Identification of Structural Proteins of *Bombyx mori* Parvo-Like Virus (China Zhenjiang Isolate)

Meng Lv Qin Yao Yong Wang Xiaoyong Liu Haijun Liu Guoping Huang
Keping Chen Junhong Zhang Xiaogang Li

Institute of Life Sciences, Jiangsu University, Zhenjiang, PR China

Key Words

Bombyx mori · Parvo-like virus · Viral structural protein

Abstract

Objective: *Bombyx mori* parvo-like virus is a type of virus containing two single-stranded linear DNA molecules (VD1, VD2). In the present work, the structural proteins of *B. mori* parvo-like virus (China Zhenjiang isolate) (BmDNV-Z) were identified. **Methods:** The virions were purified by cesium chloride density gradient centrifugation from the feces of *B. mori* larvae infected with BmDNV-Z and analyzed by SDS-PAGE, Western blotting and MALDI-TOF/mass spectrometry. **Results:** There were seven polypeptides of the virions, named P1 to P7, which reacted with the anti-virion serum. In addition, two polypeptides, P5 and P6, were recognized by the antibody against a viral protein encoded by VD1-ORF3. **Conclusion:** The results showed that the viral polypeptides were mainly composed of 7 proteins named P1 to P7. Western blotting with anti-virion serum indicated that structural proteins P5, P6 and P7 had molecular weights of around 50, 55 and 130 kDa, respectively. They were the major immunogens of the virion. Among them, P5 and P6 were coded by VD1-ORF3 and P7 was encoded by VD2-ORF1. Taken together, three viral structural proteins encoded by VD1-ORF3 and VD2-ORF1 were identified in BmDNV-Z for the first time.

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Introduction

Bombyx mori parvo-like virus (BmDNV) causes flacherie diseases of silkworm and impairs silk production. The infection of *B. mori* larvae with BmDNV leads to thorax transparency and diarrhea. Since the symptoms caused by this virus are very close to those caused by *B. mori* densovirus type I (termed BmDNV-1 Ina isolate), BmDNV was initially called *B. mori* densovirus type II which includes two isolates: Japan Yamanashi isolate (BmDNV-2) and China Zhenjiang isolate (BmDNV-Z).

The genome of BmDNV is characterized by two nonhomologous single-stranded linear DNA molecules (called VD1 and VD2) in the genome, and each strand is packed in separate virus particles [1]. The complete nucleotide sequence of the BmDNV-Z genome has been determined and the sequencing data were assembled into two contiguous sequences, the 6,543nt VD1 (GenBank accession number DQ017268) and the 6,022nt VD2 (GenBank accession number DQ017269). By analysis of the genome sequence of BmDNV-Z, we found that VD1 contained four ORFs (VD1-ORF1, VD1-ORF2, VD1-ORF3, and VD1-ORF4) and VD2 had two ORFs (VD2-ORF1 and VD2-ORF2) (fig. 1).

Up to now, specific classification is still not available for the BmDNV. In the 8th Report of the International Committee on Taxonomy of Viruses, BmDNV-2 was ex-

Fig. 1. The genome organization of BmDNV-Z. Horizontal bar represents the genomic DNA molecules. Boxes above the horizontal bars represent the ORFs found in the genome sequence and those below the horizontal bars represent ORFs found in the complementary strand.

cluded from the family *Parvoviridae*, because its genome includes two single-strand linear molecules and encodes its own DNA polymerase, characteristics which are not possessed by typical parvoviruses [2]. In 2001, Li and co-workers found that some amino acids in the conserved domain of viral protein (VP) 1 unique part (VP1up) matched the critical amino acids in the catalytic site of secretory phospholipase A2 (sPLA2) [3], and that PLA2s form a superfamily of key enzymes involved in physiological and pathological processes, such as lipid membrane metabolism, signal transduction pathways, inflammation and acute hypersensitivity, and degenerative findings could provide a basis to study the proposed diseases [4–6]. However, the conserved domain of PLA2 was absent in the VP1up of BmDNV-Z [Yao et al., in preparation]. This demonstrated that BmDNV-Z is different from the classic DNV with respect to physiological and pathological processes.

