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Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus

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White spot syndrome virus (WSSV) is one of the most virulent pathogens causing high mortality in shrimp. In the present study, an open reading frame (termed the p22 gene) was revealed from a WSSV cDNA library. The gene was expressed as a fusion protein with glutathione *S*-transferase (GST) in *Escherichia coli* and purified. Specific antibody was raised using the purified fusion protein (GST–P22). Temporal analysis showed that the p22 gene was a late gene. After binding between purified WSSV virions and anti-GST–P22 IgG followed by labelling with gold-labelled secondary antibody, the gold particles, under a transmission electron microscope, could be found along the outer envelope of WSSV virions. This experiment suggests that the p22 gene encodes an envelope protein of the virus.

Introduction

White spot syndrome is a major shrimp disease worldwide. The disease has also been found in other invertebrate aquatic organisms, such as crab and crayfish (Flegel, 1997). The disease agent is an enveloped, non-occluded and rod-shaped virus, shrimp white spot syndrome virus (WSSV) (Chou *et al.*, 1995; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Chang *et al.*, 1996; Inouye *et al.*, 1996; Chen *et al.*, 1997; Tapay *et al.*, 1997). There is little genetic variation among WSSV isolates from all over the world (Lo *et al.*, 1999). Its morphology, nuclear localization and morphogenesis are reminiscent of insect baculoviruses (Durand *et al.*, 1997), but the family that WSSV belongs to is still unknown. WSSV has a wide host range among crustaceans and a high infection and mortality rate, and has caused large economic losses in the shrimp industry.

In 1997, the WSSV genomic DNA was successfully purified from *Penaeus japonicus* in our laboratory (Yang *et al.*, 1997), and the genomic DNA and cDNA libraries were constructed. The virus contains a 305 kb double-stranded circular DNA (Yang *et al.*, 2001). Studies on WSSV genes and their regulation will be helpful for the diagnosis and control of the virus infection. However, in contrast with the insect baculoviruses, some of the best-studied viruses, only a few genes from WSSV have been reported (van Hulten *et al.*, 2000a, b, c; Tsai *et al.*, 2000a, b; Zhang *et al.*, 2000, 2001).

By analysis and comparison of the WSSV genomic DNA and cDNA libraries, an open reading frame (ORF; termed the p22 gene) that probably encodes a structural protein was identified. This study is aimed at characterizing the gene.

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The GenBank accession number of the sequence reported in this paper is AF308164.

Methods

■ **Sequencing and computer analysis of the p22 gene.** Total RNA and viral DNA were isolated from WSSV-infected tissues of *P. japonicus* from Xiamen, China (Yang *et al.*, 1997). The mRNA was reverse transcribed into cDNA, and the cDNA was cloned into pBluescript SK+ (Stratagene) with the restriction enzyme *Eco*RV and transformed into *Escherichia coli* XL-1 Blue. DIG-labelled WSSV DNA (Dig DNA labelling and detection kit, Boehringer Mannheim) was used as a probe to screen the cDNA library. The clones containing 800–2000 bp inserts were selected from the cDNA library by digestion with *Eco*RI + *Hin*dIII. The corresponding plasmids were prepared and purified with resin (Sambrook *et al.*, 1989). They were sequenced with pBluescript ks and sk primers in an automatic DNA sequencer. The generated DNA sequences and the deduced amino acids sequences were analysed using DNASIS and PROSIS software (Hitachi Software Engineering, version 4), respectively. The deduced amino acid sequences were searched against the GenBank database with BLAST (Altschul *et al.*, 1997).

■ **Expression and purification of the p22 gene in *E. coli*.** The p22 gene was cloned and expressed in pGEX-4T-2-pLysS as a fusion protein with glutathione *S*-transferase (GST) (Pharmacia Biotech). The p22 gene was amplified using the synthesized forward primer 5′ CAC*GGATCC*ATGGAATTTGGCAACCT 3′, with a *Bam*HI site (italic), and the reverse primer 5′ AGA*CCCGGG*TTACTTCTTCTTGATTT 3′, with a *Sma*I site (italic). The amplified DNA and plasmid vector pGEX-4T-2 were digested with *Bam*HI + *Sma*I. After purification and ligation of DNA fragments, the p22 gene was inserted into the pGEX-4T-2 vector downstream of GST. The resulting plasmid was named pGEX22. The competent cells of *E. coli* pLysS were transformed by pGEX22, and colonies containing transformants were screened by PCR. pGEX22 was confirmed by digestion with *Bam*HI + *Sma*I and DNA sequencing. The following treatments were conducted for the expression of the p22 gene:

