Partial characterization of an entomopoxvirus isolated from grasshoppers
by Herbert Carl Kussman

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in MICROBIOLOGY
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Abstract:
Arphia poxvirus (APV), an entomopoxvirus originally isolated from Arphia conspersa, is also infective in Camnula pellucida and Xanthippus corallipes. Infection occurs in fat body of the host causing premature death. Inclusions appear oval to ellipsoid and, when mature, measure approximately 11-13 μm in diameter. Virions are brick-shaped, about 300 nm in width by 400 nm in length, and contain a large double-stranded DNA core surrounded by a triple-layered intimal membrane. The Arphia poxvirus was found to be distinct from two other entomopoxvirus from grasshoppers, the grasshopper inclusion body virus (GIBV) and Phoetaliotes poxvirus (PPV), as determined by differences in host range electrophoretic patterns of viral proteins, and antigen-antibody gel diffusion reactions.

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Date March 1, 1978

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by

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ABSTRACT

Arphia poxvirus (APV), an entomopoxvirus originally isolated from *Arphia conspersa*, is also infective in *Camnula pellucida* and *Xanthippus corallipes*. Infection occurs in fat body of the host causing premature death. Inclusions appear oval to ellipsoid and, when mature, measure approximately 11-13 μm in diameter. Virions are brick-shaped, about 300 nm in width by 400 nm in length, and contain a large double-stranded DNA core surrounded by a triple-layered intimal membrane. The *Arphia* poxvirus was found to be distinct from two other entomopoxvirus from grasshoppers, the grasshopper inclusion body virus (GIBV) and *Phoetaliotes* poxvirus (PPV), as determined by differences in host range electrophoretic patterns of viral proteins, and antigen-antibody gel diffusion reactions.

Chapter 1

INTRODUCTION

According to Bellet et al. (2), there are three major groups of inclusion viruses that cause disease in insects: the baculoviruses, which consist of the nuclear polyhedrosis viruses and the granulosis viruses; the cytoplasmic polyhedrosis viruses; and the entomopoxviruses. Most of the early studies of viral diseases in insects have centered on the nuclear polyhedrosis viruses and the cytoplasmic polyhedrosis viruses. Baculoviruses contain deoxyribonucleic acid (DNA) as genetic material and replication occurs in the nucleus of the host cell. Entomopoxviruses also contain DNA, but replication occurs in the cytoplasm of infected cells. The genetic material of cytoplasmic polyhedrosis virus is ribonucleic acid (RNA) and this virus replicates in the cytoplasm.

Hurpin and Vago (13) isolated the first entomopoxvirus. The original characterizations of this and other entomopoxviruses were based on electron microscopic examinations (3, 10, 24, 26) that provided information on the structure of the inclusion bodies with associated protein matrices, information on the structure of the occluded virions and insight into the development of virions and inclusions (6, 8, 10, 13, 26). More recent investigations established the techniques necessary for the extraction of the virions from the inclusion matrix

in order to determine the chemistry and structure of the virions (1, 16, 19, 25). These studies have established that the virions of entomopoxviruses are brick-shaped, each possessing a central core of DNA surrounded by a limiting membrane consisting of three distinct layers (6, 9). The DNA is a large, complex molecule containing the genes responsible for the production of at least four enzymes involved in replication (23, 19). As additional examples of the general characteristics of entomopoxviruses, Landridge and Roberts (16) have shown by electron microscopy that the DNA molecule of Amsacta poxvirus was 64.5 μm long and Arif (1) established that the bouyant density of the DNA of the Choristoneura entomopoxvirus was 1.6838 gm/ml. As pointed out by Bergoin et al. (3) and McCarthy et al. (20), the entomopoxviruses of insect and the vertebrate poxviruses exhibit many similar characteristics.

The above studies were performed on entomopoxviruses isolated from Lepidoptera (1, 9, 19, 25), Coleoptera (6, 13), and Diptera (4, 7, 26). The first entomopoxvirus reported from grasshoppers (Acrididae: Orthoptera) was isolated from Melanoplus sanguinipes by Henry and Jutila (11), at which time they suggested it to be a nuclear polyhedrosis virus. More recently, Henry et al. (12) established that this virus has the characteristics of an entomopoxvirus, and referred to it as the grasshopper inclusion body virus (GIBV). GIBV has been

subjected to electron microscopic studies, and Landridge and Roberts (15) have reported that the DNA is 62.8 μ long with a molecular wt. of 123.2 X 10^6 daltons. Two similar entomopoxviruses have been isolated from different species of grasshoppers; one from Arphia conspersa has been labelled as the Arphia poxvirus (APV), and a second from Phoetaliotes nebrascensis called the Phoetaliotes poxvirus (PPV). Except for some preliminary electron microscope work, comparative studies have not been completed.

