



Homology of the spheroidin gene from entomopoxviruses isolated from *Melanoplus sanguinipes* and *Amsacta moorei*
by Suzanne Wilson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:

Melanoplus sanguinipes entomopoxvirus (MsEPV) is being considered as a biological control agent against grasshoppers. This virus, as well as other entomopoxviruses (EPVs), is occluded in a proteinaceous matrix called spheroidin. Two Lepidoptera EPV spheroidin genes (CbEPV and AmEPV) have been sequenced and homology between the genes coding the 115 kDa spheroidin subunit has been found. In performing this study I determined whether or not homology existed between the spheroidin genes belonging to *Melanoplus sanguinipes* and *Amsacta moorei* entomopoxvirus.

In order to achieve this, a Southern blot was run using PCR-produced MsEPV probes to locate the spheroidin gene. Fragments of the MsEPV spheroidin protein were sequenced, and degenerate primers were constructed based on this information as well as on homologous areas found on AmEPV. These primers were used in a DNA thermal cycler, with MsEPV as a template. The resultant products were labeled and used as radioactive probes against MsEPV and AmEPV restriction digests.

A western blot was used for further confirmation of spheroidin protein similarity. MsEPV and AmEPV spheroidin proteins were exposed to anti-AmEPV polyclonal antibodies to determine if cross-reactivity occurred.

These two methods demonstrated a degree of homogeneity between the two genomes, if not between the spheroidin genes themselves. The Southern blot showed hybridization between the MsEPV probe and an AmEPV fragment, although not the fragment which contained the spheroidin gene. The western blot did show cross-reactivity between the anti-AmEPV and MsEPV spheroidin peptides.

Unlike baculoviruses, which show a great deal of homology, irrespective of whether or not the viral hosts are the same, EPVs which infect different orders are only marginally homologous. While the western blot indicated the presence of similar antigenic determinants between the two EPV spheroidin subunits, the Southern blot failed to indicate homology between the spheroidin genes. Homology, at least to a minor degree, does exist between the total genomes.

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ISOLATED FROM MELANOPLUS SANGUINIPES
AND AMSACTA MOOREI**

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Suzanne Wilson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

September 1994

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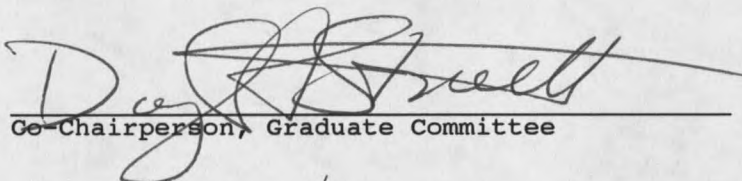
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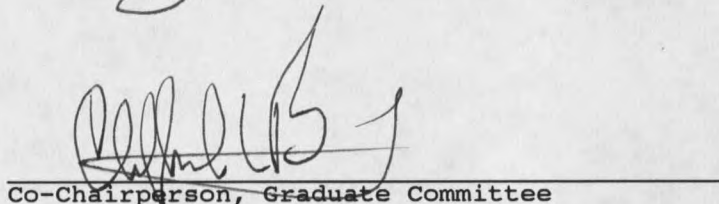
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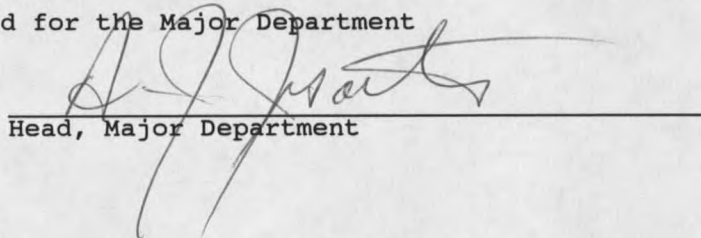

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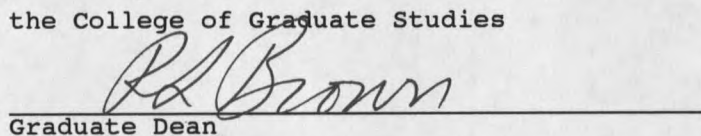
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ACKNOWLEDGEMENTS

I wish to express my thanks and appreciation to Elaine Oma for her assistance and guidance throughout this project. I also would like to give special thanks to Dr. Richard Hall for providing me with materials, guidance, and encouragement. Additional appreciation goes to Norma Irish for her technical assistance. Finally, I wish to acknowledge Drs. D. A. Streett, C. Bond, and N. Reed for serving on my graduate committee.

