Identification of Nonviral Infection-Specific Polypeptides in the Midgut of the Silkworm, Bombyx mori, Infected with B. mori Densovirus Type 2

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Two unique polypeptides with apparent molecular weights (MWs) of 45,500 (45.5K) and 44K were identified in the purified BmDNV-2 preparation from the pupal midgut of the silkworm, Bombyx mori, that had previously been infected with B. mori densovirus type 2 (BmDNV-2) at a larval stage. Immunoblot analysis and peptide mapping showed that both 45.5K and 44K polypeptides were serologically and structurally distinct from the structural polypeptides of BmDNV-2, suggesting that these two polypeptides were not related to the polypeptides for virion structure. Comparison of directly determined NH2-terminal amino acid sequences of 45.5K and 44K polypeptides with those deduced from the nucleotide sequences of BmDNV-2 genomic DNAs implied that they were not coded by viral genome. Based on the fact that 45.5K and 44K polypeptides were related serologically and structurally but distinct in their NH2-terminal amino acid sequences, 45.5K polypeptide seemed to contain entire or nearly entire amino acid sequence of 44K polypeptide and a unique NH2-terminal amino acid extension. The 45.5K and 44K polypeptides were found only in the midgut of BmDNV-2-infected silkworm, and were not detected in fat body, Malpighian tubule, ovary and hemolymph of the same infected insects. In the BmDNV-2-infected midgut, conspicuous accumulation of these two polypeptides is achieved only at two specific times, one being 2-3 days postinoculation and the other at larval-pupal transformation onward. These results suggest that 45.5K and 44K polypeptides are involved in the degeneration and discharge of virus-infected midgut cells.

Key words: Silkworm, Bombyx mori, B. mori densovirus type 2, infection specific polypeptides, midgut

INTRODUCTION

Bombyx mori densovirus type 2 (BmDNV-2) is an autonomously replicating insect virus within the family Paroviridae (cf., Watanabe et al., 1986). This virus contains two linear, single-stranded DNA molecules of about 6.6 and 6.1 kilobases (kb) that are encapsidated within an icosahedral protein capsid of about 24 nm in diameter (Kawase et al., 1984; Kurihara et al., 1984; Bando et al., 1992, 1995). The protein capsid consists of six major polypeptides with apparent molecular weights (MWs) of 120,000 (120K), 118K, 53K, 51K, 49K and 46.5K (Kawase et al., 1984; Sotoshiro and Kobayashi, 1995). Such molecular features of BmDNV-2 differ from that of any other virus species from the subfamily Densovirinae within the family Paroviridae, in which four structural polypeptides encapsidate a single molecule of linear, single-stranded DNA of 4-6 kb in length (cf., Murphy et al., 1995).

Unlike the majority of virus species from the subfamily Densovirinae, BmDNV-2 is monorganotropic and multiplies preferentially in columnar cells of the midgut epithelium of infected larvae (Seki and Iwashita, 1983). Following the infection with BmDNV-2, cells in the midgut epithelium increase in number to cause the epithelium to fold (Watanabe and Kurihara, 1988), and nuclei of columnar cells become hypertrophied which make them strongly positive to methylgreen and Feulgen reactions, probably due to the accumulation of BmDNV-2 DNA (Seki and Iwashita, 1983; Iwashita et al., 1994). At the late stage of infection, infected columnar cells degenerate into gut lumen and, complete virions in the degenerate infected cells are discharged through feces (Seki and Kawase, 1985). Light microscopy has shown no evidence for BmDNV-2 multiplication in the epithelial cells of pupal midgut that are regenerated during larval-pupal transformation (Iwashita et al., 1994).

BmDNV-2 infection in the silkworm larvae has been shown to be chronic (Kobayashi et al., 1986; Watanabe and Kurihara, 1988), and most of the larvae infected at fifth instar survive to pupae and adults without any severe external symptoms (Sotoshiro et al., 1996). The larvae infected with BmDNV-2 at the final instar thus provides an opportunity to analyze intracellular biological events involved in virus re-
plication in the animals with different developmental physiology. By employing this system, previous studies have shown that relative proportion of individual viral structural polypeptides in the BmDNV-2-infected midgut does not change significantly during larval-pupal-adult development, which contrast the situation in many other parvovirus systems (cf., Cotmore and Tattersall, 1987), including Galleria mellonella DNV-infected G. mellonella larvae (Tijssen and Kurstak, 1981). Previous studies have also shown that relative proportion of individual structural polypeptides in the BmDNV-2 virions purified from whole larvae is variable from preparation to preparation and differs from that in the BmDNV-2-infected midgut (Sotoshiro and Kobayashi, 1995; Sotoshiro et al., 1996), suggesting that one or more of the viral structural polypeptides is generated by proteolytic cleavage of primary translation products.