Statement of Objectives

The purposes of this study were:

(1) To further characterize, both biochemically and biophysically, the entomopoxvirus (APV) isolated from A. conspersa.

(2) To determine morphological and chemical relationships between APV and the other entomopoxviruses, GIBV and PPV, isolated from grasshoppers.

Chapter 2

MATERIALS AND METHODS

Experimental Inoculations

The two methods used to inoculate grasshoppers were per os and injection. For per os inoculations, grasshoppers were fed directly a 5 µl drop of a viral suspension that was placed on the mouth parts of third-instar nymphs. Also grasshoppers were inoculated indirectly by placing a 5 µl drop of a viral suspension on a small lettuce disc (5.0 mm diameter). The drop was allowed to dry and the disc was fed to a third instar grasshopper. Grasshoppers were inoculated by injecting approximately 5 µl of a viral suspension using a 0.25 tuberculin syringe with a 27 gauge needle. Injections were made intrathoracically into third-instar grasshoppers. Viral concentrations between 10^1 to 10^3 inclusions per 5 µl were used for all inoculations.

Isolation of Virus from Grasshoppers

Preliminary diagnosis of viral infection was conducted by homogenizing grasshoppers in Thomas tissue homogenizers equipped with teflon pestles and examining the homogenates with a light microscope using phase contrast optics. Homogenates that contained viral inclusions were pooled, passed through two layers of cheesecloth or gauze, and placed in 50 ml centrifuge tubes and centrifuged at 2,500 x *g* for 15 min. The sediment was washed, repelleted (2x), and stored

in distilled water at -10°C until needed.

Purification of Viral Inclusion Bodies by Sucrose Density Gradient Centrifugation (SDG)

Discontinuous sucrose gradients of 60, 55, 50, 45, 40% (weight/volume) were prepared in 16 ml cullulose nitrate centrifuge tubes. Viral preparations from grasshopper tissues were layered onto the top of the gradients and were centrifuged (64,680 x *g*) for 2 h at -5°C in a Beckman L3-40 preparative ultracentrifuge using a SW 27.1 swinging bucket rotor. After centrifugation, the band containing the inclusions was removed using a Pasteur pipette and a propipette and was washed twice with distilled water. This entire centrifugation process was repeated 3 times.

Breakdown of Viral Inclusion Bodies

Density gradient purified preparations containing 10^{10} - 10^{12} viral inclusions were pelleted and resuspended in about 5 ml of a solution of 0.1M sodium carbonate and 0.1M sodium thioglycolate in distilled water, adjusted with 0.3N sodium hydroxide to a pH of 11.5. The preparation was incubated overnight at 27°C, after which time the preparations were placed onto preformed linear sucrose gradients (55, 45, 35, 25, 20% w/v) and centrifuged at 64,680 x *g* for 4 h. At the end of this time, the virions were collected from the sucrose

tubes by inserting a 22 gauge needle, with a 10 cc syringe attached, through the tube and drawing off the virions. The virions were then pelleted at 64,680 x *g* for 30 min. and resuspended in sterile distilled water. Penicillin (1,000 units/tube) was then added, and the preparations were frozen until needed.

Preparations of Materials for Electron Microscopic Examination

Grasshopper fat tissues were fixed in 4% glutaraldehyde in phosphate buffer for 24 h at 4°C, washed in buffer for 14 min., then postfixed in asmium tetroxide (2%) for 2 h at 4°C. Tissues were dehydrated through a graded acetone series (50, 70, 85, 95, 100%), placed in two changes of propylene oxide (100%), placed in two solutions of propylene oxide-epon araldite in a ratio of 2:1 and then 1:1, and embedded in epon araldite. Tissue blocks were hardened in a vacumn oven for 51 h and sectioned on a Reichert OM U2 ultramicrotome. Sections were stained with aqueous uranyl acetate for 3 min. and Reynold's lead citrate for 1 min.

For electron microscopic examination, the virions freed from inculsions by the above method were placed on formvar coated grids and were either negatively stained with 2% phosphotungstic acid for direct examination or shadow casted with platinum at a 3:1 (length to height) ratio for indirect determination of size and structure. All examinations were made with a Zeiss EM-9 microscope.

Photomicrographs from the electron microscope were made on Kodak 4489 Electron Microscope film.