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ABSTRACT

Melanoplus sanguinipes entomopoxvirus (MsEPV) is being considered as a biological control agent against grasshoppers. This virus, as well as other entomopoxviruses (EPVs), is occluded in a proteinaceous matrix called spheroidin. Two Lepidoptera EPV spheroidin genes (CbEPV and AmEPV) have been sequenced and homology between the genes coding the 115 kDa spheroidin subunit has been found. In performing this study I determined whether or not homology existed between the spheroidin genes belonging to *Melanoplus sanguinipes* and *Amsacta moorei* entomopoxvirus.

In order to achieve this, a Southern blot was run using PCR-produced MsEPV probes to locate the spheroidin gene. Fragments of the MsEPV spheroidin protein were sequenced, and degenerate primers were constructed based on this information as well as on homologous areas found on AmEPV. These primers were used in a DNA thermal cycler, with MsEPV as a template. The resultant products were labeled and used as radioactive probes against MsEPV and AmEPV restriction digests.

A western blot was used for further confirmation of spheroidin protein similarity. MsEPV and AmEPV spheroidin proteins were exposed to anti-AmEPV polyclonal antibodies to determine if cross-reactivity occurred.

These two methods demonstrated a degree of homogeneity between the two genomes, if not between the spheroidin genes themselves. The Southern blot showed hybridization between the MsEPV probe and an AmEPV fragment, although not the fragment which contained the spheroidin gene. The western blot did show cross-reactivity between the anti-AmEPV and MsEPV spheroidin peptides.

Unlike baculoviruses, which show a great deal of homology, irrespective of whether or not the viral hosts are the same, EPVs which infect different orders are only marginally homologous. While the western blot indicated the presence of similar antigenic determinants between the two EPV spheroidin subunits, the Southern blot failed to indicate homology between the spheroidin genes. Homology, at least to a minor degree, does exist between the total genomes.

CHAPTER 1

INTRODUCTION

Grasshoppers and Biological Control

Grasshoppers and locusts have caused problems in agriculture since the time of the first historical records. They are currently one of the most important pests on rangeland in the western United States. Although there are around 600 species of grasshoppers within the United States, only about a dozen of these frequently occur in high densities on rangeland. While some of these species may actually be considered beneficial, most are considered competitors for grasses and forbs normally consumed by livestock (Hewitt and Onsager, 1983). The impact of large infestations in the western United States on our economy directed Congress in 1936 to charge the U.S.D.A. with assisting in grasshopper control efforts (Pfadt and Hardy, 1987).

Initially, aerially applied liquid insecticides were the primary tool for grasshopper control. However, due to the concern about effects on non-target organisms, ground water contamination, and environmental impact, integrated pest management has increasingly becoming the preferred alternative. This involves the use of traditional chemical methods in conjunction with methods using microbial agents, known as biological control.

Biological control is a non-chemical method of insect population control where the action of natural enemies, i.e., parasites, predators, and pathogens, is utilized. Integrated into a program with restricted use of chemicals, biological control has shown to be an effective means of decreasing an insect population, sometimes over several generations, without the negative effects of massive pesticide use.

Among the biological control agents used on grasshoppers are several pathogens that were first discovered naturally occurring in various grasshopper species. Included are species of protozoa, fungi, and viruses. Among the protozoa responsible for grasshopper disease are species of microsporidia, amoebae, and gregarines, with microsporidia being the one most commonly used in biological control. Fungal diseases could be caused by organisms found in either the division Deuteromycetes or the order Entomophthorales. The causative agent for a grasshopper viral infection, however, could be either a crystalline array virus, or, more commonly, an entomopoxvirus (Streett and McGuire, 1990).

Entomopoxviruses are the most frequently found viruses in grasshoppers, as well as in a variety of other insect species. These viruses make excellent biological control agents because of their occlusion body, a protein matrix which encloses several hundred viral particles. The occlusion body enables the virions to withstand environmental hazards and assures viral persistence. Horizontal transmission can occur through cannibalism, scavengers, and birds (Tanada and Kaya, 1993). Their narrow spectrum of infection, however, while protecting desirable non-target hosts, can also act as a constraint to this biological control agent (Streett, 1987).