Generally, a parvovirus has 2–4 capsid proteins coded by one ORF with molecular weights ranging from 40 to 80 kDa. Reports on structural proteins of BmDNV-2 are inconsistent. In 1984, Kurihara et al. [7] purified BmDNV-2 from the infected midgut of *B. mori* larvae by ultracentrifugation in sucrose buffer solution and separated the proteins with SDS-PAGE. They found that there were 4 proteins with molecular weights of 51, 53, 116 and 121 kDa. In 1995, Sotoshiro and Kobayashi [8] purified BmDNV-2 from the infected larval bodies by cesium chloride gradient centrifugation and found that there were 6 polypeptides with molecular weights of 46.5, 49, 51, 53, 118 and 120 kDa. However, the polypeptides of 118 and 120 kDa could not be detected by the anti-BmDNV-2 antiserum.

In the present study, BmDNV-Z virions were purified from the feces of infected *B. mori* larvae by cesium chloride density gradient centrifugation and examined under electron microscope. Proteins of the virions were separated by SDS-PAGE and identified by mass spectrometry. The isolated proteins were verified by Western blotting using polyclonal antibody against virion and recombinant VP expressed in *Escherichia coli*.

Materials and Methods

Virus Propagation and Purification

The virus was purified as described previously [9] from the feces of BmDNV-Z-infected 4th instar larvae of *B. mori* strain Huaba. The virus was finally purified by cesium chloride density gradient centrifugation.

Electron Microscopy

The purified viral particles were treated with a negative contrast staining method. The samples were dropped on a copper screen with supportive film and were stained immediately for 2–3 min with 3% phosphotungstic acid after it dried up. The stained virions were observed under a transmission electron microscope (Tecnai-12, Phillips).

SDS-PAGE

20 μ l of the purified virion sample was mixed with 4 μ l of 5 $\times$ SDS-PAGE loading buffer solution containing 250 mM Tris-HCl (pH 6.8), 10% SDS, 0.5% bromphenol blue, 50% glycerin and 5% β -mercaptoethanol, and boiled for 10 min. Electrophoresis was performed at 60 V for 30 min and then at 100 V for 90 min at room temperature. The protein molecular weight standard was from TaKaRa (Dalian, China). The gel was stained with 0.25% Coomassie blue R-250 for 3 h and then destained for 6 h using a mixture of 10% acetic acid and 5% ethanol.

In-Gel Digestion

Gel slices were collected manually using a pipette with a trimmed polypropylene tip. The proteins were digested with trypsin using modified procedures [10]. Briefly, the proteins in gel slices were destained by incubating the gels in ultra-pure water, 50 mM ammonium bicarbonate, 50% acetonitrile and 100% acetonitrile on a shaker and reduced with dithiothreitol and alkylated with iodoacetamide, and then treated with freshly prepared 20 μ g/ml trypsin (Promega) solution containing 40 mM ammonium bicarbonate and 10% acetonitrile at 37 $^{\circ}$ overnight. The gels were extracted with 5% formic acid in 1:1 water/acetonitrile. All solvents and reagents were purchased from Sigma.

MALDI-TOF Mass Spectrometry Identification

1 μ l of each trypsin-digested peptide was mixed with 1 μ l of 10 mg/ml α -cyano-4-hydroxycinnamic acid (Sigma) saturated with 50% acetonitrile containing 0.1% trifluoroacetic acid, and applied onto a microtiter plate anchor chip (Bruker). A matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer was used to identify the trypsin-digest-

ed peptides by using the standard peptides for calibration according to the manufacturer's instructions (Ultraflex TOF/TOF, Bruker).

Protein Identification

Mascot software (Matrix Science, Ltd.) was used to search the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>) protein databases and a local self-defined database containing protein fragments encoded by VD1-ORF4 to identify the target proteins. The parameter setting was as follows: missed cleavages as 1, fixed modification as acetylation of carbamidomethyl (C), variable modification as oxidation of methionine (M) or Gln → pyro-Glu (N-term Q), and mass tolerance as 0.2 Da. Peptide was identified with a Mowse score >79 ($p < 0.05$).

