

Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp

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ABSTRACT: A non-occluded baculovirus-like agent recently isolated by this laboratory from moribund *Penaeus japonicus* shrimps obtained from China and named Chinese baculovirus (CBV) was purified and some of its properties characterized. Under the electron microscope, negatively stained virus particles were rod-shaped, enveloped, and measured 322 to 378 nm in length and 130 to 159 nm in diameter. The nucleoprotein core exhibited a unique striated structure and measured 316 to 350 nm in length and 65 to 66 nm in diameter. The striations appear to be the result of the stacking of ring-like structures. These rings consisted of 2 rows of 12 to 14 globular subunits. Each globular subunit measured approximately 10 nm in diameter. SDS-PAGE gels of purified virus preparations showed, among several, 4 prominent protein bands with approximate molecular weights of 19, 23.5, 27.5 and 75 kDa. The structural viral proteins were identified by western blot analysis using polyclonal hyperimmune serum made against purified CBV. The 19, 27.5, and 75 kDa structural proteins were determined to be non-glycosylated components associated with the viral envelope. The 23.5 kDa protein, also non-glycosylated, was identified with the capsid structure. Viral genomic DNA digested with *Hind* III restriction endonuclease revealed at least 29 different fragments with a conservatively estimated total size of at least 183 kb.

KEY WORDS: Chinese baculovirus · Baculovirus · Penaeid shrimp

INTRODUCTION

A baculovirus-like agent was recently isolated by this laboratory from moribund pink shrimp *Penaeus japonicus* obtained from a shrimp farm in China. The isolate, labeled Chinese baculovirus (CBV), was found to cause 100% cumulative mortality 3 to 4 d post-infection in experimentally infected adult *P. stylirostris* (blue shrimp) and *P. vannamei* (white shrimp), the 2 principal species of penaeid shrimp cultured in Hawaii and the Western Hemisphere (Tapay et al. 1997, Lu et al. 1997). Non-occluded baculovirus-like agents that closely resemble CBV have also been reported to infect *P. monodon* in Thailand (Wongteerasupaya et al. 1995), *P. monodon*, *P. japonicus* and *P. penicillatus* in Taiwan (Chou et al. 1995) and *P. japonicus* in Japan (Inouye et al. 1994, Takahashi et al. 1994, Inouye et al. 1996). These viruses have been implicated in massive disease outbreaks and mortalities among penaeid

shrimp. Further characterization of the different isolates will help us to better understand these viruses and their relationships to each other.

In this paper the propagation, purification, and characterization of some of the properties of CBV are described. This virus represents a potential threat to shrimp farming, particularly broodstocks in Hawaii, because of the highly lethal nature of the infection, its wide species range, and increasing geographical distribution.

MATERIALS AND METHODS

Virus propagation. The virus was propagated in white shrimp (50 to 60 g *Penaeus vannamei*) by intramuscular injection of filtrates [200 µl of a 4% w/v homogenate in TNE (0.05 M Tris, 0.1 M NaCl, 1 mM EDTA), clarified at 2000 × g for 20 min, and filtered through a 0.45 µm filter] prepared from CBV-infected shrimp gill tissue. Two to four days after injection,

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hemolymph was collected from moribund specimens (by ablation of the telson and uropod), mixed with an equal volume of 20% (w/v) sodium citrate, and stored at -20°C .

Virus purification. To purify the virus, various tissues were harvested from frozen infected shrimps. The tissues were suspended in TNE at 10% w/v and homogenized [Brinkmann Polytron 3000 (Kinematica AG) with 90/Polytron PT-DA 3012/2TS] (Brinkmann Instruments Inc., NY, USA). Tissue debris was pelleted at $2500 \times g$ for 15 min and the supernate further clarified by centrifugation at $4946 \times g$ for 10 min. Virus was pelleted at $58\,200 \times g$ for 30 min, resuspended in 30:1 volume of TNE (i.e. 30 parts of original volume to 1 part of new suspension), layered on top of a 20–60% (w/v) continuous sucrose gradient, and centrifuged at $58\,200 \times g$ for 2 h. The diffuse virus band was collected by side puncture, diluted 1:5 in TNE, and pelleted at $58\,200 \times g$ for 30 min. The virus pellet was resuspended in 0.5 ml of TNE, layered on top of a 30 to 50% (w/v) continuous CsCl gradient and centrifuged at $192\,000 \times g$ for 19 h at 4°C . The bands of complete virions and nucleoprotein cores were collected, diluted 1:5 in TNE, and pelleted at $192\,000 \times g$ for 15 min at 4°C . The pellets were resuspended in 0.5 ml TNE and checked for purity under the electron microscope (EM).

Electron microscopy. For EM examination, virus samples were mounted on formvar-coated, carbon-stabilized copper grids (200 mesh), negatively stained with 2% (w/v) uranyl acetate, and examined under a Zeiss EM10/A electron microscope.

SDS-PAGE. The structural proteins of the virus were analyzed by 12.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (1970). Samples were electrophoresed for 40 min at 200 V and the gels stained using the silver stain plus kit (Biorad, CA, USA).