Determination of Total Protein of Inclusions and Virions

Suspensions of 6.56×10^6, 3.05×10^6, 1.53×10^6, and 7.55×10^5 inclusions per ml were prepared and checked by direct counts using a Neubauer hemacytometer. One ml of each preparation was centrifuged, the water decanted, and the pellet dissolved in 0.5 ml of 1N NaOH overnight in cellulose nitrate tubes. Protein determinations were then carried out using a modification of the Lowry method (14, 21). Standard curves were obtained using 13 concentrations of bovine serum albumin, ranging from 600 µg per 0.5 ml to 5 µg per 0.5 ml, and a blank prepared in 1N NaOH. These were prepared by first dissolving crystalized bovine serum albumin in a small amount of distilled water and adjusting to volume with 1N NaOH. Ten absorbance readings were made for each dilution of the standard and these were averaged and plotted on normal graph paper. The logs of both concentration and absorbance reading averages were also determined and plotted on 2 X 2 cycle log graph paper. These results were used as linear correlation models, from which an equation was derived using a Wang calculator, thus allowing determination of the protein concentrations of inclusions without graphing error. In the established equation $X = (\text{Log } Y - 4.352624)/.73098732$, X equals the protein concentration and Y is the absorbance

reading. Eleven absorbance readings were obtained for each viral inclusion concentration from which the highest and lowest were deleted and the average was based on the remaining 9 readings. Using these averages as the values of Y, the protein concentrations (X) are then determined, after which the total protein per inclusion sample was assessed according to a 10^3 inclusion baseline. Total protein in preparations of virions released from similar concentrations of inclusions by the method reported above also were determined by the modified Lowry technique. The estimate of total protein in inclusion preparations and in viral preparations were compared to estimate percentage of the total protein in inclusions and in virions.

Electrophoresis of Proteins

Viral inclusions, purified by density gradient centrifugation, were suspended in 0.1M sodium carbonate and 0.1M sodium thioglycolate (pH 11.5) overnight at 25°C, after centrifugation at 2,500 x *g* for 5 min. to remove undissolved inclusions and fragments, the supernatant was decanted and precipitated by the addition of 2 to 3 volumes of 10% trichloroacetic acid. The precipitate was pelleted by centrifugation at 900 x *g* for 5 min., the clear supernatant decanted, and washed once with 5% trichloroacetic acid and twice with 100% acetone. The precipitate was then dissolved in a mixture of 3 ml of 1% sodium dodecyl sulphate (SDS), 3 ml spacer gell buffer (1:8 dilution), 0.1 ml

of 2-mercaptoethanol, and 2 ml of 10 µl/l bromphenol purple solution. The solution was brought to 10% with respect to sucrose (40%) and heated to 100°C in a water bath until the precipitate dissolved completely.

Some viral inclusions were placed into 10% SDS overnight. Sucrose was added to a final concentration of 10% along with bromphenol blue as a marker (18). The solutions were layered into troughs cut in 5% polyacrylamide gel as a spacer gel and electrophoresed through 13% polyacrylamide gel at 25 milliamps for 4 h. The gels were stained in 25% Coomassie blue in 9% acetic acid, 50% methanol for 2 h, destained in 9% acetic acid in 5% methanol overnight, and examined by locating the blue staining areas. Gels were stored in 7.5% acetic acid in 5% methanol.

Preparation and Collection of Immune Sera

Sera containing antibodies to Arphia poxvirus inclusions (APV-I), grasshopper inclusion body virus inclusions (GIBV-I) or to virions extracted from APV inclusions were obtained from rabbits after 4 weekly inoculations according to the following regimen:

First week - Inclusions or virions with Freund's complete adjuvant (Colorado Serum Co.) - 4 ml

Second week - Inclusions or virions with Freund's incomplete adjuvant (Grand Island Biological Co.,) - 4 ml

Third week - Inclusions or virions with Freund's incomplete adjuvant - 2 ml

Fourth week - Inclusions or virions with Freund's incomplete adjuvant - 2 ml

Injections were given intradermally above the axillary lymph nodes, using a 22 gauge needle. Five days after the final injection 50 ml of blood were withdrawn. The blood was transferred to 40 ml plastic centrifuge tubes and after 1 h at 22°C (room temperature) the clot was released from the sides of the tubes with a metal rod and the tubes were incubated overnight at 4°C. The serum was drawn off with a syringe, transferred to serum bottles and placed in storage at -10°C.

Preparation of Ouchterlony Plates

Antigenic relatedness among the viruses was assessed by the double-diffusion in two dimensions (Ouchterlony) method (22). Four ml of sodium barbitol buffer (1.03% w/v sodium barbitol plus 1.25% v/v 1N HCl in distilled water) containing 1% purified agar (Difco Corp.) and 0.1% sodium azide were added to 60 X 15 mm petri dishes (Falcon plastics). Six peripheral wells, evenly spaced, were located 8 mm from the edge of a central well. Antigen and serum were added to the wells and the plates were incubated 4 days at 4°C, after which they were washed overnight in distilled water and stained with a solution of 0.1% Coomassie blue dye in acetic acid:ethanol:distilled

water (1.0:4.5:4.5) for 2 h, then destained with a solution of acetic acid:ethanol:distilled water (1.0:2.5:6.5) for 4 h. Reaction zones were located using an indirect light box.