Entomopoxviruses

The orders of insects known to harbor entomopoxviruses (EPVs) include Lepidoptera, Coleoptera, Diptera, and Orthoptera (Francki et al., 1991). Within the order Orthoptera, nine different species of grasshoppers have been shown to harbor EPVs. Six of these viruses have been characterized by restriction endonuclease analysis (Streett et al., 1986), and appear to be unrelated. One of these species of grasshoppers, *Melanoplus sanguinipes*, harbors the virus *Melanoplus sanguinipes* entomopoxvirus (MsEPV), about which this study has been

done. The other virus in the homology analysis with MsePV is AmEPV (*Amsacta moorei* entomopoxvirus), which infects a Lepidoptera species.

MsePV and AmEPV are classified as being in the family Poxviridae, subfamily Entomopoxvirinae, and the genus B. The family Poxviridae consists of a group of which the main characteristics include having a single molecule of dsDNA, with a molecular weight in the range of 130-390 kbp, the presence of occlusion bodies, and a narrow host range. The subfamily Entomopoxvirinae refers to insect-borne poxviruses which possess four enzymes, and where multiplication occurs in the cytoplasm of fat cells or hemocytes. The four enzymes include nucleotide phosphohydrolase (NPH), acidic deoxyribonuclease, neutral deoxyribonuclease, and DNA-dependant RNA polymerase (Arif, 1984). The B genus includes entomopoxviruses found in the orders Lepidoptera (butterflies and moths) and Orthoptera (grasshoppers), with a G+C content of approximately 26%.

This genus classification is based on several characteristics. The three probable genera, A, B, and C, are separated based on morphology of the virions, host range, and molecular weight of the genome. While genera A and B both have ovoid shaped virions, the molecular weight range of genus A is 260-370 kbp, versus approximately 225 kbp in genus B. Genus A is found primarily in the order Coleoptera, while genus C is found in Diptera species and has a brick-shaped virion (Francki *et al.*, 1991).

Entomopoxvirus Spheroidin Gene

The characterization of the genome of EPVs has shown a similarity in molecular weight, structure (linear, double-stranded), and G+C content (18-26%). These viruses are all occluded in a proteinaceous matrix called an occlusion body (OB) or spheroid. The OBs are composed

of a single polypeptide subunit, or spheroidin, with a molecular weight range of 100-115 kDa (Bilimoria and Arif, 1979).

Due to the strong promoter potential of the spheroidin gene, the spheroidin protein and gene have been studied extensively in EPVs found in both *A. moorei* and *Choristoneura biennis*, Lepidopteran species. In each case, the spheroidin gene has been located and, in the case of AmEPV, sequenced (Hall and Moyer, 1991). Bilimoria and Arif (1979) first determined the molecular weight of the *C. biennis* entomopoxvirus (CbEPV) spheroidin protein to be 102 kDa. Yuen *et al.* (1990), however, arrived at a molecular weight of 49 kDa, explaining that the 49 kDa protein formed dimers, creating, after post-translational modifications, the 102 kDa protein. The polyhedrin of baculoviruses, which are functionally analogous to the spheroidin of EPVs, are relatively conserved (Rohrman, 1986). It would therefore be logical to assume that the spheroidin protein found in EPVs, especially when the virus infects the same insect order (Lepidoptera), would have a similar molecular weight and possible homology. Hall and Moyer (1993), however, found that there was no homology between the 115 kDa spheroidin protein from AmEPV and the CbEPV 49 kDa protein. There was, however, homology between the AmEPV spheroidin protein and the approximately 102 kDa CbEPV protein.

There have been several indications of homology between other genes found in EPVs infecting the same order. Hall and Moyer (1993) determined that the NPHI gene for AmEPV was 89% homologous with the same gene in CbEPV at the amino acid level. The thymidine kinase gene was also found to be conserved between the same two viruses, with 63% identity and 10% similarity at the protein level (Lytvyn *et al.*, 1992). Banville *et al.* (1992), on the other hand, did not find homology between the spheroidin proteins or genes of these two viruses. This was due to the assumption that the CbEPV spheroidin protein was the 50 kDa fragment versus the 115 kDa fragment proposed by Hall and Moyer (1993).

