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# Morphogenesis and Ultrastructure of a Baculovirus in the Juveniles of *Penaeus chinensis*

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## Abstract

A non-occluded baculovirus was observed and purified from the hepatopancreas of juveniles of *Penaeus chinensis* with white spots on the carapace. The nucleic acid of the baculovirus was demonstrated as DNA. The enveloped virion is a stocky rod-shaped particle and has a tail, which is an envelope extension. The nucleocapsid is a helical cylinder formed by two cap structures at both ends and 13 helical bands in the middle. One helical band is made up of 11 capsomeres formed by 3-row-parallel protein subunits. An ultrastructure model of nucleocapsid has been proposed. Viral morphogenesis begins in the nuclei and its process is composed of four stages: the formation of viral stroma, the formation of membrane, the assembly of viruses, and the formation of complete virions. Sometimes two or three nucleocapsids use one membrane to assemble during the viral assembly. This may result to: one membrane fragment envelops respectively each nucleocapsid so that two complete virions are formed each of which is one nucleocapsid in one envelope; or virions are formed, which are two or three nucleocapsids in one envelope.

## Introduction

More than 18 kinds of viruses of penaeid shrimp, half of which are baculoviruses, have been found since Couch (1974) found the first. The most common characteristics of the disease caused by most of the baculoviruses

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are the presence of white spots on the carapace and high mortality. Electron-microscopic observations of sections and viral suspension reveal rod-shaped enveloped non-occluded virions. However the baculovirus are named differently, such as Baculoviral Hypodermal and Haematopoietic Necrosis Virus (HHNBV) by Huang et al. (1994) Rod-shaped Virus of *Penaeus japonicus* (RV-PJ) by Inouye et al. (1994) and White Spot Baculovirus (WSBV) by Wang et al. (1995). Lightner (1996) regrouped these baculoviruses with the various members of the WSBV group under the name White Spot Syndrome (WSS). However, in the 6$^{th}$ report of the ICTV (Murphy et al. 1995), the previous classification of these viruses as baculoviruses was canceled and these viruses were left unassigned (Durand et al. 1997). For clarity, we have decided to name the baculovirus, non-occluded baculovirus in this paper.

In this study, we purified and observed a rod-shaped, enveloped, non-occluded baculovirus from shrimps. We found that the ultrastructure and the morphogenesis of the baculovirus differed from the other viruses in certain respects and report the differences.

## Materials and Methods

Approximately 40-day old juveniles of *P. chinensis* were obtained from a large-scale hatchery in Zhejiang province, China. Before the experiment started, all juveniles of shrimp were kept in 82 l glass tanks containing filtered, aerated sea water (25‰) at ambient temperature (20 to 27°C) for at least 17 days to allow them to adapt to laboratory conditions. The shrimps were divided into two groups, 80 in each group. In one group each shrimp was fed on hepatopancreas of dead *P. monodon*, purchased from the market and infected with white spots on the carapace. In the other group, which served as the negative control group, each shrimp was fed on artificial diet.

Heads of infected *P. monodon* and infected juveniles of *P. chinensis* were homogenized at 5000 r·min$^{-1}$ for 5 min. The homogenate was frozen for 20 min and thawed for 10 min. The procedure was repeated twice to disrupt infected cells. The homogenate was clarified by low speed centrifugation at 2198 × g for 20 min and followed by centrifugation at 8792 × g for 20 min. The supernatant fluid was centrifuged at 85860 × g for 2 h. The pellet was resuspended in 2 ml PBS buffer. The suspension was further separated in 20 to 50% sucrose gradient by centrifugation at 119579 × g for 3 h (Beckman SW28 rotor). After that, the milky white zone containing the viruses was removed and diluted by 3 to 5 times volume PBS buffer. The diluted fluid was centrifuged at 85860 × g for 30 min to eliminate sucrose. The pellet was suspended in 2 ml PBS and then examined under an electron microscope by negative staining.

The purified virus suspension was treated with proteinase K (final concentration: 200 μg·ml$^{-1}$) and SDS (final concentration: 0.5%) at 37°C for 2 h. After adding an equal volume of phenol, the mixture was shaken gently at room temperature. After centrifugation, the aqueous layer was extracted

with phenol/chloroform and the nucleic acids were precipitated with 1/10 volume of 3M sodium acetate buffer and 2.5 volumes of ethanol overnight at -20°C. After washing with 70% alcohol and centrifugation, the final pellet was resuspended in TE.

The samples of nucleic acids were treated with DNase and RNase, used according to the manufacturer's instructions. Samples of nucleic acids were electrophoresed in 0.5% agarose gels and stained with ethidium bromide and observed under vu light. The λ–DNA was digested with EcoRI and HindIII and co-electrophoresed for use as molecular markers.