A, pGEX22-pLysS (containing the p22 gene) induced;
B, pGEX22-pLysS (containing the p22 gene) non-induced;
C, pGEX-4T-2-pLysS (the vector only as a control) induced;
D, pGEX-4T-2-pLysS (the vector only as a control) non-induced.

After incubation at 37 °C overnight, pGEX22-pLysS and pGEX-4T-2-pLysS were inoculated into new media at the ratio 1:100. When the OD_{600} was 0·6, the bacteria were induced with the lactose analogue IPTG and grew for an additional 6 h at 37 °C. The induced and non-induced bacteria were analysed by SDS–PAGE.

The recombinant pGEX22-pLysS was incubated and induced in 1000 ml LB media. The induced bacterium was spun down (4000 ***g***) at 4 °C, suspended in ice-cold PBS (containing 1% Triton X-100, 1 mM PMSF, 4 mM benzamidine, 10 μg leupeptin and 10 μg aprotinin) and sonicated for 30 s on ice. The sonicate was mixed with glutathione–agarose beads (Sigma) and incubated at 4 °C for 2 h. The beads were washed with ice-cold PBS and incubated in reducing buffer (50 mM Tris–HCl, 10 mM reduced glutathione, pH 8·0) at room temperature for 10 min. After centrifugation at 1000 ***g*** for 5 min, the supernatant was collected and detected by SDS–PAGE.

■ **Preparation of antibody.** The purified GST–P22 fusion protein was used as antigen to immunize mice by intradermal injection once every 2 weeks over an 8-week period. Antigen (5 μg) was mixed with an equal volume of Freund's complete adjuvant (Sigma) for the first injection. Subsequent injections were conducted using 5 μg of antigen mixed with an equal volume of Freund's incomplete adjuvant (Sigma). Four days after the last injection, mice were exsanguinated, and antisera collected. The titres of the antisera were 1:20000 as determined by ELISA. ELISA was performed essentially as described by Harlow & Lane (1988). The immunoglobulin (IgG) fraction was purified by protein A–Sepharose (Bio-Rad) (Sambrook *et al*., 1989) and stored at −70 °C. The optimal dilution of purified IgG, after serial dilutions, was 1:1000, as determined by ELISA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was obtained from Sigma. Antigen was replaced by PBS in negative control assays.

■ **Transcriptional analysis of p22 gene**

Shrimp infection with WSSV. The infected tissue from *P. monodon* shrimp with a pathologically confirmed infection was homogenized in TN buffer (20 mM Tris–HCl and 400 mM NaCl, pH 7·4) at 0·1 g/ml. After centrifugation at 2000 ***g*** for 10 min, the supernatant was diluted to 1:100 with 0·9% NaCl and filtered (0·22 μm filter). 0·2 ml of the filtrate was injected intramuscularly into each healthy shrimp (determined by PCR) in the lateral area of the fourth abdominal segment. At various times post-infection, four specimens were selected at random and their haemolymph was collected. The collected haemolymph samples were immediately frozen and stored at −70 °C.

RT–PCR. Total RNA was isolated from WSSV-infected shrimp haemolymph according to the manufacturer's instructions (Macherey-Nagel). Then RT–PCR was performed with primers 5′ *AGACCCGGGTTACTTCTTCTTGATTT* 3′ and 5′ *AGACCCGGGTTACTTCTTCTTGATTT* 3′ using a TITANIUM One-step RT–PCR kit (Clontech Laboratories). The RT–PCR cycles were as follows: 1 h at 50 °C; 5 min at 94 °C; 30 s at 94 °C, 30 s at 65 °C, 1 min at 68 °C, 30 cycles; 2 min at 68 °C.