Studies to find homology between EPVs that infect the order Orthoptera have been less intensive, concentrating on restriction enzyme patterns and Southern blot hybridization of the whole genome. Streett *et al.* (1986) found the restriction enzyme pattern for each of six EPVs isolated from grasshoppers to be unique. The Southern blot hybridization under stringent conditions showed homology between MsEPV and only three other grasshopper EPVs. Less stringent conditions allowed expression of homology to the other grasshopper EPVs, but no homology was detected against AmEPV. Langridge *et al.* (1983) demonstrated similar findings, with no hybridization between MsEPV and AmEPV.

Homology Between EPVs Which Infect Different Insect Orders

Although MsEPV and AmEPV do not infect insects that are in the same order, the viruses are in the same genus (B), and show many morphological and biochemical similarities. It is conceivable that homology exists between the two EPV genomes, especially at the spheroidin gene, and that by using a fragment of MsEPV DNA as a probe, and low stringency conditions, this homology could be detected.

The purpose of this study was to determine the presence of homology between the spheroidin genes of MsEPV and AmEPV. To do this, peptides from MsEPV spheroidin were sequenced, degenerate primers were derived using both this sequencing information and an assumption of homology with AmEPV, and the primers were used to create a PCR product. This product was then labeled with ^{32}P and used as a probe against a Southern blot containing restriction fragments of MsEPV and AmEPV.

A western blot was run to determine if immunological similarities existed between spheroidin peptides from the two viruses. This experiment also served to resolve the issue as to which of the two major spheroidin bands (115 kDa or 50 kDa) of MsEPV spheroidin reacted with antiserum made against the larger AmEPV band. Exposure of this blot to

AmEPV polyclonal antibodies was done to determine if cross-reactivity occurred.

CHAPTER 2

MATERIALS AND METHODS

The Production of MsEPV in Grasshoppers

A non-diapause strain of *Melanoplus sanguinipes* grasshoppers was raised in the rearing facility at the Rangeland Insect Laboratory, USDA, ARS, Montana State University, Bozeman, MT, according to the procedure outlined by Henry (1985). A grasshopper occlusion body virus (MsEPV), first isolated from field grasshoppers in 1969 (Henry et al., 1969), was propagated in *M. sanguinipes*. Grasshoppers were infected with MsEPV by the method described by Oma and Streett (1993), and the infected cadavers were stored at -25°C until needed.

Isolation of Spheroids from Grasshoppers

A diagnosis of viral infection was made by homogenizing grasshoppers individually in Thomas tissue homogenizers equipped with teflon pestles, using a grinding buffer consisting of 10 mM Tris, 1 mM EDTA, and 10 mM 2-mercaptoethanol (Mitchell et al., 1983). The homogenate was then examined using phase contrast microscopy. Homogenates containing spheroids were pooled and passed through two layers of nylon organdy. The filtered homogenate was then washed 5 times with distilled water by pelleting in an IEC clinical centrifuge at 763 g for 10 min.

The spheroid preparation was then layered on a 50-65% (w/w) continuous sucrose gradient that had been prepared the day before to allow for equilibration at 4°C. The gradients were centrifuged in a Beckman L7 ultracentrifuge (SW 28.0 rotor) at 22,000 rpm for one hr. The desired band of purified spheroids was located at the 55-60%

interface, removed, washed repeatedly with distilled water to remove the sucrose, and counted on a hemacytometer.

Release and Isolation of Virions

Virion release buffer (VRB), pH 11.2, was prepared fresh using 0.3 M Na₂CO₃, 0.5 M NaCl, 0.03 M EDTA (free acid), and 0.1 M 2-mercaptoethanol. The occlusion bodies were pelleted, and the VRB was mixed with the pellet at a ratio of 3 ml/2x10⁸ OB's. This suspension was incubated in a 30°C water bath for 20 min and the suspension examined using phase contrast microscopy to determine that at least 95% of the occlusion bodies were degraded and the virions released. Released virions were then layered on continuous 40-55% (w/w) sucrose gradients preequilibrated at 4°C overnight, and centrifuged in a Beckman L7 ultracentrifuge (SW 28.0 rotor) at 22,000 rpm for one hr.