To help understand molecular mechanisms for the generation of viral structural polypeptides, we have made an attempt to isolate purified BmDNV-2 virions from pupal midgut which had previously been inoculated with BmDNV-2 at a larval stage, and to compare their structural polypeptides with those of purified virions from infected whole larvae. During the electrophoretic analysis of polypeptides in the purified virion preparations from the pupal midgut, we found two previously unidentified polypeptides which were serologically and structurally distinct from BmDNV-2 structural polypeptides (Sotoshiro et al., 1997). In this paper, we describe that these two polypeptides are detectable exclusively in the infected midgut and only at specific times during virus infection and development of the infected silkworm.

**MATERIALS AND METHODS**

**Virus, animal and virus inoculation**

BmDNV-2 isolated by Seki and Iwashita (1983) was used. The silkworm races used were F1 hybrids between Shunrei and Shogestu, and Kinshu and Showa. Silkworm larvae were aseptically reared at 25°C on an artificial diet and day-1 fifth-instar larvae were inoculated per os with BmDNV-2 for 24 h at 25°C as described previously (Kobayashi et al., 1988; Sotoshiro et al., 1996). The infected larvae were reared aseptically on an artificial diet, or conventionally on mulberry leaves. The infected larvae which fed on mulberry leaves were used only for virus purification from the pupal midgut. The larvae survived and metamorphosed to pupae and adults were kept at 25°C.

**Virus purification**

Purification of BmDNV-2 was carried out essentially as described previously (Nakagaki and Kawase, 1980; Choi et al., 1990). The purification procedure consisted of repeated differential centrifugations, fluorocarbon (1, 1, 2-trichlorotrifluoroethane) treatment, ammonium sulfate precipitation, centrifugation on a 40% (w/w) sucrose cushion, and 10-40% (w/v) continuous sucrose density gradient centrifugation. About 30 g of whole midgut from BmDNV-2-infected, day-5 pupae at 16 days postinoculation (pi) was employed for the virus purification.

**Immunoblot analysis**

Immunoblot analysis was performed according to the method of Choi et al. (1989). The MWs of polypeptides on the membrane was estimated by comparing their mobilities with those of marker polypeptides (Kaleidoscope Prestained Standards, Bio-Rad Laboratories, CA). Procedure for the preparation of anti-BmDNV-2 antiserum has been described previously (Kobayashi and Choi, 1990). Antisera against 45.5K and 44K polypeptides were prepared by immunizing the mice with 45.5K and 44K polypeptides purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), essentially as described previously (Ikeda et al., 1991).

**Peptide mapping by limited proteolysis**

The 45.5K and 44K polypeptides, and 53K BmDNV-2 structural polypeptide were resolved on a 10.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. The gel pieces containing each polypeptide were excised from the gel and were equilibrated in 50 mM Tris-HCl, pH 6.8. The polypeptides on the gel were digested with appropriate amount of *Staphylococcus aureus* V8 protease (ICN ImmunoBiologics, IL) as described previously (Kobayashi and Inagaki, 1989; Choi et al., 1992). The digested products on the gel were visualized by silver staining using 2D-silver stain “Daiichi” (Daiichi Pure Chemicals, Tokyo, Japan).

**SDS-PAGE**

Midgut, Malpighian tubule, fat body and ovary of BmDNV-2-infected pupae at 17 days pi were dissected out and these tissues except for hemolymph were homogenized in three volumes (v/w) of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Hemolymph with hemocytes was homogenized without added TE buffer. The infected tissues and purified virion preparations were mixed with the same volume of 2 x gel-loading buffer (gel-loading buffer: 62 mM Tris-HCl,
pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled for 5 min, and stored at -20°C until used. SDS-PAGE was performed according to the method of Laemmli (1970). The MWs of polypeptides on the gel were estimated using low-molecular-weight calibration kit (Pharmacia, Uppsala, Sweden).

NH₂-Terminal amino acid sequencing
The 45.5K and 44K polypeptides were resolved by SDS-PAGE, and blotted onto a polyvinylidene difluorido membrane (ProBlot; Applied Biosystems, CA). The NH₂-terminal amino acid sequences of the polypeptides on the membrane were determined by automated Edman degradation (Edman and Begg, 1967) using Model 476A sequencer equipped with Model 610A data analysis system (Applied Biosystems, CA).

