Separation and Structure of Components of Nuclear Polyhedrosis Virus of the Silkworm

TOSHIYUKI KHOSAKA, MICHIO HIMENO, AND KONOSHIN ONODERA

Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan

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Morphology of structural components of nuclear polyhedrosis virus (NPV) particles of the silkworm (*Bombyx mori* Linné) was studied by electron microscope using negative staining. NPV particles isolated from polyhedra could be separated into five structural components by centrifugation in sucrose density gradients. The lowest band (band I) was found to consist of thick rod-shaped particles (330 by 80 nm) with knobby surfaces and with occasional protrusion at one end. The second band from the bottom (band II) was shown to consist mainly of slender rod-shaped particles (360 by 60 nm), in which internal structures were visible as a dense mass. Regular striations were also seen on the surface of these particles. By treatment with mercaptoethanol, these particles were drastically damaged, and in some cases the internal substances were partially released, producing empty inner membranes of various degrees of disintegration. In bands III and IV, both empty spherical and empty rod-shaped membranes were present. Band III was rich in empty spherical membranes which were shown to be the outer membranes of thick rod-shaped particles. The empty rod-shaped membranes, the inner membranes, were mainly located in band IV and have cross striations on the surface. It is remarkable that the uppermost band (band V) consisted purely of small spherical particles, somewhat heterogeneous in size and shape (around 20 to 25 nm in diameter), indicating the particles to be the degradation product of the virus particles. Similar particles could also be observed within the empty inner membranes.

Although the structure of nuclear polyhedrosis virus (NPV) of insects has been investigated by a number of workers, and was found to consist of complex rod-shaped particles, the fine structure of the particles still remains obscure. Earlier observations on the shadowed preparations of virus particles isolated from polyhedra by treatment with weak alkali (1, 2, 3) have led to the working model that several spherical subunits with a diameter of about 40 nm were enveloped with a set of membranes (outer and inner membranes) to form rod-shaped particles (4). On the contrary, Krieg (8) observed that the rod-shaped particles have a helical structure similar to that of tobacco mosaic virus and concluded that the subunits do not represent viral developmental stages but artifacts produced by alkaline degradation of rod-shaped particles. Such structural components were also found in negatively stained virus preparations but were interpreted as discs or groups of discs of the inner membranes (5).

Examination of ultrathin sections of polyhedra (3, 9, 10), however, gave no evidence for supporting the existence of a subunit structure in rod-shaped particles. More recently, Kozlov and

Alexeenko (7) observed the central helical core in virus rods treated with chloroform or urea and suggested that the structure of the inner core is similar to threadlike bacteriophage *fd*.

In a previous report (6), it was shown by thin sectioning of tissue infected with NPV of the silkworm that, at the earlier stages of the maturation process, the flexuous rod particles loosely packed with inner membrane first appeared in the cell nucleus and have laterally striated internal structures. These particles were, then, enveloped with the outer membrane to form a compact rigid structure before embedding in polyhedral protein.

In the present study, the fine structure of virus particles isolated from polyhedra was investigated by separating viral structural components by means of sucrose density gradient centrifugation and by examination of each component by electron microscopy.

MATERIALS AND METHODS

Preparation of polyhedra. The sources for preparation of polyhedra were the hemolymph, collected shortly before death, of experimentally diseased fifth instar larvae of the silkworm. The polyhedra were

collected from the hemolymph by settling out for a few days in the cold. The sediment containing polyhedra was placed on and centrifuged through a 50% sucrose layer at $8,000 \times g$ for 15 min at 2 C. As a result, the polyhedra were recovered as a pellet at the bottom of the centrifuge tube, whereas the majority of the impurities remained over the sucrose layer. After washing several times by cycles of suspending in distilled water and centrifugation ($3,000 \times g$, 15 min, 2 C), the polyhedra were further treated with massive doses of lipase and trypsin for 2 hr at room temperature to remove any residual impurities. Finally, the polyhedra were again washed extensively with distilled water and stored as dry powder in the cold.

Isolation of virus. Virus particles were isolated from polyhedra by treatment with Na_2CO_3 solution of various concentrations (from 0.005 M to 0.05 M) containing 0.05 M NaCl for 1 to 3 hr at 5 C. After removal of impurities by low-speed centrifugation, centrifuge. The pelleted virus particles were suspended the suspension was centrifuged for 1 hr at $105,000 \times g$ at 5 C in the RP 30 rotor of a Hitachi 55P-2 ultracentrifuge in ~ 0.067 M phosphate buffer (PB), pH 7.8, and purified by another cycle of low- and high-speed centrifugation.