The cloudy virion layer, located at the 50-55% interface of the sucrose gradient, was removed, diluted in distilled water, and pelleted in a Beckman 28.1 rotor for 30 min at 22,000 rpm. After decanting and air drying the pellets, each was resuspended in 0.5 ml TE buffer (10 mM Tris, 1 mM EDTA), pH 7.6, and stored at -25°C. Each pellet represented approximately 1x10⁹ occlusion bodies.

Phenol Extraction and DNA Purification

Six 0.5 ml virion suspensions were thawed and 20 µl of proteinase K (0.4 mg) and 20% SDS were added to each tube. After gently mixing, the suspensions were incubated in a 37°C waterbath for 16 hr.

An equal volume of TE-buffered phenol-chloroform, pH 8.0, previously warmed to 37°C, was added to the virion suspension, and gently rocked for 2 min. The suspensions were centrifuged in a Hermle Z230M microcentrifuge for 4 min at 15,000 rpm. The aqueous layer was removed, an equal volume of TE-buffered phenol-chloroform was added, and the extraction was repeated 5 times. A final wash using chloroform-

isoamyl alcohol (24:1) was done to remove phenol residue. A dialysis buffer of a 1:10 dilution of TE buffer, pH 7.6, was made and the DNA solution was dialyzed with 6 changes for a minimum of 6 hr each. After dialysis, the DNA solution was concentrated in a Speed-Vac concentrator for approximately 4 hr for a final volume of 60 μ l and concentration of approximately 200 μ g/ml. The concentration and purity of the MsEPV DNA was determined on a Beckman DU®-64 spectrophotometer at 260 and 280 nm (Sambrook *et al.*, 1989).

Sequencing of Spheroidin Protein

A sample of spheroids was purified on a sucrose gradient (see "Isolation of Spheroids from Grasshoppers" above) to prepare a sample for spheroidin peptide sequencing. A 5×10^8 /ml spheroid preparation in sterile distilled water was sent to M-Scan, West Chester, PA, for protein sequencing.

Half of the sample was pelleted and dissolved in sample buffer consisting of 0.25 M Tris-HCL, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 2% (w/v) bromophenol blue. The material was then loaded onto 2 mini-SDS-PAGE gels (1.5 mm thick). The 105 kDa band was removed and the protein electroeluted. A trypsin digest was performed because of a suspected amino terminal block. The peptides were then separated on a reversed phase column equilibrated with a phosphate buffer at pH 7.0. Elution was performed with acetonitrile. Selected fractions were then separated on the same reversed phase column equilibrated with 0.1% TFA (trifluoroacetic acid). Peptides were sequenced in a ABI model 477A sequencer using standard programs.

Designing Primers

An analysis was made for homology between the above peptide sequences and the sequenced spheroidin genes found in AmEPV using the NIH-DNA software (Prtaln). These programs are based on the algorithm

described by Wilber and Lipman (1983). The AmEPV spheroidin gene sequence was retrieved from Genbank data base under accession # M77182. Degenerate primers (17mer) and their complementary primers were designed and ordered from Genosys Biotechnologies, Inc., The Woodlands, Texas, using as a guideline the presence of homology with AmEPV and a minimum number of permutations.

Creation of a Probe Using the DNA Thermal Cyclor

Polymerase chain reactions with custom-designed degenerate 17mer primers were performed as described in "PCR Protocols" (Innis *et al.*, 1990). Amplification buffer composition included: 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μM each of dATP, dGTP, dTTP, and dCTP. The primers were added at 20 pM per permutation. The MsePV DNA template (see above) was added at 60 ng/100 μl reaction mixture, optimized as determined by serial dilution. The specific reaction conditions for 34 cycles were as follows: 1 min at 94°C for denaturation, 1 min at 37°C for annealing, and 2 min at 72°C for extension. Finally, the samples were incubated for 11 min at 72°C to complete extensions (Hall and Moyer, 1993), and the samples were left on a soak cycle at 4°C. The Amplitaq™ DNA polymerase was added at 2.5 U after the first denaturation temperature had risen above 75°C ("hot start"--see D'Aquila *et al.*, 1991).