Preparation of antibodies. Polyclonal hyperimmune serum against CBV was prepared in New Zealand white rabbits according to the following immunization schedule: 52 μg of CBV proteins in Freund's complete adjuvant were injected intradermally and subcutaneously on Day 0. Subsequent booster injections of 52 μg CBV protein in Freund's incomplete adjuvant were given on Days 13, 27, and 41. The rabbits were exsanguinated on Day 63. Immunoglobulin G was purified from rabbit antisera using recombinant bacterial protein-G columns (Gamma-Bind G, Genex Corp., Gaithersburg, MD, USA) and adsorbed to normal shrimp hemolymph proteins to remove cross-reacting antibodies.

Western blotting. For western blotting, purified CBV virions were electrophoresed on 12.5% SDS-PAGE gel at 200 V for 40 min and blotted onto nitrocellulose membranes (Schleicher and Schuell, NH, USA) at 100 V

for 40 min. The nitrocellulose (NC) membranes were then washed with PBS (137 mM NaCl, 1.4 mM KH_2PO_4 , 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 2.7 mM KCl), blocked with 5% skim milk/PBS for 1 h, washed with PBS, and then treated with 1:1000 rabbit anti-CBV 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 1:2500 goat anti-rabbit HRPO (horseradish peroxidase) (Kirkegaard and Perry Laboratories, MD, USA) diluted in 1% skim milk/PBS for 1 h. The NC paper was washed 3 \times with PBST and then incubated with the TMB (3,3',5,5'-tetramethylbenzidine; Kirkegaard and Perry Laboratories, MD, USA) substrate. All incubations were done at room temperature.

Triton and DNase I treatment. The locations of the different viral structural proteins were analyzed by treatment of the complete virions with 1% Triton X-100/TNE for 1 h at 37°C , and treatment of the nucleoprotein core with DNase I (1 unit per 10^9 particles) for 1 h at 37°C . Triton-treated samples were centrifuged at $140\,000 \times g$ for 5 min in a Beckman Airfuge (Beckman Instruments, CA, USA) to pellet the virus particles and separate it from components that were dissolved in Triton. All samples were separated by SDS-PAGE gel electrophoresis and visualized by either silver staining or western blotting.

Glycoprotein detection. Carbohydrate moieties of purified CBV proteins western blotted onto nitrocellulose membrane were labeled using the ECL (enhanced chemiluminescence) glycoprotein detection system (Amersham Corp., IL, USA). A parallel western blot was also done using anti-CBV IgG (1:1000 dilution) to confirm the presence of all the CBV proteins.

Preparation of viral nucleic acid. The CBV DNA was purified from CsCl banded virus using the QIAamp Tissue Kit (QIAGEN Inc., CA, USA). Aliquots of the DNA (200 ng in 17 μl) were digested with excess (10 units) *Hind* III for 1 h at 37°C . The digest, together with undigested controls, was then electrophoresed on 0.4% agarose (TBE) at 10 V overnight or 0.7% agarose (TBE) at 60 V for 3 h.

RESULTS

Purification of CBV

Although CBV particles were successfully purified from gill tissues, epidermal tissues, head soft tissues, and hemolymph, the cleanest preparations (Figs. 1 & 2) were obtained from the hemolymph. Furthermore, virus purification from the hemolymph did not require the homogenization step which was necessary for all other tissues.