Sucrose density gradient centrifugation. The purified virus was suspended in a small amount of ~ 0.067 M PB (pH 7.8), and Tween 80 was added to give a final concentration of 0.05%. The suspension was layered onto 30 to 50% gradient of sucrose in the same buffer, and centrifuged for 2 hr at 25,000 rev/min, 5 C, in a swinging bucket rotor. After the run, fractions were collected through a needle by puncturing the bottom of the tube, and measured as absorbance at 260 nm.

Electron microscopy. The structural components thus separated were removed from sucrose either by high-speed centrifugation or by filtration through a short column (1 by 20 cm) of Sephadex G-25, equilibrated with ~ 0.067 M PB, pH 7.2. The pellets obtained in the former case were resuspended in a small volume of the same buffer. Each drop of the samples was mixed on dental wax with one drop of 2% phosphotungstic acid adjusted to pH 6.8, and the specimens were examined immediately in a JEM 7A electron microscope.

RESULTS

As described by Bergold (4), NPV of insects is easily purified, practically free of host materials, because polyhedra can readily be obtained in a very pure form for the following reasons. (i) They are highly characteristic macromolecules several microns in diameter with high density (2), and (ii) although proteinaceous in nature, they are resistant to the usual proteolytic enzymes. In addition, polyhedra consist only of virus particles besides polyhedral protein matrix (3, 10).

Examination of isolated virus particles in the electron microscope revealed the presence of several structural components. To separate these

FIG. 1. Sucrose density gradient profile of NPV structural components of *B. mori*. Virus particles were isolated from polyhedra by treatment with 0.01 M Na_2CO_3 solution containing 0.05 M NaCl for 1 hr at 5 C. A 0.3-ml amount of virus suspension in ~ 0.067 M PB (pH 7.8) with 0.05% Tween 80 was layered onto 4.4 ml of 30 to 50% sucrose gradient and centrifuged at 25,000 rev/min for 2 hr at 5 C in a Hitachi SW 40 rotor. Fractions were collected as described in the text. Sedimentation is from right to left.

components, sucrose density gradient centrifugation was found to be useful. Under the conditions used, virus particles, isolated by dissolving polyhedra with 0.01 M Na_2CO_3 solution containing 0.05 M NaCl for 1 hr, could be separated into five bands in 30 to 50% sucrose density gradients by centrifugation in the SW 40 rotor at 25,000 rev/min for 2 hr at 5 C (Fig. 1).

It is shown in Fig. 2 that the lowest band (band I) is found to consist of thick rod particles, measuring 330 by 80 nm in average size, which have an irregular knobby surface structure and occasionally a terminal protrusion at one end. When enlarged, however, further details could not be obtained, since the staining merely outlined the particles.

Band II (Fig. 3a) consists mainly of slender rod particles, 360 nm long and 60 nm wide. As seen in Fig. 3b, this particle appears to have transverse regular banding on the surface, in which a dense mass of internal structure can also be seen. Of these particles, some were observed to be distorted in shape and some were partially degraded. When treated with 1% mercaptoethanol, the slender rods were drastically damaged, resulting in partial release of the internal substance in some particles and producing empty rod membranes with various degrees of disintegration (Fig. 4).

Bands III and IV were fairly close together so that the two bands could not be easily distinguished. For further separation, these two bands were combined and recentrifuged in 20 to 40% sucrose gradient at 25,000 rev/min for 2 hr at 5 C.

FIG. 2. Virus particles, negatively stained with 2% phosphotungstic acid, from the lowest band (band I) in the gradient. Knobby surfaced rod-shaped particles, 330 by 80 nm in average size, are seen. A terminal protrusion is observed at one end of some particles. $\times 50,000$.

FIG. 3. (a) Virus particles found in band II. These slender rod-shaped particles, about 360 nm long and 60 nm wide, are partially penetrated by the stain and are somewhat elongated longitudinally and distorted in shape. $\times 160,000$. (b) Slender rod-shaped particles. On the surface, regular banding is visible. Note that an internal substance can also be seen inside the particle. $\times 160,000$.