For transmission electron microscopy (TEM), hepatopancreas of infected *P. chinensis* was fixed in cold (4°C) 2.5% glutaraldehyde in phosphate buffer at pH 7.2 for 2 h. After rinsing in phosphate buffer, the samples were post fixed in 1% osmium tetroxide and dehydrated through a series of graded ethanol, then transferred to propylene oxide for 20 min and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. A JEM-100 SX electron microscope was used throughout the observation.

## Results

### *Infection of P. chinensis juveniles*

Juveniles of *P. chinensis* fed on the hepatopancreas of infected *P. monodon* were anorexic, slow moving on the fourth day, and 50% of the shrimps died on the seventh day. Using light microscopy, white spots were found on the removed carapace. Survival rate of the control group was 100%.

### *Analysis of nucleic acid*

The 0.5% agarose gel electrophoretic analysis of viral nucleic acid showed one fragment. The nucleic acid could be digested with DNase, but not with RNase. Based on this result, the nucleic acid of the baculovirus was demonstrated as DNA (Fig. 1).

Fig. 1. Agarose gel electrophoresis of viral nucleic acid
a. λ – DNA - EcoRI/ HindIII
b. viral nucleic acid treated by Dnase
c. viral nucleic acid
d. viral nucleic acid treated by Rnase

### *Ultrastructure*

After negative staining, purified viruses from *P. monodon* and *P. chinensis* were examined by TEM. The pellet of the centrifugation at 85860 × g contained enveloped virions, free nucleocapsids and free viral envelope fragments. After sucrose gradient centrifugation, the milky white zone was observed and examined by TEM after negative staining. It contained many free nucleocapsids and envelope fragments, the gradient of which is equal to 1.18 $g \cdot cm^3$ (20°C) of sucrose.

The enveloped virus was a rod-shaped particle with a tail formed by an apical enveloped extension, which was 270 to 300 × 90 to 120 nm. The ratio of length to diameter varied from 2.25:1 to 3.1:1 with an average of 3:1. The size of the tail was 300 to 1000 × 15 to 20 nm. The thin parts of the tail were electron-dense and some of its thick parts were electron-clear (Fig. 2). At the virion-tail connector, there was an elliptical projection which was 34 to 56 × 24 to 37 nm with two dark bands at both ends. Some complete virions with cross–hatched appearance (Fig. 3) were noted.

The nucleocapsid was slightly flat and straight at one end while round at the other. The free rod-shaped nucleocapsid was 290 to 320 × 50 to 70 nm and a helical cylinder, with helical bands displaying an angle of 90° to the long axis of the particle. One "D" cap-shaped structure was at each end of the cylinder, which was 20 nm wide. Between the cap-shaped structures were 13 helical bands, about 18 nm wide, and about 2 nm apart. This cused the nucleocapsids to display a superficially segmented appearance (Fig. 4)

One helical band was made up of 11 capsomeres, each of which was a structural unit of capsid protein. One capsomere was made up of three protein subunits, one of them lay at the middle of the helical band, called the middle subunit (MS); while the other two lay at the edge of the helical band, called the edge subunit (ES). The protein subunits were arranged in "X" shape (Fig. 5). One MS can combine four ES around it while one ES can combine two MS in the same helical band and interact with the other two ES in the neighboring helical band as well. The "D" cap-shaped structure was made up of 3-row protein subunits and the amount of protein subunits composed of the upper layer was less than that of the other two.

Fig. 2. Virus showing the long envelope extension at the apex of the particle × 80000

Fig. 3. Complete virion with a cross-hatched appearance × 100000

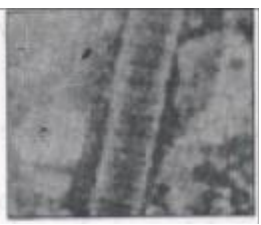

Fig. 4. Negatively stained free rod-shaped nucleocapsid × 100000

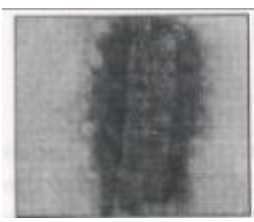

Fig. 5. Nucleocapsid from dead *P. monodon* and larval *P. chinensis* showing the composition of the capsomere and the structure of the helical cylinder × 80000

Fig. 6. Degraded nucleocapsid showing a worm-shaped particle × 100000

We also observed another kind of loose nucleocapsid, which was a worm-shaped particle, 360 to 370 × 95 to 100 nm in size with two round ends (Fig. 6). The helical cylinder was a right-hand helical from the degraded nucleocapsid (Fig. 7). The degraded nucleocapsid was enlarged (13 to 28% and 36 to 100% the length and diameter, respectively) than those of the normal nucleocapsids. The distance between two helical bands was also enlarged.