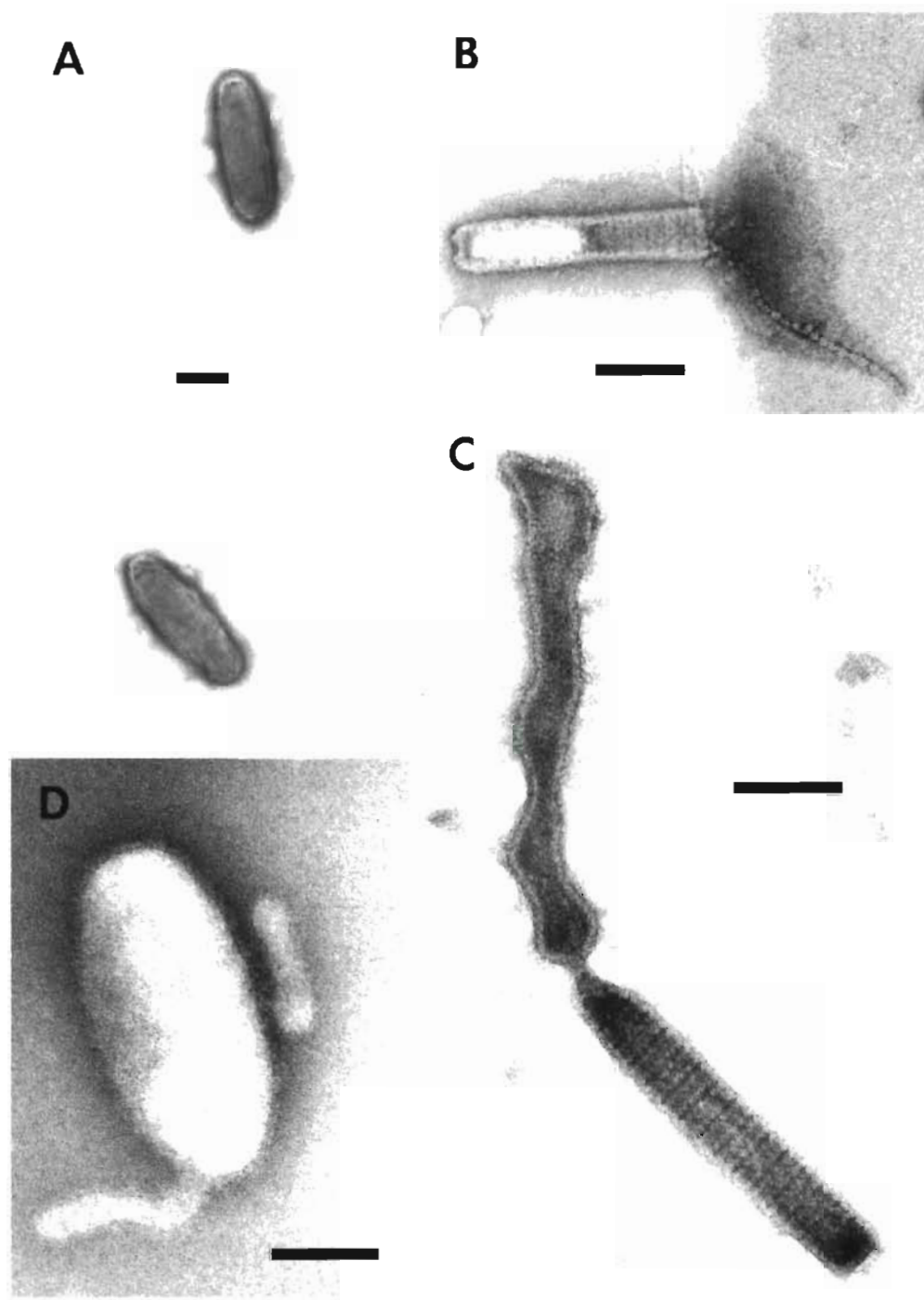


Fig. 1 Electron micrographs of negatively stained CBV particles. (A) Whole virus particles partially penetrated by the stain revealing the nucleocapsid. (B) Partially degraded nucleocapsid with the globular subunits unwound. (C) Virus particle with the outer envelope almost completely removed from the nucleocapsid. (D) Whole virus particle showing a tail-like extension from the outer envelope. All scale bars = 100 nm

Isopycnic centrifugation in CsCl was found to be superior to sucrose gradients for the optimal purification of CBV particles and nucleoprotein cores. The complete virions banded at 1.22 g ml^{-1} and the nucleoprotein cores banded at 1.31 g ml^{-1} in CsCl.

Structure of CBV

Electron microscopical examination of negatively stained complete CBV particles revealed a rod-shaped nucleoprotein core enclosed in a tight-fitting capsid