After the run, however, only single a diffuse band appeared in the gradient. When fractionated and measured by optical density at 280 nm, two widely diffused, overlapping bands could be resolved, indicating a considerable size variation of these components. Examination of these bands in the electron microscope revealed that both empty spherical and empty rod-shaped membranes were present.

Band III is rich in empty spherical and fragmented membranes (Fig. 5). Even at higher magnification, further details could not be resolved in these membranes. During examination of band I, such membranes could frequently be seen free from the thick rod particles to give the appearance of slender rod-shaped particles (Fig. 6). The empty spherical membranes are thus considered to be the outer membrane of the thick

FIG. 4. Slender rod-shaped particles treated with 1% mercaptoethanol. Drastic degradation is apparent. One particle is releasing its internal substance, and another one has partially released. $\times 200,000$.

FIG. 5. Membraneous materials found in band III. Empty spherical and fragmentary membranes are observed. $\times 50,000$.

rods. Similar findings have been reported in the isolated virus preparations from some species of NPV polyhedra (2, 7). The empty rod-shaped membranes were mainly located in band IV. On the surface, regular striations are clearly visible (Fig. 7) as can be seen with the slender rod-shaped particles (Fig. 3b). It is noted that the same observation has been made by Kozlov and Alexeenko (7) with NPV of the silkworm. It seems obvious that these membranes are the inner membrane of the thick rod-shaped particles.

The uppermost band (band V) appeared at the sucrose-suspension interface as a sharp band and was found to consist purely of small spherical particles with a diameter of about 20 to 25 nm (Fig. 8). It must be noted that the particles are somewhat heterogeneous in size and shape. When analyzed in a 10 to 40% sucrose density gradient by centrifugation for 3 hr at 25,000 rev./min, these particles behaved as a broad diffuse band, indicating size heterogeneity. It is also noteworthy that within the empty inner membranes found in band IV, similar particles are frequently observed (Fig. 9).

DISCUSSION

NPV of the silkworm was shown to be degraded in a progressive fashion from thick rod particles to subviral structural components by the action of alkali.

The thick rod particles observed in the lowest band (Fig. 2) are apparently identical to those found in ultrathin section of polyhedra (3, 9, 10) and were elucidated to consist of an internal substance enveloped with two layers of membrane, outer and inner membranes (Fig. 3a, 3b, 5, and 7). The outer membrane may serve to retain the compact rigid structure of virus rods, since the slender rods, which had lost the outer membrane, were elongated longitudinally and frequently were distorted in shape during the process of electron microscopy (Fig. 3a). This is supported by the results obtained by thin sectioning of the tissue infected with NPV of the silkworm (6).

It is evident that when slender rods degrade, further breakages may occur preferentially at the end of the particles to release internal substances (Fig. 4) because the structure of the empty inner

FIG. 6. Outer membranes are freeing from thick rod-shaped particles as empty spherical membranes or fragmented membranes. This sample was taken from band I. $\times 50,000$.

FIG. 7. Empty rod, inner membranes from band IV. Regular striations are clearly seen on the surface. $\times 150,000$.

membrane was well preserved except at the ends of the membrane (Fig. 7). Although the internal substance which appeared as a dense mass (Fig. 4) may represent a highly organized structure and is possibly helical in configuration, as suggested

previously (5, 7), no direct evidence could be obtained.

The facts that the small spherical particles which seemed heterogeneous in size and shape (Fig. 8) were consistently found and shown to

FIG. 8. Small spherical particles from the uppermost band (band V). It is remarkable that these particles are somewhat heterogeneous in size and shape. $\times 186,000$.

FIG. 9. Membraneous materials found in band IV. It must be noted that within the empty inner membranes particles similar to those shown in Fig. 8 are observed. $\times 192,000$.

behave as a broadly diffused band in sucrose gradients indicate that the component does not represent any structural unit but is a degradation product of virus particles. Similar particles are frequently observed within the empty inner membrane after various degrees of disintegration. This strongly suggests that the component (Fig. 8) might be the degradation product of the internal substance.

The small spherical viral subunits observed earlier in the shadowed preparation of virus particles (1, 8) may be interpreted as corresponding to those shown in Fig. 8. It is probable that metal shadowing caused misunderstanding of the component as a viral subunit.

The infectivity of each fraction was examined, and it has been found that bands I and II show the activity of NPV. The detailed accounts will be reported elsewhere.

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