Western blot. The infected shrimp haemolymph samples (dilution 1:10) from various times were analysed in a 12% SDS–PAGE gel. Proteins were visualized using Coomassie brilliant blue staining. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad) in electroblotting buffer (25 mM Tris, 190 mM glycine, 20% methanol) for 3 h. The membrane was immersed in blocking buffer (3% BSA, 20 mM Tris, 0·9% NaCl, 0·1% Tween 20, pH 7·2) at 4 °C overnight followed by incubation with a polyclonal mouse anti-GST–P22 IgG or mouse anti-GST IgG for 3 h. Subsequently, HRP-conjugated goat anti-mouse IgG (Sigma) was used and detection was performed with substrate solution (4-chloro-1-naphthol, Sigma).

■ **Shrimp WSSV and immuno-electron microscopy**

Intact WSSV. The infected tissue from *P. monodon* shrimp was homogenized and centrifuged as described above and the supernatant was injected (1:100 dilution in 0·9% NaCl) intramuscularly into healthy crayfish (*Cambarus clarkii*) from Singapore in the lateral area of the fourth abdominal segment. Four days later, haemolymph freshly extracted from infected crayfish was layered on the top of the 10–40% (w/v) continuous sodium bromide gradient and centrifuged at 110000 ***g*** using an RP40-T rotor in the Prespin Ultracentrifuge (Shimadzu model MSE-75) for 2 h at 4 °C. Virus bands were collected by side puncture, diluted 1:10 in TNE buffer (50 mM Tris–HCl, 100 mM NaCl and 1 mM EDTA, pH 7·4) and pelleted at 119000 ***g*** for 1 h at 4 °C. The resulting pellets were resuspended in TNE. Virus samples were examined under a transmission electron microscope (JEOL 100 cxII, Japan) for purity (Huang *et al*., 2001).

Nucleocapsid of WSSV. WSSV virus particles were treated with 0·5–1·0% Triton X-100 for 30 min at room temperature, and then centrifuged at 119000 ***g*** using an SW 41-Ti rotor (Beckman Coulter). The pellet was resuspended in 0·1 × TNE buffer and centrifuged at 119000 ***g***. After several repeats to completely remove Triton X-100, the resulting WSSV nucleocapsids were resuspended in TNE. Samples were examined under a transmission electron microscope (JEOL 100 cxII, Japan).

Immuno-electron microscopy. The purified WSSV virion suspension and nucleocapsids were mounted on carbon-coated nickel grids (200 mesh), respectively and incubated for 1 h at room temperature. After washing with PBS, the grids were blocked with 3% BSA for 1 h. The grids were rinsed with PBS and incubated in mouse anti-GST–P22 IgG or mouse anti-GST IgG or pre-immune serum of mouse for 1 h at room temperature followed by washing with PBS. Then 15 nm gold-labelled anti-mouse IgG raised in goat (Sigma) was added to the grids and incubated for 1 h at room temperature. After negative staining with 2% phosphotungstic acid, the specimens were examined under a transmission electron microscope.

Results

The structure of the p22 gene

Based on the lengths of many structural protein genes of insect baculoviruses, it was inferred that the sizes of the