Electron microscopy
The virus preparations at a concentration of 1.5 A₂₆₀, which were finally purified by 10-40% (w/v) sucrose density gradient centrifugation following 40% (w/w) sucrose cushion centrifugation, were negatively stained with 2% phosphotungstic acid, pH 7.2, and were observed under an electron microscope. The diameter of purified virion preparation was estimated by comparing it with that of tobacco mosaic virus, whereby the diameter is 18 nm.

Immunohistochemistry
The whole body of BmDNV-2-infected and uninfected larvae was immersion-fixed for 2 h at room temperature in 4% paraformaldehyde in PBS (0.775% NaCl, 0.15% K₂HPO₄, 0.02% KH₂PO₄, pH 7.6) containing 0.5% glutaraldehyde and 0.2% picric acid, and dissected midgut was fixed for 20 h at 4°C in 4% paraformaldehyde in PBS containing 0.5% glutaraldehyde. The fixed midgut was dehydrated in increasing ethanol series, embedded in paraffin, and cut into 8 μm-sections. Following the removal of paraffin in xylene and rehydration in 90% ethanol, the sections were treated with 3% H₂O₂ for 20 min to block the staining of endogenous peroxidase and then with 10% preimmune goat serum in PBS to block nonspecific immunoreactions. The sections were incubated sequentially in anti-45.5K polypeptide antiserum (1: 1,000 in PBS containing 3% bovine serum albumin) overnight at 4°C and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1: 2,000) for 2 h at room temperature. After a wash in PBS, the HRP-conjugated antibody complex was visualized in a solution of dianiminobenzidine (DAB) as a coloring reagent.

RESULTS
Polypeptide analysis of purified BmDNV-2 virion preparation from infected pupal midgut
Day-1 fifth-instar larvae were inoculated with BmDNV-2, and infected larvae were reared on mulberry leaves. At 16 days postinoculation (pi) (day-5 pupae), infected pupal midguts were dissected out and processed for virus purification. The virus purification procedure established for whole larvae infected with BmDNVs (Nakagaki and Kawase, 1980; Choi et al., 1990), was applied to the BmDNV-2-infected pupal midgut, and virus particles were finally isolated by sucrose density gradient centrifugation. The highest A₂₆₀ peak on the sucrose density gradient was designated as peaks 1 (data not shown).

Polypeptides from peak 1 was analyzed by SDS-PAGE (Fig. 1). It contained two major polypeptides with MWs of 45.5K and 44K and several minor polypeptides with lower and higher MWs. The relative amount of 45.5K and 44K polypeptides was approximately the same, whereas the relative amount of minor polypeptides varied with each other. The MWs of three of the minor polypeptides were identical to the MWs of three of the six BmDNV-2 structural polypeptides (53K, 49K, and 46.5K) previously identified in the BmDNV-2 virions purified from infected larvae (Kawase et al., 1984; Sotoshiro and Kobayashi, 1995), indicating that peak 1 contained small but significant amount of BmDNV-2 virions.

Serological characteristic of 45.5K and 44K polypeptides
Polypeptides corresponding to 45.5K and 44K polypeptides detected in the present study have not been identified in BmDNV-2 virions purified from infected larvae (Kawase et al., 1984; Sotoshiro and Kobayashi, 1995). To see whether 45.5K and 44K polypeptides are related to viral structural polypeptides, polypeptides in peaks 1 were analyzed by immunoblotting using antisera against purified BmDNV-2 virions (Fig. 2). The immunoblot showed that BmDNV-2 structural polypeptides were not detected with antiserum against each of the 45.5K and 44K polypeptides. Fig. 3 also showed that anti-45.5K polypeptide antiser-
um reacted not only with 45.5K polypeptide but also with 44K polypeptide, and anti-44K polypeptide antiserum reacted with 45.5K polypeptide as well as with 44K polypeptide, indicating that the 45.5K and 44K polypeptides were serologically related. Another immunoblotting with purified BmDNV-2 virions showed that none of the six BmDNV-2 structural polypeptides reacted with antisera against 45.5K and 44K polypeptides (data not shown), indicating that 45.5K and 44K polypeptides were not related to BmDNV-2 structural polypeptides.

