bind G, Genex Corp., Gaithersburg, MD, USA) and adsorbed to shrimp muscle tissue to remove IgG cross-reacting with normal shrimp antigens.

Western blotting. After SDS-PAGE, YHV proteins were blotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) at 100 V for 1 h. The nitrocellulose (NC) membranes were then washed with phosphate-buffered saline (PBS; 8.0 g NaCl, 0.2 g KH_2PO_4 , 1.15 g Na_2HPO_4 and 0.2 g KCl, dissolved in 1.0 l distilled water), blocked with 5% skim milk/PBS for 1 h, washed with PBS, and then treated with 1:500 rabbit anti-YHV IgG diluted in 1% skim milk/PBS for 1 h. The NC paper was washed with PBST (0.05% Tween-20/PBS) twice and once with PBS. The NC paper was then treated with 1:2000 goat anti-rabbit HRPO (horseradish peroxidase) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted in 1% skim milk/PBS for 1 h. The NC paper was washed 3 times with PBST and then incubated with TMB (3,3',5,5'-tetramethylbenzidine; Kirkegaard and Perry Laboratories) substrate. All incubations were done at room temperature (r.t.).

Glycoprotein detection. Carbohydrate moieties of purified YHV proteins were labelled on the membrane using the ECL (enhanced chemiluminescence) glycoprotein detection system (Amersham Corp., Arlington

Heights, IL, USA). A parallel western blot was also performed using anti-YHV IgG (1:500 dilution) to confirm the presence of all the YHV proteins.

Purification of viral nucleic acid. Viral RNA was extracted from purified YHV using the Qiagen Total RNA Kit (Qiagen Inc., CA, USA). Aliquots of the YHV RNA were then electrophoresed together with ribosomal RNA (18s & 28s eukaryotic), VSV (vesicular stomatitis virus) RNA, and DNA high molecular weight standards on 0.5% agarose (TBE) at 30 V for 3 h. The gel was treated with RNase ($1\mu\text{g ml}^{-1}$, 1 h, 37°C) to determine susceptibility of the YHV RNA to digestion.

In vitro translation of viral nucleic acid. The purified YHV RNA was used as the template in an ECL *in vitro* translation labelling kit (Amersham Corp.). The biotin-labelled translation products were analyzed by western blot using streptavidin-HRPO (1:1000 dilution), and developed using the TMB substrate. Brome mosaic virus (BMV) mRNA was used as positive control.

Hemagglutination. Serial dilutions of purified preparations of YHV and VSV (vesicular stomatitis virus) in 0.85% saline were mixed with equal volumes (50 μl) of 0.5% chicken red blood cells (RBC) and then incubated for 1 h at r.t. The highest dilution at which maximum agglutination occurred was noted and used for computing the hemagglutination (HA) titer.

RESULTS

Purification of YHV

Of the purified YHV prepared from the gills, head soft tissues, and hemolymph of experimentally infected shrimp, the hemolymph yielded the cleanest preparation of virus as determined by electron microscopy. Samples prepared from gill and head soft tissues were often contaminated with vesicles and other membranous materials which proved difficult to separate from the virus particles. YHV had a buoyant density of 1.19 to 1.20 g ml^{-1} in a continuous sucrose gradient.

Morphology of YHV

By electron microscopy, negatively stained banded preparations of YHV particles revealed enveloped, bacilliform, particles measuring $50\text{--}60 \times 190\text{--}200\text{ nm}$ (Fig. 1A, B). The internal helical nucleocapsid (Fig. 1C, arrowhead) was closely surrounded by an envelope studded with prominent peplomers or spikes. The virus particle appeared highly flexible as indicated by a large number of pleomorphic forms (Fig. 1A). Several particles were damaged, suggesting a fragile structure.

Fig. 1. Morphology of negatively stained YHV as seen under the electron microscope: Scale bars = 100 nm. (A) Electron micrograph demonstrating the flexibility and fragility of the bacilliform viral particles. (B) Higher magnification showing the very prominent peplomers or spikes covering the surface of the virus. (C) A partially disrupted viral particle with part of the envelope torn away, revealing the helical nucleocapsid structure (arrowhead)

Analysis of the viral structural proteins

SDS-PAGE analysis of the YHV particle indicated that it was composed of at least 4 structural proteins with the following estimated molecular weights (kDa): 170, 135, 67, and 22 (Fig. 2A, arrowheads). The band located just below the 170 kDa band was considered an artefact because unlike the other 4 bands it was not consistently observed in SDS-PAGE gels of other purified YHV preparations.

Western blot analysis using polyclonal anti-YHV IgG also revealed these same 4 bands (Fig. 2B, arrowheads). Of the 4, the 135 kDa band was present in the highest concentration and was also the most reactive to the anti-YHV antibodies. The anti-YHV IgG did not react with the structural proteins of rhabdovirus of penaeid shrimp (RPS) or VSV. Neither did anti-RPS IgG react with the structural proteins of YHV (data not shown).