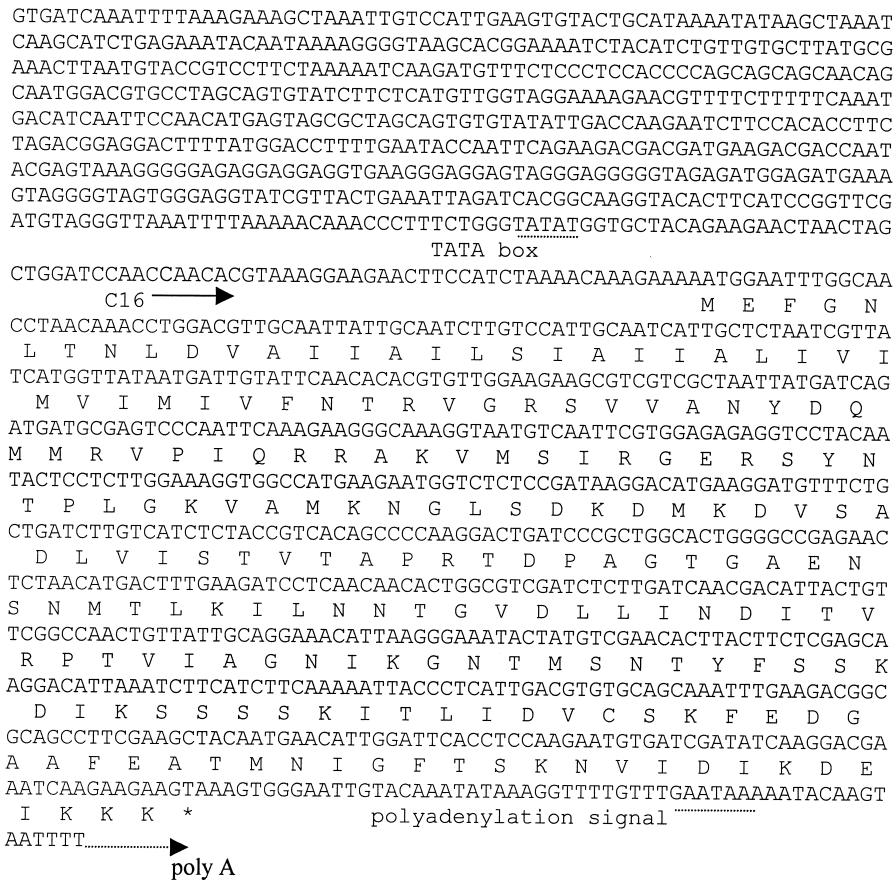

Fig. 1. Nucleotide sequence of the genomic region containing the C16 clone and the deduced amino acid sequence (one-letter code) of the p22 gene. The transcription initiation site of C16 and the position of the poly(A) are indicated by solid and dashed lines with arrows, respectively. The putative TATA box and polyadenylation signal are marked with dashed lines.

transcripts for the envelope and nucleocapsid protein genes of WSSV were probably 1000 nucleotides or so. Clones with 800–2000 bp inserts were screened and sequenced from the cDNA library of WSSV. An ORF of 612 bp was revealed in clone C16 (Fig. 1). Sequence comparison of C16 with the genomic DNA of WSSV showed that it was identical with that of a fragment of genomic DNA except for its poly(A) (Fig. 1). This confirmed that C16 was transcribed from the genomic DNA of WSSV.

The ORF in C16 encoded a 204 amino acid protein (P22) with a theoretical molecular mass of 22 kDa, hence it was termed the p22 gene (GenBank accession no. AF308164). A typical TATA box (TATAT) and a putative polyadenylation signal (AATAAA) were presented 39 bp upstream of the transcription initiation site of C16 and 21 bp upstream of the poly(A), respectively (Fig. 1). The base sequence surrounding the methionine start codon (AAAATGG) of the p22 gene was consistent with the Kozak rule for efficient eukaryotic translation initiation (PuNNATGPu) (Kozak, 1987). The amino acid sequence of the P22 protein was subjected to BLAST in GenBank. It was identical to a nucleocapsid protein of WSSV (van Hulten *et al.*, 2000b).

Expression and purification of the p22 gene

The p22 gene was cloned into a pGEX-4T-2 vector and expressed as a GST fusion protein. After induction with IPTG at 37 °C, induced and non-induced pGEX22-pLysS (containing the p22 gene) and pGEX-4T-2-pLysS (the vector only) were analysed by SDS–PAGE (Fig. 2). A band (about 52 kDa) corresponding to the GST–P22 fusion protein (GST 26 kDa + P22 26 kDa) was observed in the induced pGEX22-pLysS (Fig. 2, lane 3). No protein was found at the same position in the induced and non-induced pGEX-4T-2-pLysS (Fig. 2, lanes 4 and 5). This showed that the p22 gene was expressed. The induced recombinant pGEX22-pLysS was purified using affinity chromatography. A purified fusion protein, GST–P22, was obtained (Fig. 2, lane 6).