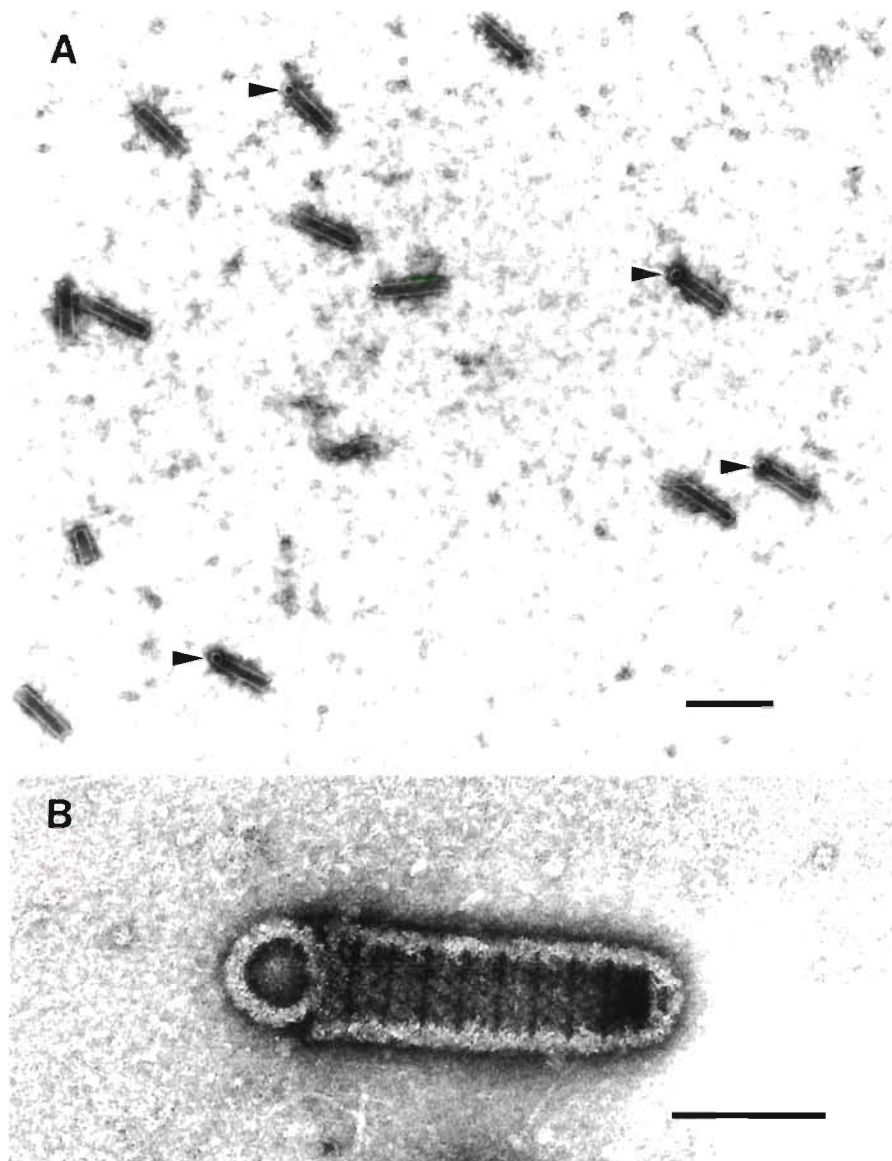


Fig. 2. Electron micrographs of negatively stained CBV nucleoprotein cores. (A) Lower magnification showing ring-like structures (arrowheads) peeling away from several nucleoprotein cores (scale bar = 500 nm). (B) Higher magnification showing a close-up view of the ring-like structure still attached to the nucleoprotein core (scale bar = 100 nm)

layer and a loose-fitting outer membrane (Fig. 1A, C). Because of the loosely fitting outer membrane, the diameter of the whole virus was as large as 130 to 159 nm and provided an ellipsoidal shaped structure (Fig. 1A, D) occasionally exhibiting tail-like membranous extensions (Fig. 1D).

Electron microscopical examination of negatively stained nucleoprotein cores revealed some 14 to 15 conspicuous vertical striations located periodically along the long axis of the rod-shaped core and about 22 nm apart (Fig. 1C). These striations are probably the result of the stacking of ring-like structures making up the nucleoprotein core structure (Fig. 2A, B). These rings consist of 2 rows of 12 to 14 essentially globular structures each approximately 10 nm in diameter (Fig. 3). Degraded nucleocapsids occasionally exhib-

ited globular structures that seem to be attached to each other like beads on a string (Fig. 1B). The total length of the nucleoprotein core is 316 to 350 nm with a diameter of 65 to 66 nm.

CBV structural proteins

Analysis of the structural proteins of the virus by SDS-PAGE gel electrophoresis of purified whole virus particles revealed at least 4 prominent and consistent proteins that were approximately 19, 23.5, 27.5 and 75 kDa in size (Fig. 4A, lane 1, asterisks). The additional 60 kDa band seen in lane 1 of Fig. 4A was not virus-specific since it was not observed in other purified virus samples (data not shown). A very high mole-

cular weight band (>200 kDa) associated with the nucleoprotein core was also observed (Fig. 4A).

Western blot analysis of complete virions employing polyclonal antibody against CBV revealed the 4 prominent (19, 23.5, 27.5 and 75 kDa) bands but not the high molecular weight band (Fig. 4B). Pre-immune rabbit serum did not react with any of these protein bands (data not shown). All 4 protein bands were of virus origin as confirmed by time course *in vitro* and *in vivo* virus replication experiments (Nadala et al. 1997).

Fig. 3. Electron micrograph of negatively stained CBV nucleoprotein cores. A computer-modified image of the boxed part of the nucleocapsid illustrates the arrangement of the globular subunits. Scale bar = 100 nm