Prokaryotic Expression of VP and Preparation of Antibody

The VP coding region was amplified from BmDNV-Z genomic DNA as template using the forward primer BmDNV-VP-F 5'-AAGGCCTAATATTATGGGTAGAGTACTTGGCT-3' and reverse primer BmDNV-VP-R 5'-ATAAGAATGCGGCCGCTTATTATTGAAAACCAACAAGC-3', with *Bam*H I and *Xho*I restriction sites at their 5'-ends, respectively. The target fragment was cloned into the expression vector pMAL-c2x (New England Biolabs, Inc.) to construct the recombinant expression plasmid pMAL-c2x-VP, which was then transformed into *E. coli* TB1 and was induced to express the VP by using 1.0 mM isopropyl- β -D-thiogalactopyranoside for 4 h at 37°. The recombinant protein was purified with the affinity column of a maltose-tagged purification system (New England Biolabs, Inc.), and the maltose-binding-protein-fused VP was used to immunize healthy rabbits and produce antibodies. The antibodies were purified with a protein A affinity column (Millipore).

Western Blot Analysis

Western blot analysis was performed according to the method of Towbin et al. [11] and Burnette [12]. The polypeptides resolved by SDS-PAGE were transferred to polyvinylidene fluoride membrane (Millipore). Viral structural polypeptides on the membrane were incubated with rabbit anti-BmDNV-Z virion serum and antibody against VP which was encoded by VD1-ORF3 and then incubated with the goat anti-rabbit IgG antibody conjugated with peroxidase to display color.

Results

Observation of Virions with the Electron Microscope

Electron micrograph of tungsten-stained virions showed that the viral solution contained a lot of small spherical particles (fig. 2). The viral particles were about 20 nm in diameter. No obvious large particles were seen, indicating that the prepared viruses were not contaminated with cell debris.

Analysis of VP with SDS-PAGE and Western Blot

SDS-PAGE analysis showed that the viral particles prepared from feces of the sick *B. mori* larvae infected

Fig. 2. Electron micrograph of silkworm parvo-like virus.

with BmDNV-Z had 7 proteins (fig. 3a, lane 2). The molecular weights of P1 to P4 were between 29.0 and 44.3 kDa, those of P5 and P6 between 44.3 and 66.4 kDa, and that of P7 about 120 kDa. Western blot analyses using anti-BmDNV-Z serum showed that P1 to P7 could be recognized by anti-virion serum, suggesting that those polypeptides were major immunogens of the virion (fig. 3b). Western blot analysis with antibody against VP indicated that P5 and P6 were coded by ORF3 in VD1 (fig. 3c).

Analysis of VP with MALDI-TOF/Mass Spectrometry

VP bands P1 to P7 shown in SDS-PAGE (fig. 3a) were analyzed by MALDI-TOF/mass spectrometry. High-quality peptide mass fingerprints were obtained (fig. 4). After searching and comparing with the theoretical trypsin-digested peptides of existing proteins in databases, it was found that P5 and P6 were encoded by the VD1-ORF3 of BmDNV-Z and P7 was encoded by the VD2-ORF1 of BmDNV-Z. P4 had a limited sequence identity with a fragment of BmDNV-Z VD1-ORF4 (fig. 5). Molecular masses of peptides from trypsin-digested P3 showed no significant hits both in the NCB database and the local BmDNV-Z, VD1-ORF4 database. P1 and P2 were the triacylglycerol lipases of *B. mori*. The database search results are listed in table 1.

Fig. 3. SDS-PAGE and Western blot analyses of proteins from BmDNV-Z virions. **a** Lane 1: protein molecular weight marker; lane 2: polypeptides from BmDNV-Z virions named P1 to P7. **b** Western blot analysis with anti-BmDNV-Z serum. **c** Western blot analysis with anti-MBP-VP antibody.

Fig. 4. Peptide mass fingerprints of P7, P5 and P4.

Fig. 5. Matched peptides of P7, P6, P5 and P4 are shown in shaded areas.

Table 1. MALDI-TOF/mass spectrometry identification of proteins from BmDNV-Z virions

No.	Score	NCBI accession number	Mass Da	Protein name	Sequence coverage, %
1	90	gi114050855	32,242	triacylglycerol lipase of <i>B. mori</i>	40
2	112	gi114050855	32,242	triacylglycerol lipase of <i>B. mori</i>	49
3	55	gi38046528	24,339	VP1 of foot-and-mouth disease virus-type Asia 1	32
	10	Q4U3C3_BMDNV	42,799	BmDNV-Z VD1-ORF4 (668–1,038aa)*	7
4	80	gi123217725	46,116	Pol protein of human immunodeficiency virus 1	30
	8	Q4U3C3_BMDNV	39,637	BmDNV-Z VD1-ORF4 (463–865aa)*	12
5	179	gi28932924	51,541	structural protein of <i>B. mori</i> densovirus Zhenjiang	58
6	193	gi28932924	55,185	structural protein of <i>B. mori</i> densovirus Zhenjiang	53
7	128	gi189007679	133,876	structural protein of <i>B. mori</i> densovirus Zhenjiang	22

* The local BmDNV-Z, VD1-ORF4 database was used for comparison. This database contains only 4 polypeptide fragments in accordance with 4 functional domains of a protein encoded by VD1-ORF4.