Temporal analysis of p22 gene transcription

RT–PCR was used to detect the p22 gene-specific transcript in the total RNAs extracted from the haemolymph of adult *P. monodon* at various infection stages (0, 6, 18, 24, 30, 36 and 48 h post-infection) with WSSV. The transcript was first

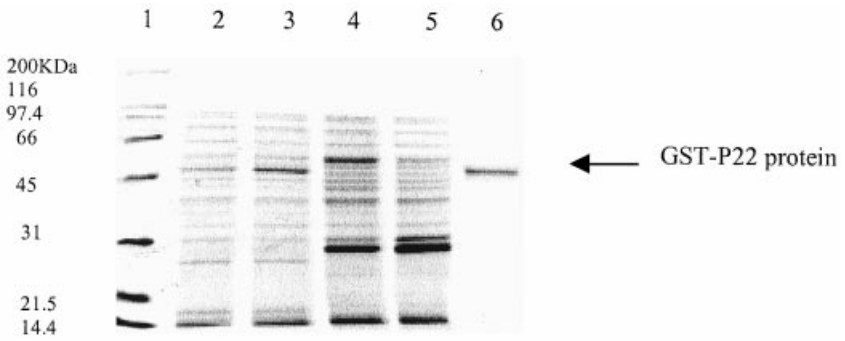

Fig. 2. SDS–PAGE of expressed and purified proteins encoded by the p22 gene. Lanes 1, marker; 2, pGEX22 (containing the p22 gene)-pLysS, non-induced; 3, pGEX22-pLysS, induced; 4, pGEX-4T-2 (the vector only as a control)-pLysS, non-induced; 5, pGEX-4T-2-pLysS, induced; 6, purified fusion protein (GST–P22).

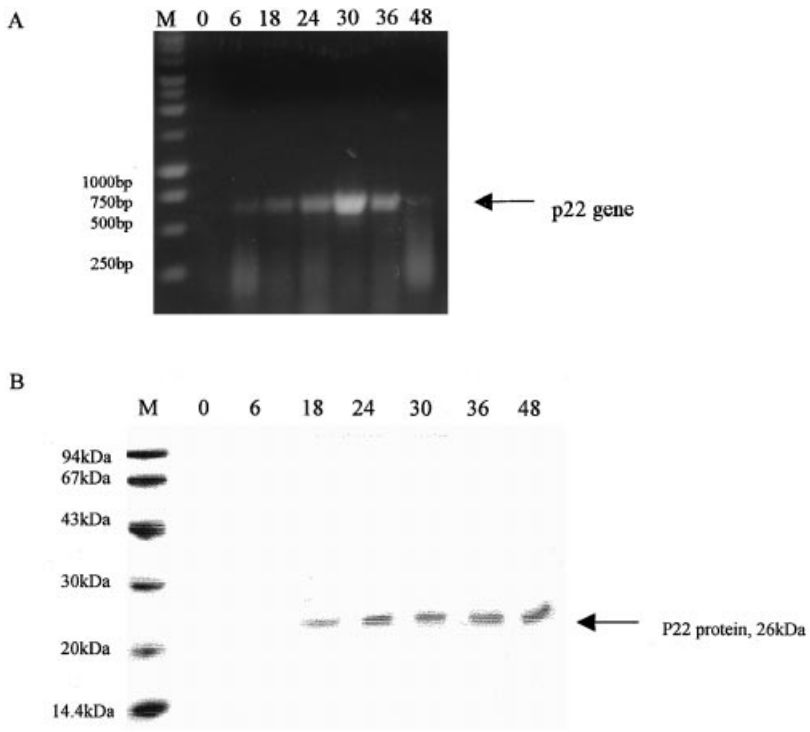

Fig. 3. Temporal analysis of p22 gene transcription. (A) RT–PCR with the p22 gene-specific primers using the total RNA extracted from the haemolymph of healthy and infected shrimp. (B) Western blot with anti-GST–P22 antibody using the haemolymph of healthy and infected shrimp. Lane headings show time post-infection in hours. M, DNA marker (A), protein marker (B).

slightly detected at 6 h p.i. and maximally at 30 h p.i. (Fig. 3). This suggested that the p22 gene was a late gene.

The haemolymph both before and after (at 6, 18, 24, 30, 36 and 48 h) artificial infection with WSSV was transferred onto a nitrocellulose membrane for the detection of P22 protein from WSSV with Western blot. The P22 protein was first detected at 18 h p.i. (Fig. 3), and maximally at 24 h p.i. It revealed that the P22 protein was expressed at the late stage.