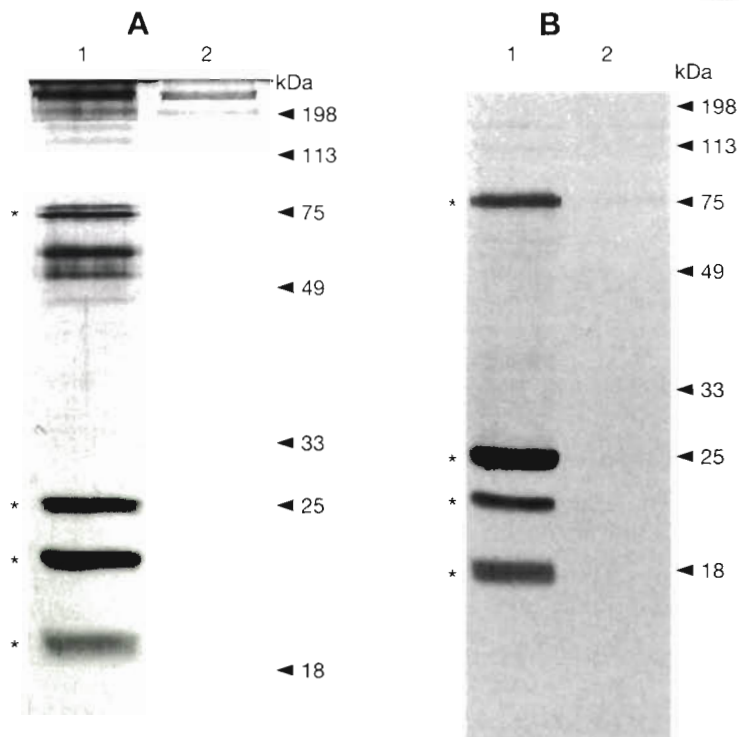
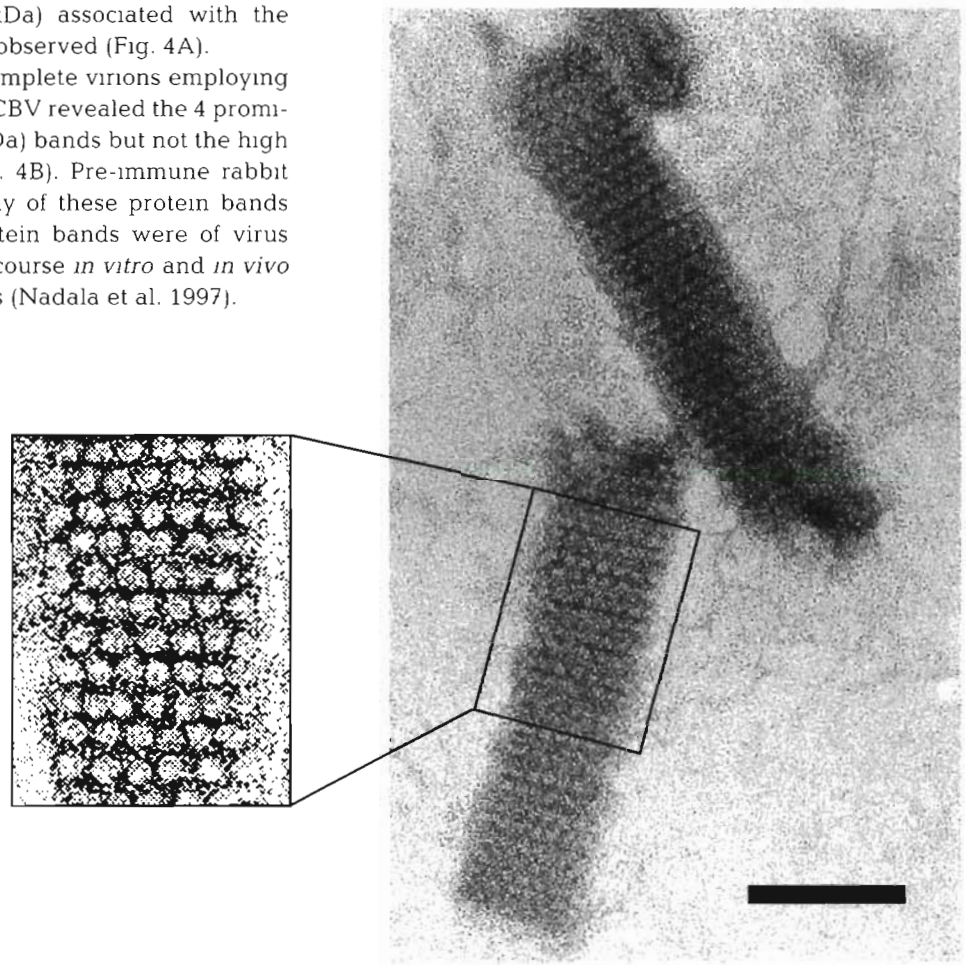


Fig. 4 The structural proteins of CBV as revealed by 12.5% SDS-PAGE gel electrophoresis and western blot. Arrowheads indicate the relative positions of the molecular weight markers. Asterisks indicate the positions of the 4 prominent structural proteins of CBV. (A) Silver-stained gel. Lane 1: purified whole virus; Lane 2: purified viral nucleoprotein core. (B) Western blot. Lane 1: purified whole virus; Lane 2: purified viral nucleoprotein core.

SDS-PAGE and western blot analysis of complete virions treated with Triton X-100 showed that the 27.5 kDa protein was easily removed from the virion when the outer lipid membrane was dissolved (Fig. 5A, B). In contrast, the 23.5 kDa protein remained with the nucleoprotein core. However, prolonged treatment with Triton X-100 along with repeated pelleting and re-suspension resulted in stripping of the 23.5 kDa protein from the nucleoprotein core (Fig. 4A, B). Although less distinct (the bands are very faint), data in Fig. 5A also suggested that the 19 kDa protein was located in the outer lipid membrane of the virus. Likewise, Fig. 5B suggested that the 75 kDa protein (barely visible in lanes 2 and 5) was also located in the outer lipid membrane.