Discussion

The two single-stranded linear DNA molecules (VD1 and VD2) from BmDNV-Z are separately packed into a nonenveloped icosahedral capsid. The capsid proteins play important roles in protecting the genome and also in determining host specificity by interaction between structural proteins and host receptors [13]. In the present study, we found 7 polypeptides in viral particles from feces of the infected *B. mori* larvae. Of them, 3 structural proteins, P5, P6 and P7, constituted the structural proteins of BmDNV-Z viral particles, which was not fully consistent with previous studies.

Structural protein P7 is coded by VD2-ORF1. Hayakawa et al. [14] suggested that VD2-ORF1 coded the largest viral structural protein. They expressed the polypeptides encoded by C-terminal 600 nucleotides of VD2-ORF1, and the product could be recognized by the anti-serum against BmDNV-2 virions, which is consistent with our finding. In our study, we found that P7 was coded by the entire sequence of VD2-ORF1, as shown from the MALDI-TOF/mass spectrometry analysis, and demonstrated that VD2-ORF1 is the gene that encodes the largest structural protein of BmDNV-Z for the first time.

Structural proteins P5 and P6 were encoded by VD1-ORF3 of the viral genome. Our results from Western blot analysis showed that P1 to P7 could react with antibody against viral particles, suggesting that P1 to P7 were components of the viral particles. The result of P5 and P6 being able to react with the antibody against VP indicated that P5 and P6 were encoded by VD1-ORF3 and are the major structural proteins of BmDNV-Z. According to the matched peptides of P5 and P6 (fig. 5), we speculated that P6 and P5 were coded from the first and second methionine of VD1-ORF3, respectively. Indeed, the observation that several structural proteins translate from one ORF is very common in parvoviruses. For instance, VPs of densovirus of *Galleria mellonella*, *Junonia coenia* and *Mythimna loreyi* are all translated from one ORF by a leaky scanning mechanism [15–17].

P4 might be encoded by VD1-ORF4 of BmDNV-Z which shows high sequence identity with VD1-ORF1 of BmDNV-2. In 2000, Hayakawa et al. [14] speculated that amino acids 329–1,115 of the structural protein encoded by VD1-ORF1 of BmDNV-2 contained conserved sequences among various DNA polymerases. They also expressed the 1,000 nucleotides of the N-terminus of VD1-ORF1 and showed that the anti-BmDNV-2 serum recognized the N-terminus of VD1-ORF1. In our study, P4 could be recognized by the antiserum against BmDNV-

Z. Mascot searches of the protein database initially identified P4 as a HIV-1 DNA polymerase protein. This protein has a very high sequence identity with the DNA polymerase encoded by VD1-ORF4. A comparison with sequences in the self-defined database showed that the coverage between P4 and the VD1-ORF4 DNA polymerase segment (amino acids 463–865) reached 12%. Therefore, we speculated that P4 might be the spliced product of the protein encoded by VD1-ORF4. However, P4 might not be the viral structural protein but another protein that had been packaged into the virus and had other roles. An examination of the polypeptide obtained by Hayakawa et al. [14] from the expression of the N-terminal 1,000 nucleotides of BmDNV-2 VD1-ORF1 indicated that the polypeptide's C-terminus is the basic region with an isoelectric point of around 8.16. We speculated that this basic region had a similar function as histone in eukaryotic chromosomal organization and therefore was the viral nucleoprotein. The composition of BmDNV particles is different from the parvovirus particles. The parvovirus particles contain polyamines such as spermine and spermidine. Therefore, further in-depth researches are required to confirm whether P4 protein is a viral nucleoprotein.