### *Morphogenesis*

Morphogenesis of the baculovirus was studied by observing the ultrathin sections. The average size of virions was 110 × 295 nm. The nucleocapsid was a cylinder about 80 × 245 nm in the average, and the membrane was 10 nm thick (Fig. 8). The viral stroma and numerous membrane fragments were present in early-infected nuclei. The membrane fragments all gathered around the viral stroma (Fig. 9). The membrane appearances were vesicles or line-shaped (Fig. 10).

In highly infected nuclei, many assembling viruses were observed. The virions formed with viral stroma moved to the membranes and were enveloped. Virus assembly occurred in different ways. The most common was that one membrane fragment enveloped one nucleocapsid. Some empty capsids that were completely or partly enveloped (Fig. 11) were observed. The empty

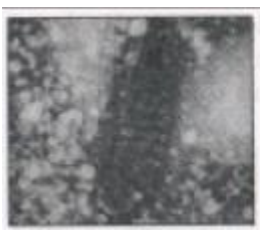

Fig. 7. The large worm-shaped nucleocapsid showing a right-hand helical cylinder × 100000

Fig. 8. Envelope baculovirus in nuclei of infected hepatopancreas and some showing the characteristic envelope extension (arrow) at the apex of the particle × 25000

Fig. 9. The membrane fragments and the viral stoma gathered around the membrane in nuclei of infected hepatopancreas × 30000

Fig. 10. The membrane showing the characteristic vesicles or line-shape in the nuclei of infected hepatopancreas × 50000

Fig. 11. Some empty capsids completely or partly enveloped × 100000

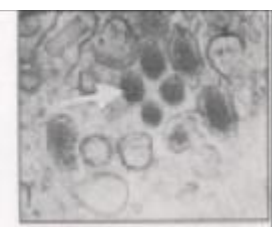

Fig. 12. Full nucleocapsids without an envelope × 50000

capsids were about 50 nm in diameter, which was less than the diameter of the true nucleocapsid. We also noted unenveloped nucleocapsids in the nuclei (Fig. 12).

In some instances, two or three nucleocapsids used one envelope to assemble (Figs.13 and 14). One result was that one membrane fragment enveloped each nucleocapsid respectively. When the nucleocapsid was almost enveloped, the membrane split so that two complete virions were formed each of which was one nucleocapsid in one envelope (Fig. 15). The other result was that a virion formed with two nucleocapsids in one envelope (Fig. 16). The envelope between the two nucleocapsids of the virus sank and split in the middle (Fig. 17).

In some viruses, two cores were wrapped in one capsid. When separated, the middle parts of the nucleocapsids became slim, and looked like a dumbbell (Fig 18). Results show that 1.4 to 5.2% of all the viruses were two nucleocapsids in one envelope.

## Discussion

After sucrose gradient centrifugation, free nucleocapsids were observed in the suspension, while enveloped virions were not. This may be because osmotic shock degraded the envelope. The cross-hatched appearance of a complete virus was formed by peplomers that were projections of envelope protein. If nucleocapsids degraded differently, the nucleocapsids displayed different superficial appearances. One end of the nucleocapsid would be slightly flat and the other would be round. At the same time, the capsids

Figs. 13 and 14. Two or three nucleocapsids using one membrane to assemble × 80000

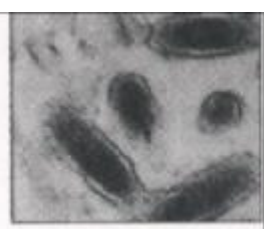

Fig. 15. Membrane enveloping respectively each nucleocapsid × 80000

Fig. 16. Two nucleocapsids wrapped in one envelope × 50000

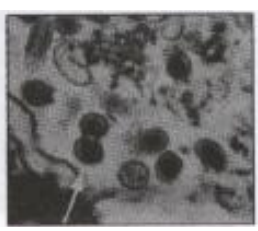

Fig. 17. Envelope between two nucleocapsids of these viruses sinking and splitting in the middle part × 50000

Fig. 18. Two viral cores wrapped in one capsid were separated, capsids were split, envelopes were also split × 80000

were usually observed flat and straight at both ends. These structures were attributed to degradation of the two cap structures at both ends. The WSBV described by Durand et al. (1997) had the same structure. The protein subunits of nucleocapsid were arranged in a cross, making the nucleocapsid structure more stable.