Electron microscopy of structural entity in peak 1

Structural entities in peak 1 were stained negatively with phosphotungstic acid and observed under an electron microscope. As shown in Fig. 4, particles in peak 1 were somewhat heterogenous in shape and size, and had two types of morphology. The smaller particles (21-23 nm in diameter) were solid and had a spherical shape with rigid appearance. The morphology of the smaller particles coincided with that of BmDNV-2 virions, although the diameter of present estimation was somewhat smaller than those estimated by Kawase et al. (1984) and Kurihara et al. (1984). The larger particles (28-32 nm in diameter) were also spherical in shape but had an appearance of deformability and flexibility. The size of the larger particles was roughly comparable to that of IFV, a B. mori picornavirus, but unlike IFV (cf., Ayuzawa, 1972; Kawase et al., 1980), the surface of larger particles was mottled and fuzzy. Larger particles were observed more frequently than smaller ones, and the ratio of larger particles estimated on electron micrographs was 62.9% (438 larger vs 258 smaller particles in a given area).
Peptide mapping of 45.5K and 44K polypeptides by limited proteolysis

Peptide mapping was performed by limited proteolysis with *S. aureus* V8 protease to analyze structural relationship between 45.5K and 44K polypeptides, and between these two polypeptides and BmDNV-2 structural polypeptides. As shown in Fig. 5, over twenty digests were detected in common for 45.5K and 44K polypeptides when digested with 2 and 10 ng of *S. aureus* V8 protease, while the pattern of digests of 45.5K and 44K polypeptides differed completely from those of 53K BmDNV-2 structural polypeptide. These results indicated that 45.5K and 44K polypeptides shared a common primary amino acid sequence, while these two polypeptides were not structurally related to any of the four smaller BmDNV-2 structural polypeptides (53K, 51K, 49K, and 46.5K) which shared a common primary amino acid sequence to each other (cf., Sotoshiro and Kobayashi, 1995).

NH₂-terminal amino acid sequence of 45.5K and 44K polypeptides

To see whether 45.5K and 44K polypeptides are derived from BmDNV-2 genome, NH₂-terminal amino acid sequences of these polypeptides were determined. The given sequences were NH₂-W(S)-A-N-R-S-R-P-I-V-R(N)-I-A-A-R--- for 45.5K polypeptide and NH₂-T(W)-L-K-S-I-L-R-P-I-L-N-E-L-A-G--- for 44K polypeptide. These amino acid sequences were not conclusive, yet it was evident from the present data that 45.5K and 44K polypeptides retained distinct NH₂-terminal amino acid sequences. It also became clear that NH₂-terminal amino acid sequences of 45.5K and 44K polypeptides were not coded by BmDNV-2 genomic DNAs, when these NH₂-terminal amino acid sequences were compared with the amino acid sequences deduced from nucleotide sequence of BmDNV-2 genome (Bando et al. 1995; Nakagaki, personal communication).

Association of 45.5K and 44K polypeptides with infected midgut

To examine whether 45.5K and 44K polypeptides accumulate in the tissues of BmDNV-2-infected silkworm, midgut, Malpighian tubule, fat body, ovary, and remaining carcass were each dissected out from the infected pupae at 18 days pi (day-6 pupae) and polypeptides were analyzed by immunoblotting using a 1:1 mixture of antisera against the 45.5K and 44K polypeptides. The immunoblot showed that 45.5K and 44K polypeptides and their presumed degradation products were present in the midgut, but they were not detected in any of other tissues examined (Fig. 6). These polypeptides were also absent in the hemolymph from the same BmDNV-2-infected pupae (data not shown).
As shown in Fig. 7A, both 45.5K and 44K polypeptides were first detected clearly in the midgut epithelium at 3 days pi and declined quickly to an undetectable level at 4 days pi. These two polypeptides again appeared clearly at 10 days pi (1 day before the larval-pupal ecdysis) in both midgut epithelium and contents and persisted throughout the experiment (Figs. 7A, B).

The data in Fig. 7 showed that the accumulation of 45.5K and 44K polypeptides in BmDNV-2-infected midgut occurred first transiently at 3 days pi and then persistently from larval-pupal transformation onward, regardless of the larval ages of virus inoculation.