Photographs of plates were made using a 35 mm camera, with bellows attachment, and a Vivitar No. 25A red filter on Kodak Panatomic X black and white film (ASA 32). Indirect lighting was applied from beneath.

Feulgen Reaction for Nucleic Acid Type Determination

Nucleic acid type, deoxyribonucleic acid or ribonucleic acid, was determined by use of the Feulgen reaction. Purified inclusions were fixed in Arnoy's fixative, hydrated, incubated in 1N HCl, stained in Schiff's Reagent (0.01% basic fuchsin w/v, 0.02% Na_2HOP_4 w/v, 0.2% 1N HCl, v/v in distilled water, counterstained in fast green, mounted, and examined (21). Viral inclusions containing DNA stain purplish-red by this technique. RNA containing virions are not stained by this technique.

Acridine Orange Staining to Determine Strandedness of DNA

Virions extracted from APV inclusions were stained with the fluorochrome acridine orange to establish if the DNA was double- or single-stranded. Purified virions were dried on a cover slip, fixed in Carnoy's fixative, hydrated, rinsed in McIlvaine's buffer (pH 4.0),

stained in 0.1% acridine orange in McIlvaine's buffer, mounted and examined with a Zeiss flourescent microscope (17). Double-stranded DNA viruses fluoresce yellow-green by this technique. Barley stripe mosaic virus (BSMV), a single-stranded RNA virus, was stained as a control. Single-stranded viruses flouresce red by the above technique.

Extraction of DNA from Virions

Deoxyribonucleic acid was extracted from the APV virions by the method of Gafford and Randall (5). Saline citrate buffer with EDTA and sodium lauryl sulfate in 95% ethanol were added to the virion suspension and the solution was carefully mixed. After 45 min. the solution was mixed with glycerol-bromphenol marker and electrophoresed through 0.7% agarose gel tubes, 12 cm long, on a Bio-Rad disc-gel electrophoresis instrument at 4 milliamps per tube for 3 h. Tubes were then stained with ethidium bromide and examined under UV light. Nucleic acid floureSces yellow-orange with this technique.

Chapter 3

RESULTS

Gross Pathology of Virus Infections

Arphia poxvirus (APV), was originally isolated from *Arphia conspersa* collected from the Spanish Creek drainage of Gallatin County, Montana. It has been experimentally transmitted to other *Arphia conspersa* and to *Camnula pellucida* and *Xanthippus corallipes*, all of which are banded-wing grasshoppers of the subfamily Oedipodinae. These experimental inoculations were per os using preparations containing either occluded (virions in inclusions) or nonoccluded (virions removed from inclusions) virions and by intrahemocoelic inoculations of preparations of purified nonoccluded virions. *Melanoplus sanguinipes*, *M. bivittatus*, *Schistocerca americana*, and *Schistocerca vaga vaga*, which belong to the traditional spur-throat grasshopper subfamily of Cyrtacanthacridinae, and *Aulocara elliotti*, of the subfamily Acridinae, were refractory to infection when inoculated either per os or by intrahemocoelic injection in that disease symptoms were not expressed by inoculated grasshoppers nor were inclusions evident when tissues were examined.

In the terminal phases of infection by APV, grasshoppers appeared sluggish and pale in color. Their abdomens were distended and they exhibited signs of ataxia. When inoculated per os as third instar

nymphs, development was slowed during the fourth or fifth nymphal instars and they often died prior to development to the adult stage. If inoculated per os with a high concentration of virus or if inoculated by injection, the nymphs often failed to develop beyond the third instar stage during which they either died or persisted in a moribund state for prolonged periods. Inclusions were detectable in susceptible tissues at approximately 14 days after per os inoculation, but gross signs of infection, particularly ataxia, were evident at about 10 days post-inoculation.

Histopathology and Viral Replication

Infection generally was restricted to the fat tissues of the grasshoppers which, upon gross dissection of infected cadavers, appeared hypertrophied and nearly filled the body cavity. The fat tissue became increasingly pale yellow in color and granular textured as opposed to the normal shiny-yellow, smooth characteristic.

Examination of thin sections of fat tissue by electron microscopy revealed that replication occurs within the cytoplasm of cells (Figure 1). The earliest stages of viral replication observed were virogenic stroma (Figure 2) that appeared as electron dense amorphic masses located within the cytoplasm at 10 days post-inoculation. These stroma contained both coarse and fine granular material that possibly represented pools of protein and DNA. Immature virions were observed