Transmission electron microscopy studies

Intact WSSV and nucleocapsids were purified from the haemolymph of WSSV-infected crayfish. As a negative control, haemolymph was also taken from healthy crayfish. Under transmission electron microscopy, many enveloped and rod-shaped virions were found in the infected samples (Fig. 4A), and no virus particles were found in the healthy crayfish samples. After the removal of the envelope, the nucleocapsid was obtained (Fig. 4B). The purified WSSV virions and nucleocapsids were incubated with anti-GST–P22 IgG or anti-GST IgG or pre-immune serum of mouse, respectively, followed by incubation with the gold-labelled secondary antibody on the carbon-coated nickel grids. After hybridization, the gold particles could be clearly found on the envelopes of WSSV virions labelled with the anti-GST–P22 IgG (Fig. 4C), but no gold particle was found on the non-

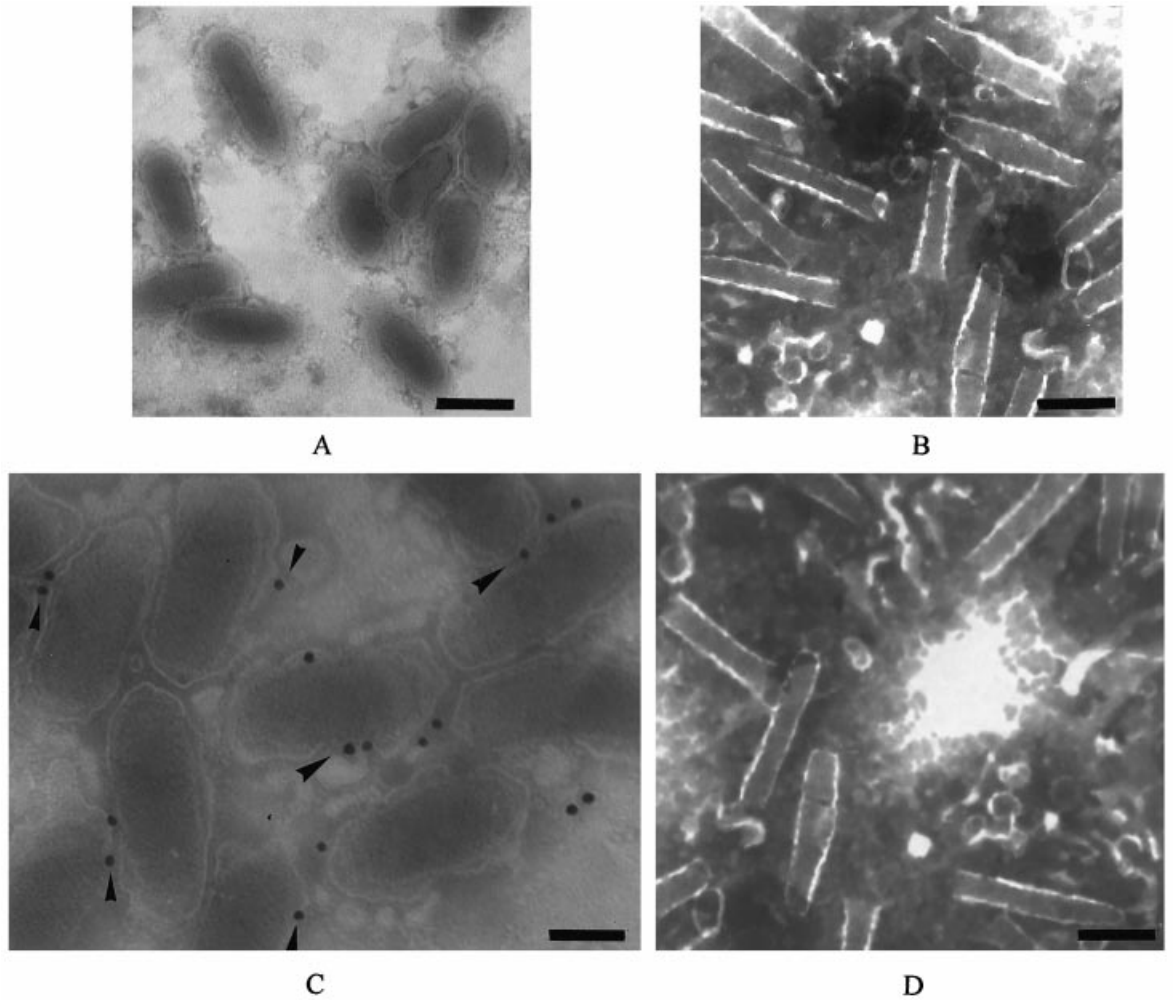

Fig. 4. Immuno-electron microscopy of purified WSSV virions and nucleocapsids with anti-GST–P22 IgG followed by gold-labelled secondary antibody. (A) Intact WSSV virions (scale bar, 185 nm); (B) nucleocapsids of WSSV (scale bar, 185 nm); (C) WSSV virions labelled with gold (scale bar, 238 nm); and (D) WSSV nucleocapsids labelled with gold (scale bar, 185 nm). Arrows indicate gold particles.

enveloped nucleocapsid with the same antibody (Fig. 4D). As controls, no gold particles could be observed on the envelope of WSSV or the nucleocapsid labelled with anti-GST IgG or pre-immune serum of mouse. This showed that the P22 protein was distributed in the envelopes of WSSV virions.

Discussion

White spot syndrome, which appeared in the 1990s, is a devastating virus disease in penaeid shrimp. WSSV, considered as a new virus at present (Chen *et al*., 1997), has not been classified by The International Committee on Taxonomy of Viruses. Characterization and sequence determination of the structural proteins are of importance for the determination of taxonomy, diagnosis and control of WSSV. In the present study, a novel gene (termed the p22 gene) has been successfully cloned by screening a WSSV cDNA library. Homology searches with the P22 protein against GenBank using BLASTA showed that the p22 gene was identical to the vp26 gene (van Hulten *et al*., 2000b). The vp26 gene was identified from the SDS–PAGE of WSSV and N-terminal amino acid sequencing. Moreover, according to the difference in protein bands between the purified intact WSSV virions and the purified WSSV nucleocapsids, the vp26 gene was inferred to be a viral gene encoding a nucleocapsid protein, without direct information regarding its intracellular