**Immunohistochemical localization of 45.5K and 44K polypeptides in infected midgut**

Day-1 fifth-instar larvae were inoculated with BmDNV-2 and immunohistochemical analysis with anti-45.5K polypeptide antiserum was carried out to localize 45.5K and 44K polypeptides in the infected midgut. The result showed that BmDNV-2-infected midgut displayed a clear immunoreaction at 3 days pi and at larval-pupal transformation onward. The immunoreaction was not detected in uninfected midgut incubated with anti-45.5K polypeptide antiserum nor in BmDNV-2-infected midgut incubated with preimmune mouse serum as a primary antibody, indicating the immunoreaction displayed in the BmDNV-2-infected midgut due to 45.5K and 44K polypeptides. At 3 days pi, the immunoreaction was only clear in a few number of columnar cells in the infected midgut epithelium, while no immunoreaction was detected in goblet cells (data not shown). At early stage of larval-pupal transformation (day-9 fifth-instar larvae, 8 days pi), the immunoreaction was localized in vacuoles and inclusion bodies (Figs. 9A, B), that probably corresponded to those appearing characteristically in the midgut epithelium of infected larvae.
Infection-Specific Protein in BmDNV-2-Infected Silkworm

At later stage of larval-pupal transformation (day-0 pupae, 11 days pi), many immunoreactive cell debris were found in the midgut contents (Fig. 9C), indicating that the BmDNV-2-infected cells, that degenerated and were discharged into midgut lumen, still retained the immunoreactivity. No immunoreaction was detected in the newly regenerated midgut cells of infected day-9 fifth-instar larvae (8 days pi), while significant numbers of regenerated midgut cells of infected day-0 pupae (11 days pi) displayed a clear immunoreaction (data not shown). These results were consistent with the results from immunoblot analysis (cf., Fig. 7).

**DISCUSSION**

In the present study, we have identified two unique polypeptides with MWs of 45.5K and 44K that accumulate in the midgut of the silkworm infected with BmDNV-2. These two polypeptides have not been identified in the virions of BmDNV-2 purified from infected larvae, and the present study by immunoblot analysis and peptide mapping demonstrates that they are serologically and structurally distinct from the structural polypeptides of BmDNV-2 (Figs. 2, 3, 5), indicating that 45.5K and 44K polypeptides are not related to the polypeptides for virion structure. The possibility that these polypeptides are viral non-structural polypeptides might be eliminated due to a relatively large amount of accumulation in the infected midgut. In addition, preliminary comparison of directly determined NH₂-terminal amino acid sequences of 45.5K and 44K polypeptides with the amino acid sequences of open reading frames deduced from nucleotide sequences of 6.1 and 6.6 kb genomic DNAs (Bando et al., 1992, 1995) suggests that the genes of these two polypeptides are not located in the BmDNV-2 genome. It is thus reasonable to conclude that 45.5K and 44K polypeptides are coded by the host cell genome rather than by the viral genome. In support of this tentative conclusion, 45.5K and 44K polypeptides also accumulate in the midgut infected with IFV, a picornavirus of B. mori (data not shown). This, in turn, suggests that the infection of different types of cells with different species of viruses results in the production of common 45.5K and 44K polypeptides, since the BmDNV-2 infects preferentially the columnar cells (Seki and Iwashita, 1983) whereas IFV infects exclusively the goblet cells of the larval midgut (Iwashita, 1965; Inoue, 1972).

When day-1 fifth-instar larvae are inoculated with BmDNV-2, the accumulation of 45.5K and 44K...
polypeptides in the BmDNV-2-infected midgut is clearly detected first transiently at 3 days pi and secondarily persistently at 9-10 days pi onward (Figs. 7, 8). An experiment in which the larvae are inoculated at day-1, -3, and -5 of fifth instar further shows that the time of the first transient accumulation of 45.5K and 44K polypeptides is fixed at 2-3 days pi that corresponds to the time when severe cytopathology is first detected clearly in the infected midgut (Watanabe and Kurihara, 1988; Iwashita et al., 1994), whereas onset of the second persistent accumulation temporally matches onset of larval-pupal transformation, regardless of the larval ages of BmDNV-2 inoculation (Fig. 8). These results indicate that the conspicuous accumulation and expression of 45.5K and 44K polypeptides in BmDNV-2-infected midgut is achieved only at specific times in response to virus infection and/or larval-pupal development.