When purified nucleoprotein core particles were treated with DNase I (Fig. 6), the very high molecular weight band (lane 1, arrowhead) normally observed even in denaturing SDS-PAGE gels disappeared. However, no new protein bands were detectable after DNase digestion except for a very low molecular weight band (lane 2, arrowhead) that seemed to migrate with the dye front.

Glycoprotein labelling experiments showed that none of the 4 prominent viral structural proteins were glycosylated (data not shown).

Analysis of the viral nucleic acids

When purified CBV DNA was digested with *Hind* III and electrophoresed in an agarose gel (0.7%), 17 bands were distinguished and reliably sized at 5500, 4361, 4150, 3700, 3400, 3000, 2900, 2450, 2027, 1557, 1350, 1050, 970, 920, 740, 610 and 530 bp (Fig. 7). When the same digest was electrophoresed on 0.4% agarose gel, 12 more bands were easily recognized and reliably sized at 22.8, 22, 15.2, 12.8, 12, 10.8, 10.2, 8.6, 8.4, 7.6, 7.4 and 6.9 kb. When combined, a total of 29 fragments were counted with a total size of about 183.9 kb.

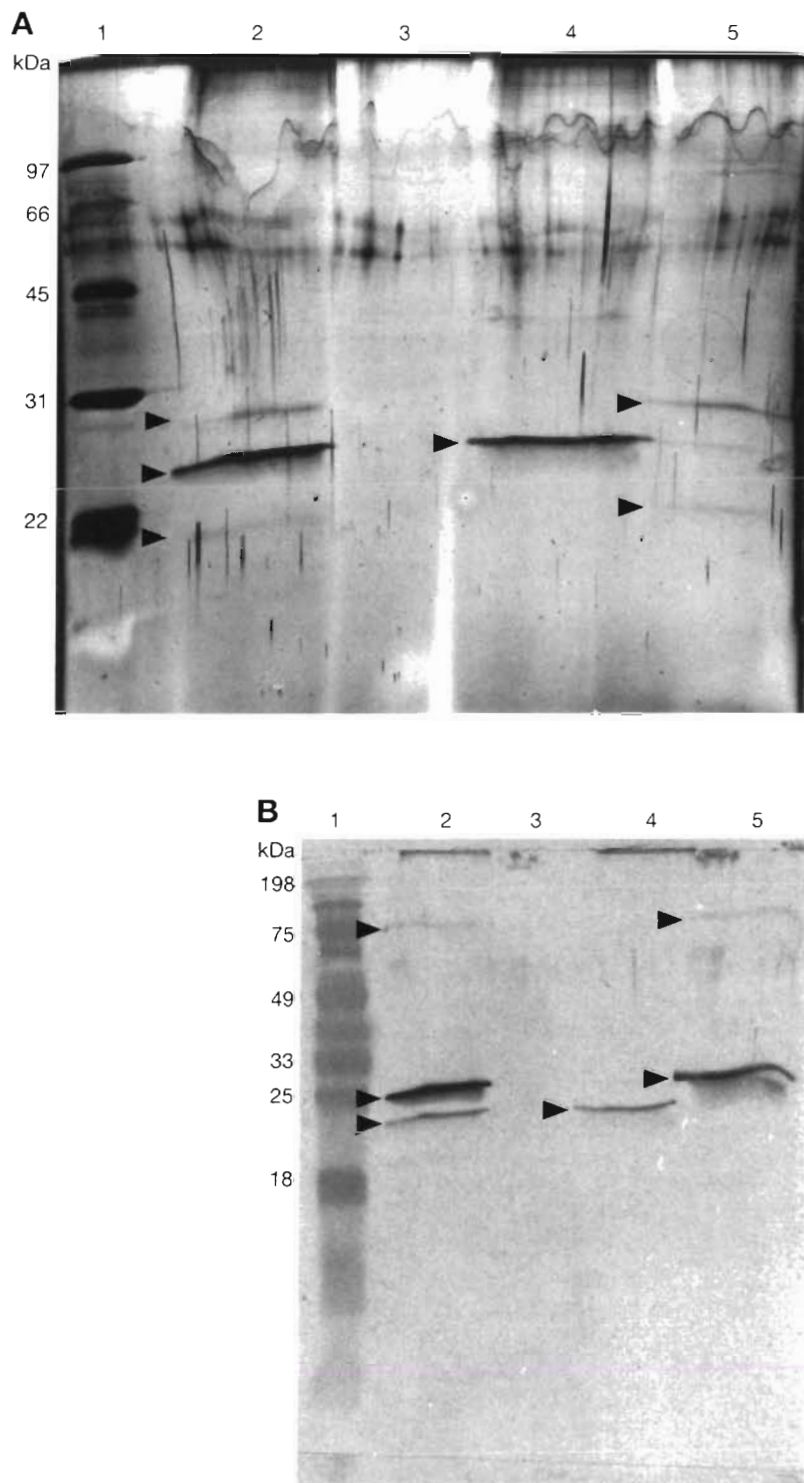


Fig. 5. SDS-PAGE gel electrophoresis and western blot of untreated and Triton-treated CBV. Arrowheads indicate the positions of the protein bands of interest. (A) Silver-stained gel. Lane 1: molecular weight markers; Lane 2: pellet of untreated virus; Lane 3: supernate of untreated virus; Lane 4: pellet of Triton-treated virus; Lane 5: supernate of Triton-treated virus. (B) Western blot. Lane 1: pre-stained molecular weight markers; Lane 2: pellet of untreated virus; Lane 3: supernate of untreated virus; Lane 4: pellet of Triton-treated virus; Lane 5: supernate of Triton-treated virus.