Glycoprotein labelling experiments showed that only the 135 kDa band was glycosylated (Fig. 3).

Analysis of the viral nucleic acid

Agarose gel electrophoresis of YHV RNA revealed a band approximately 22kb in size, when estimated by extrapolation using VSV and ribosomal RNA as standards (Fig. 4). This band was susceptible to RNase digestion (data not shown). The heterogeneous lower molecular weight component seen in Fig. 4A & B is assumed to be degraded YHV RNA. The viral RNA was found not to serve as a template for translational activities in an *in vitro* system (Fig. 5), strongly suggesting that the YHV genome had negative sense.

Hemagglutination

YHV and VSV were found to agglutinate chicken RBC yielding a hemagglutinating end-point titer of 1:256 and 1:64, respectively. Both viruses were not eluted after 24 h of incubation, suggest-

Fig. 2. Structural proteins of YHV. (A) SDS-PAGE of high molecular weight markers (lane 1), purified YHV (lanes 2 & 3), and low molecular weight markers (lane 4). Arrowheads indicate the 4 structural protein bands of the virus. (B) Western blot of purified YHV (lane 1) and prestained molecular weight markers (lane 2). Arrowheads indicate the 4 structural protein bands of the virus

Fig. 3. Western blot ECL showing the biotinylated 135 kDa glycoprotein band of YHV. Arrowheads indicate the relative positions of the prestained molecular weight markers in lane 1 as seen in the nitrocellulose paper. Lane 2 contains the labelled YHV protein band after development in the X-ray film

ing that the hemagglutination reaction was stable and that the virus lacked receptor-destroying enzymes.

DISCUSSION

YHV does not exhibit the characteristic bullet-shaped morphology typical of rhabdoviruses infecting vertebrate hosts. However, in size, shape, general ultrastructural morphology and buoyant density in sucrose gradients, it closely resembles the bacilliform rhabdoviruses of plants (Jackson et al. 1987, Payment & Trudel 1993) and the rhabdo-like virus infecting the blue crab, *Callinectes rapidus* (Yudin & Clark 1979).

The 4 proteins detected (170, 135, 67, 22 kDa) from purified YHV preparations may represent, respectively, the L (RNA transcriptase), G (spike), N (nucleocapsid), and M (matrix) proteins of the rhabdoviruses (vesiculoviruses). A fifth protein, P or phosphoprotein

Fig. 4. RNA genome of YHV. (A) Agarose gel electrophoresis of 18s & 28s eukaryotic ribosomal RNA (lane 1), YHV genome (lane 2), and high molecular weight DNA markers (lane 3). Arrowhead indicates the position of the YHV RNA band. (B) Agarose gel electrophoresis of high molecular weight DNA markers (lane 1), YHV genome (lane 2), and VSV genome (lane 3). Arrowhead indicates position of the YHV RNA band

(originally called NS), usually present in much lower concentrations in VSV and also in a few plant rhabdoviruses, was not detected (Jackson et al. 1987). The unusually large size of the putative G protein of YHV was

Fig. 5. Western blot showing the results of the *in vitro* translation experiment: 1 = control template, 2 = YHV genome, 3 = prestained markers. Arrowheads indicate the protein products of the control RNA template. No new protein products were observed in lane 2

corroborated by electron microscopic images showing very prominent peplomers emanating from the viral envelope. Designation of the 135 kDa protein as G protein was supported by data showing that this protein was glycosylated. The unusual sizes of the YHV proteins are not atypical since the viral proteins of plant rhabdoviruses differ considerably in their size as well as their electrophoretic patterns (Jackson et al. 1987). The viral origin of the 4 protein bands was further confirmed by western blots done in our laboratory showing their presence in YHV-infected but not in control uninfected shrimp tissues.

The genome of YHV was clearly RNA as shown by its susceptibility to RNase and by the ability of YHV to replicate in primary lymphoid cell cultures in the presence of the DNA-antagonist BUDR (Tapay 1996). This confirmed the findings of Wongteerasupaya et al. (1995). Furthermore, failure by the YHV-RNA to act as a template for *in vitro* translation suggested that it is of negative polarity like the rhabdoviruses. Precautions were taken to insure that the prescribed amount of purified YHV-RNA was used as template. However, more data (i.e. sequencing data) are needed to firmly establish the negative polarity of the YHV-RNA.

The genome of YHV is unusually large (22 kb) when compared to those of the other members of the rhabdovirus family. The estimated size of the YHV genome was not definitive since a non-denaturing gel was used, but it is probably valid since it was run in parallel with other known single-stranded RNA standards.

Except for its large putative G protein and large RNA genome, YHV has many of the properties of rhabdoviruses and we strongly believe that it should be provisionally classified as one until further information indicates otherwise.

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