P1 and P2 were identified as the triacylglycerol lipase of *B. mori*. During primary infection, the virus must have the essential protein to start infection and cause host change for further infection. The host cell-derived protein components of viruses can subsequently serve diverse functions during the viral life cycle. The roles of these host-derived proteins have been receiving increasing attention, specifically in connection with HIV infection and the development of AIDS in humans [18]. P1 and P2 might be the proteins being packaged into the virus to play a role in primary infection. Further research into P1 and P2 is being undertaken in our laboratory to investigate the function of the triacylglycerol lipase in virus infection.

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References

- 1 Bando H, Choi H, Ito Y, Nakagaki M, Kawase S: Structural analysis on the single-stranded genomic DNAs of the virus newly isolated from silkworm: the DNA molecules share a common terminal sequence. *Arch Virol* 1992;124:187–193.
- 2 Ito K, Kidokoro K, Sezutsu H, Nohata J, Yamamoto K, Kobayashi I, Uchino K, Kalyebi A, Eguchi R, Hara W, Tamura T, Katsuma S, Shimada T, Mita K, Kadono-Okuda K: Deletion of a gene encoding an amino acid transporter in the midgut membrane causes resistance to a *Bombyx* parvo-like virus. *Proc Natl Acad Sci USA* 2008;105:7523–7527.
- 3 Kurihara Y, Watanabe H, Maeda S, Shimizu T: Chemical characteristics of a previously undescribed densovirus isolated from the silkworm, *Bombyx mori*. *J Sericult Sci Jpn* 1984;53:33–40.
- 4 Balsinde J, Balboa MA, Insel PA, Dennis EA: Regulation and inhibition of phospholipase A2. *Annu Rev Pharmacol Toxicol* 1999;39:175–189.
- 5 Dennis EA: The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends Biochem Sci* 1997;22:1–2.
- 6 Kramer RM, Sharp JD: Structure, function and regulation of Ca²⁺-sensitive cytosolic phospholipase A2 (cPLA2). *FEBS Lett* 1997;410:49–53.
- 7 Kurihara Y, Watanabe H, Maeda S, Shimizu T: Chemical characteristics of a previously undescribed densovirus isolated from the silkworm, *Bombyx mori*. *J Sericult Sci Jpn* 1984;53:33–40.
- 8 Sotoshiro H, Kobayashi M: Identification of viral structural polypeptides in the midgut and feces of the silkworm, *Bombyx mori*, infected with *Bombyx* densovirus type 2. *J Invertebr Pathol* 1995;66:60–67.
- 9 Wang YJ, Yao Q, Chen KP, Wang Y, Lu J, Han X: Characterization of the genome structure of *Bombyx mori* densovirus (China isolate). *Virus Genes* 2007;35:103–108.
- 10 Mirza UA, Liu YH, Tang JT, Porter F, Bondoc L: Extraction and characterization of adenovirus proteins from sodium dodecylsulfate polyacrylamide gel electrophoresis by matrix-assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 2000;11:356–361.
- 11 Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* 1979;76:4350–4354.
- 12 Buennette WN: 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981;112:195–203.
- 13 Gardiner EM, Tattersall P: Evidence that developmentally regulated control of gene expression by a parvoviral allotropic determinant is particle mediated. *J Virol* 1988;62:1713–1722.
- 14 Hayakawa T, Kojima K, Nonaka K, Nakagaki M, Sahara K, Asano S, Iizuka T, Bando H: Analysis of proteins encoded in the bipartite genome of a new type of parvo-like virus isolated from silkworm – structural protein with DNA polymerase motif. *Virus Res* 2000;66:101–108.
- 15 Tijssen P, Li Y, El-Far M, Szelei J, Letarte M, Zadori Z: Organization and expression strategy of the ambisense genome of densovirus of *Galleria mellonella*. *J Virol* 2003;77:10357–10365.
- 16 Bossin H, Fournier P, Royer C, Barry P, Cerutti P, Gimenez S, Couble P, Bergoin M: *Juonia coenia* densovirus-based vectors for stable transgene expression in sf9 cells: influence of the densovirus sequences on genomic integration. *J Virol* 2003;77:11060–11071.
- 17 Fediere G, El-Far M, Li Y, Bergoin M, Tijssen P: Expression strategy of densovirus virus from *Mythimna loreyi*. *Virology* 2004;320:181–189.
- 18 Liu XY, Chen KP, Cai KY, Yao Q: Determination of protein composition and host-derived proteins of *Bombyx mori* nucleopolyhedrovirus by 2-dimensional electrophoresis and mass spectrometry. *Intervirology* 2008;51:369–376.