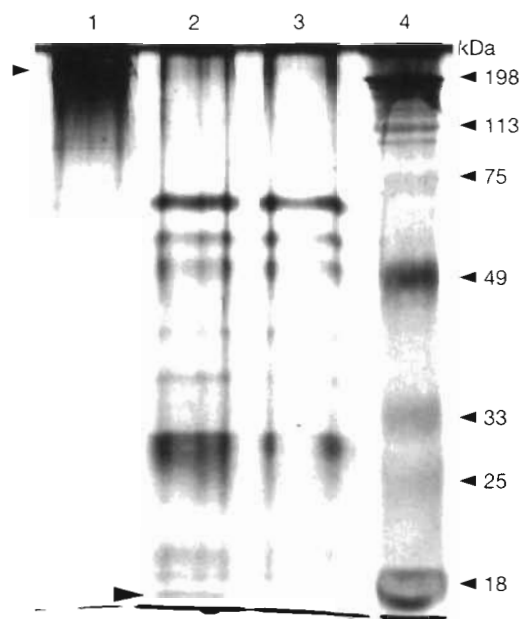


Fig. 6. SDS-PAGE gel electrophoresis of DNase I treated viral nucleoprotein cores. Silver-stained gel of purified nucleoprotein cores showing the high molecular weight band (arrowhead) in lane 1, DNase I treated nucleoprotein cores showing the new low molecular weight band (arrowhead) in lane 2, DNase I alone in lane 3, and prestained molecular weight markers in lane 4

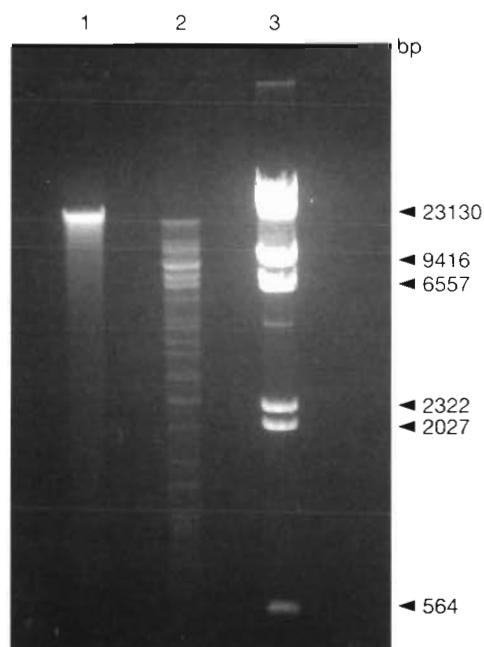


Fig. 7. Agarose gel electrophoresis of purified CBV DNA digested with *Hind* III. Lane 1: uncut CBV genomic DNA; Lane 2: the CBV genomic DNA digested with *Hind* III; Lane 3: lambda DNA digested with *Hind* III

DISCUSSION

The results above suggest that the complete CBV particle consists of a nucleoprotein core, covered with capsid protein, and enveloped in a lipid membrane. The nucleoprotein core is arranged as a series of 14 or 15 stacked rings. It apparently contains the viral DNA and a closely associated low molecular weight protein. The nucleoprotein core has a relatively complex structure and it is likely that there are other proteins that are part of it but have not been clearly demonstrated in our SDS-PAGE gels. These, along with the low molecular weight protein, need to be further characterized.

The nucleoprotein core is covered with a capsid protein of approximately 23.5 kDa. The nucleocapsid in turn is enveloped in a lipid membrane containing at least 3 major structural proteins (19, 27.5, and 75 kDa). Based on comparisons between silver-stained gels and their corresponding western blots, the 27.5 and 75 kDa outer membrane proteins seem to be the most immunogenic. However, neither protein was glycosylated. A proposed schematic cutaway diagram of CBV, indicating the tentative locations of the various viral structural proteins and the nucleoprotein core, is shown in Fig. 8.

The protein profile of CBV is unlike that of any known baculovirus. Instead of the 12 to 30 polypeptide species usually seen in SDS-PAGE analyses of baculoviruses (both occluded and non-occluded) (Bilimoria 1986), CBV has 4 prominent structural proteins plus an unknown number of polypeptides present at much lower concentration. This, plus the unique structure of its nucleocapsid, distinguishes CBV from present members of the *Baculoviridae* family. Although similar to the baculoviruses in size, general structure, and genome composition, CBV along with the former members of the *Nudibaculovirinae* subfamily await reassignment by the International Committee on Taxonomy of Viruses (Murphy et al. 1995).