We have anticipated that the expression and accumulation of 45.5K and 44K polypeptides at two specific times can be associated with common cellular events in the BmDNV-2-infected midgut that occur readily during larval-pupal transformation. In the BmDNV-2-infected midgut, two distinct pathological changes are evident at the cellular level (Watanabe and Kurihara, 1988; Iwashita et al., 1994). Infected columnar cells exhibit extensive hypertrophy of the nuclei and the cells with such cytopathology ultimately degenerate and are discharged into gut lumen, whereas a number of cells are newly regenerated, causing the replacement of infected cells by newly regenerated cells. Such cell replacement in the BmDNV-2-infected midgut occurs drastically during larval-pupal transformation and, to a lesser extent, during larval development, suggesting the expression and accumulation of 45.5K and 44K polypeptides in infected cells being related either to the regeneration or to the degeneration and discharge of midgut cells. Our immunohistochemical observation indicates that the degeneration and discharge of the infected cells are more likely to be associated with the expression and accumulation of these two polypeptides (Fig. 9), although the precise nature of the regulation mechanism awaits further investigation. The reason why the first transient expression and accumulation of these two polypeptides are confined to 2-3 days pi and last only one or two days, is not known. Also, it is not known whether the observed persistence of 45.5K and 44K polypeptides throughout the pupal-adult development in both the epithelium and contents of the BmDNV-2-infected midgut is due to continuous expression of the polypeptides or due to high stability of the polypeptides synthesized transiently at the larval-pupal transformation. Finally, it should be noted that the expression and accumulation of 45.5K and 44K polypeptides are infected-cell specific, and cannot be detected in the midgut cells of the uninfected silkworm, in spite of the fact that during larval-pupal transformation, viral midgut cells of the uninfected silkworm undergo apoptotic form of cell death to be discharged into gut lumen and replaced by newly regenerated cells that form pupal midgut epithelium (Tsujita, 1943; Waku and Sumimoto, 1971; Kobayashi and Kawase, 1976; Kobayashi et al., 1978).

Detailed molecular mechanism for the generation of 45.5K and 44K polypeptides remains to be elucidated. Immunoblot analysis and peptide mapping in the present study demonstrate that these two polypeptides are related both serologically and structurally to each other (Figs. 4, 5), suggesting that the two polypeptides are gene products with overlapping amino acid sequences. Further characterization of these polypeptides indicates that NH2-terminal amino acid sequences are distinct between 45.5K and 44K polypeptides. These results suggest that 45.5K polypeptide contains entire or nearly entire amino acid sequence of 44K polypeptide and possesses a unique NH2-terminal extension, which probably accounts, at least in part, for the observed differences in MWs on the SDS-polyacrylamide gels. It is not known whether possible posttranslational modifications such as phosphorylation and glycosylation are also responsible for the difference in MWs between 45.5K and 44K polypeptides.

We have originally found 45.5K and 44K polypeptides in the purified BmDNV-2 preparation from pupal midgut of the silkworm previously inoculated with BmDNV-2 at a larval stage. On a sucrose density gradient, majority of the two polypeptides are copurified with BmDNV-2 virions in our purification procedure (Figs. 1, 3). Considering their large amount, as compared with that of BmDNV-2 structural polypeptides, in the purified BmDNV-2 preparation (Fig. 1), the co-existence of the two polypeptides with BmDNV-2 virions does not necessarily indicate that 45.5K and 44K polypeptides are directly associated with or are incorporated into the BmDNV-2 virions. The possibility that such a large amount of these two polypeptides are present in the purified BmDNV-2 preparation as soluble impurities which are not associated with any structural entities might be excluded, since our purification procedure employed in the present study includes the centrifugation on a 40% sucrose cushion prior to the sucrose density gradient centrifugation, which has been proved to be effective to eliminate soluble impurities (Nakagaki and Kawase, 1980). It is thus possible that 45.5K and 44K polypeptides are...
components of some structure with physical properties similar to those of BmDNV-2 virions on the sucrose density gradient.

Our results provide no direct evidence concerning the structural entity that comprises 45.5K and 44K polypeptides, but several lines of circumstantial evidence derived from the electron microscopy and other analyses suggest that 45.5K and 44K polypeptides might constitute particles of approximately 28-32 nm in diameter (Fig. 4). First, these particles are distinctively different in size from the BmDNV-2 virions with the estimated diameter of approximately 24 nm (cf., Kawase et al., 1984: Kurihara et al., 1984) Second, although the diameter of these larger particles is roughly comparable to that of IFV virions of approximately 27 nm in diameter (Ayuzawa, 1972; Himeno et al., 1974; Kawase et al., 1980), no detectable amount of IFV structural polypeptides is present in the purified BmDNV-2 preparation, thus indicating the larger particles being different from IFV virions. Third, these larger particles account for more than 60% of total number of particles, coinciding with a large amount of 45.5K and 44K polypeptides in the purified BmDNV-2 preparation. Further extensive study is required to correlate 45.5K and 44K polypeptides with the larger particles present in the purified BmDNV-2 preparation.

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