localization. However, in this study, immuno-electron microscopy revealed that the gold particles were present on the outer envelopes of WSSV virions, and no gold particle was present on the non-enveloped nucleocapsids. These experiments showed that the p22 gene/vp26 gene encoded one of the envelope proteins of WSSV, but not a nucleocapsid protein as inferred by van Hulten *et al*. (2000b). Hydrophobicity analysis (PROSIS) of the amino acid sequence of the P22 protein revealed that there was a strong hydrophobic region at the N terminus. This suggested that it was responsible for the membranous structure. However,

the P22 protein from WSSV was not glycosylated using SDS–PAGE of purified WSSV followed by glycoprotein detection (the Immun-Blot kit for glycoprotein detection, Bio-Rad) (data not shown). Because there is no suitable cell line in which to culture WSSV, the function of the p22 gene remains to be determined.

The p22 gene was obtained by screening a cDNA library of WSSV isolated from *P. japonicus* in China, whereas the vp26 gene was found in the Thailand WSSV isolate from *P. monodon* (van Hulten *et al*., 2000b). In spite of the different geographical locations and species from which the isolates were obtained, the comparison of protein and DNA sequences showed that p22 and vp26 were 100% identical. Earlier studies also showed that there was little genetic variation among WSSV isolates (Lo *et al*., 1999). However, it is too soon to conclude that all WSSV geographical isolates are genetically similar, and further genetic studies are needed.

Generally, structural protein genes in the viral genomes are late genes, but some structural protein genes of insect baculoviruses can be transcribed in the early stage post-infection. Temporal analysis of the p22 gene transcript by RT–PCR showed that it was a late gene from WSSV, and the gene transcripts accumulated in the course of infection (Fig. 3). However, the conserved motif (ATAAG) presented in the late genes of insect baculoviruses could not be found in the DNA sequence corresponding to the p22 gene. This suggested that WSSV was different from the baculoviruses.

The P22 protein was one of the envelope proteins from WSSV. It could be selected to further study, in particular, its relatedness to structural proteins of other viruses, including baculoviruses. It could also be used to reveal the infectioun process of WSSV in shrimp by hybridization *in situ* and to study if the P22 protein has an effect on WSSV infection. Antibody against P22 protein might serve as a specific diagnostic reagent to detect WSSV infection in shrimp.

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