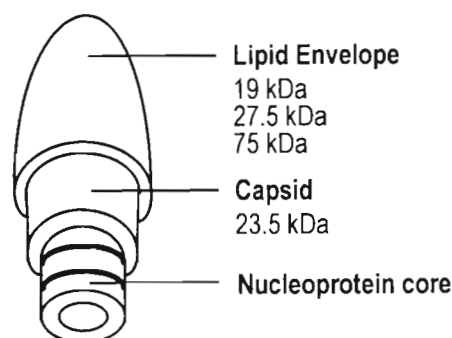


Fig. 8. Proposed schematic cutaway diagram of CBV indicating the tentative locations of the various viral structural proteins

The CBV isolate in the present study is one of several non-occluded baculovirus-like agents which have been recently reported to infect several species of penaeid shrimp and cause high mortalities in Asia and SE Asia (Inouye et al. 1994, Takahashi et al. 1994, Arimoto et al. 1995, Wang et al. 1995, Wongteerasupaya et al. 1995). The present isolate shares a unique nucleocapsid morphology (striated structure) with the white spot baculovirus (WSBV) described by Wang et al. (1995), the *Penaeus monodon* non-occluded baculovirus II (PmNOBII) described by Wongteerasupaya et al. (1995), and the penaeid rod-shaped DNA virus (PRDV) described by Inouye et al. (1996).

The estimated total size of the *Hind* III-digested fragments of CBV-DNA seemed to be larger than that reported for WSBV (183.9+ kb versus 150+ kb) by Wang et al. (1995). However, this is probably due to the fact that Wang et al. counted only 22 fragments versus our 29. At least 2 of the higher molecular weight fragments migrated very closely with other fragments and could have been easily missed. In addition, 5 smaller fragments (530, 610, 740, 920, and 970 bp) were not included. Despite the slight differences in our estimates of the sizes of individual *Hind* III fragments, the profile is virtually identical. This suggests that the WSBV isolate of Wang et al. (1995) is probably identical to our CBV.

The sizes estimated for PRDV DNA by Inouye et al. (1996) and for PmNOBII DNA by Wongteerasupaya et al. (1995) were 163.2 and 168 kb respectively. The lower estimate for DNA size compared to CBV-DNA could again be due to these authors' conservative counting of the restriction fragments. Because Wongteerasupaya et al. (1995) did not report the individual sizes of their fragments, it was not possible to make direct comparisons. However, if one compares the profile of the *Eco*R I restriction fragments of PRDV (Inouye et al. 1996) with that of PmNOBII (Wongteerasupaya et al. 1995), it is apparent that they are also nearly identical. Inouye et al. noted a difference only because Wongteerasupaya et al. had inadvertently transposed the names of the restriction enzymes in their figure (Lo et al. 1996). More recent data reported by Lo et al. (1996) indicate that WSBV *Eco*R I and *Bam*H I restriction profiles were similar to those of PmNOBII. Since we already have some evidence that WSBV is identical to CBV, this means that PRDV and PmNOBII may be the same virus as well. More restriction endonuclease analyses using a battery of enzymes are needed to confirm this close relatedness or identity among the different isolates.

The size dimensions of the virus particles appeared to vary among the different isolates. However, it is difficult to compare virus dimensions of even negatively-stained purified virus samples prepared in different laboratories.

A difference was noted between CBV and the other isolates (PRDV, WSBV, PmNOBII) in the characteristic pathological response of cultured penaeid shrimp, such as *Penaeus monodon*, *P. japonicus*, and *P. penicillatus* when infected with these viruses. Observed in such infected shrimp were a general reddish coloration and the production of conspicuous white spots on the carapace, appendages and inside surface of the body. In contrast, CBV infection of the 2 commercial species, *P. stylirostris* and *P. vannamei* cultured in Hawaii, does not result in the appearance of white spots. Also, the general reddish coloration is seen only occasionally and primarily in the extremities of the infected shrimp. These differences in responses however, may reflect a difference in the penaeid species involved and/or the fact that CBV was inoculated into the shrimps by injection at a high dosage while the other isolates underwent a natural infection.

Lastly it should be noted that the CBV was originally isolated from *Penaeus japonicus* obtained from a farm in the People's Republic of China. The virus was found to be highly pathogenic for the penaeid species cultured here in Hawaii (Tapay et al. 1997, Lu et al. 1997). Available data indicate that the CBV isolate is probably closely related if not identical to the other isolates associated with the white spot syndrome disease. A recent report employing an *in situ* DNA hybridization procedure has presented evidence to strongly suggest that these isolates may be closely related variants (Wongteerasupaya et al. 1996). However, a definitive comparison awaits further characterization of these viruses, especially protein profiles, more RFLP (restriction fragment length polymorphism) studies, nucleic acid sequencing, and serologic testing.

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