The ultrastructure of the baculovirus we observed was similar to the WSBV observed by Durand et al. (1996), SEMB virus (Wongteerasupaya et al. 1995), WSS virus (Wang et al. 1995), RV-PJ (Momoyama et al. 1995), LNBV (Chen et al. 1997) and HHNBV (Huang et al. 1995). They also had the segmented superficial appearance of the nucleocapsids and an envelope extension of the virions.

Based on the ultrastructure of the baculovirus observed, we propose an ultrastructure model for WSSV of penaeid shrimp, the main points of which are:

1). The nucleocapsid is a helical cylinder.

2). The nucleocapsid is composed of one cap structure at each end and 13 helical bands between the two cap structures.

3). One capsomere is made up of three protein subunits, one of which is the middle subunit; the other two are the edge subunits. The three protein subunits are arranged in ">" shape.

4). The width of the helical band and the amount of capsomeres in one helical band are different in the different baculoviruses. This may be the species characteristic (Fig. 19).

Through this model, we can interpret the formation of the segments. In one helical band, the ES and the MS arrange compactly, thus little stain could penetrate them. This part was an electron-clear band and forms a light color striation. The ES in one helical band were far from the ES of the neighboring helical band, and the action between them is weak, so much stain can penetrate them. This part was an electron-dense band and formed a dark color striation.

In infected nuclei, membrane fragments were so near the viral stroma that the virus could quickly assemble. To maximize the use of the membrane material in the nuclei, two or three nucleocapsids assembled with one envelope. These acts adapt to the characteristics of the baculovirus, enormous and capable of quick replication during morphogenesis.

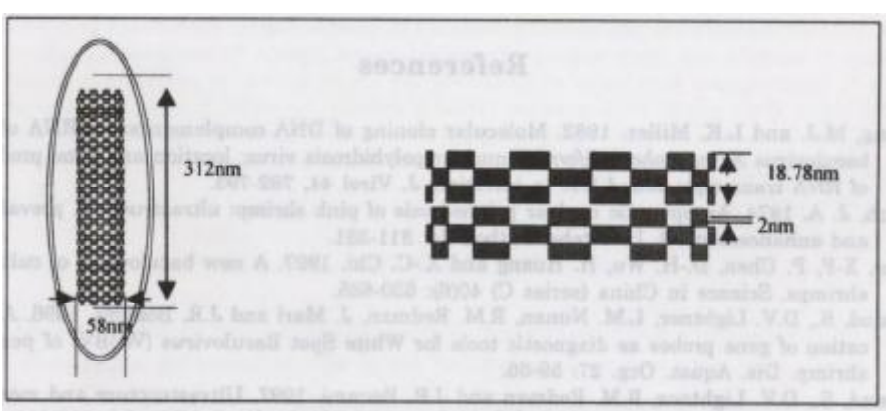


Fig. 19 The ultrastructure model of baculovirus of penaeid shrimp

Morphogenesis of WSSV described by Durand et al. (1997) has many things in common with the baculovirus we observed. 1) Viral stroma and membrane were formed *de novo* in the nucleoplasm. 2) Empty and enveloped capsids were observed, and their diameter was smaller than the diameter of the true nucleocapsids. This was probably enveloped with the membrane after formation and before densification. The phenomenon often occurs in insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV). The study of DNA genes of AcNPV showed that many gene groups control viral replication, and the translation of virus mRNA is still active in the latter phase of replication (Adang and Miller 1982, Esche et al 1982, Smith et al. 1982). We infer that the structure is formed by surplus structural proteins synthesized by virus in the nuclei.

The morphogenesis of baculovirus we observed begins in the nuclei. There are a lot of membranes in the nucleoplasm of the infected nuclei, but in a normal nuclei, there are no membranes. What does the membrane form? We suggest that the membrane is coded by viral gene and formed by using materials of the host. What controls the splitting of the envelope that enveloped the two nucleocapsids? This needs further study.

This baculovirus morphogenesis possesses some particular characteristics. We noted that two or three nucleocapsids use one membrane fragment to assemble and there are some virons with two nucleocapsids in one envelope. This structure is common in occluded-body baculovirus of insect (Hou 1990), but this phenomenon in shrimp has been reported only by us (Wang et al. 1997). At that time, we only observed that the complete virions were two nucleocapsids with one envelope but did not observe the phenomenon that more than one nucleocapsid was assembling with one envelope. This time we also observed two cores in one capsid.

Our results suggest that there are two kinds of division-like processes in which the envelope and the nucleocapsids were split: 1) Two nucleocapsids wrapped in one envelope separated gradually and then the envelope split. 2) Two cores wrapped in one capsid separated gradually and then capsids and envelope split. Hou (1990) thinks that sometimes several genomes of the baculovirus string together. The efficient assembling procedure adapts to enormous and quick replication of the baculovirus.

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