A horizontal 1.6% agarose gel was made by dissolving agarose (Seakem®GTG) in 1X TAE buffer (40 mM Tris, 0.02 N acetic acid, 1 mM EDTA, pH 8.0). A fifth of each reaction mixture containing the products resulting from all of the primer combinations was electrophoresed (4.1 V/cm, 1.5 hr). The bands were visualized by ethidium bromide staining. The fragment size was determined using a φx174/HaeIII digest as a standard and Gel™ software, which uses the least-squares analysis of Schaeffer and Sederoff (1981). Two products, A and B, were selected based on their size being within 10% of the predicted size on the AmEPV

spheroidin gene. The rest of the reaction mixtures for A and B were aliquoted into 4 lanes each on additional 1.6% agarose gels, and after electrophoresis, the bands were excised and the DNA electroeluted from the gel. The DNA was precipitated out, using 2X volume of 95% ETOH, and a 1/10 volume of 5 M ammonium acetate. The DNA pellet was reconstituted with 50 μ l TE buffer (pH7.6).

Southern Blots

Gel Electrophoresis

A 0.3% agarose gel (SeaKem® Gold) in 1X TAE buffer (0.4 M Tris, 0.2 N acetic acid, 0.01 M EDTA [disodium], pH 8.0) was set up with a 1% agarose support layer. Restriction digests of AmEPV/BamHI (Research Laboratories) and MsEPV/BsiCI (USB) were prepared as suggested by the manufacturers. AmEPV DNA was prepared from infected IPLB-LD-652 cells and provided by Dr. Richard L. Hall, Department of Immunology and Medical Microbiology, University of Florida, Gainesville, Florida. After addition of sample buffer (100 mM EDTA, 1% SDS, 50% glycerol, and 0.1% bromophenol blue), the restriction fragments were incubated at 65°C for 10 min and separated by electrophoresis for 25.5 hr, 2.4 V/cm in 1X TAE buffer (40 mM Tris, 0.02 N glacial acetic acid, 1 mM EDTA, pH 8.0), along with BRL High Molecular Weight Standards. Molecular weight estimation was determined with Gel™ software.

Capillary Transfer

A bidirectional capillary transfer to nitrocellulose filters was made according to Smith and Summers (1980), modified with a third wash of a 0.5 M Tris-HCL (pH 7.4), 3.0 M NaCl solution to neutralize the gel before transfer. The transfer occurred for approximately 16 hr, and then the blots were washed for 5 min in 10X SSC buffer (0.15 M citric acid, 1.5 M NaCl, pH 7.0), and air dried. Control dots (1 μ l/dot) were added, including 200 ng each of MsEPV DNA and the 2 PCR products created

using degenerative primers (see "Creation of a Probe Using the DNA Thermal Cycler"). The blots were baked under vacuum for 2 hr at 80°C.

Radiolabeling of Viral DNAs

Three radiolabeled probes were made, using the nick translation kit protocols distributed by Amersham as described by Rigby *et al.* (1977). DNA from MsEPV and the two PCR products were sonicated with a cell disrupter for 6 min at 80% duty cycle (Heat systems-Ultrasonics Inc., Model W-375) and radiolabeled with [$\alpha^{32}\text{P}$] dCTP. A minimum DNA concentration of 200 $\mu\text{g/ml}$ was used. Unincorporated dNTPs were removed from the probes by passage through 10 cm columns of Sephadex G-50 in TNE buffer (30 mM Tris-HCl, 25 mM EDTA, 100 mM NaCl, pH 8.1).

DNA Hybridization

Low stringency conditions (20% formamide) were used in prehybridization and hybridization procedures. The $\alpha^{32}\text{P}$ -labeled DNA probes and 1% salmon sperm DNA were denatured by heat shock (i.e. boiled for 5 minutes and suddenly cooled), and combined with 20% formamide, 0.75 M sodium citrate, 0.75 M NaCl, 2% 1 M NaPO_4 , (pH7.0), 1X Denhardt's (1 mM EDTA, 0.0004% Ficoll 400, 0.0004% PVP-360, 0.0004% BSA), and 0.1% SDS on ice. Hybridization was done at 42°C. The blots were washed 4X (15 min each) in a low stringency wash solution (0.03 M sodium citrate, 0.3 M NaCl, 0.1% SDS). The blots were dried and autoradiographed on Kodak film at -70°C for 3 days. It was noted whether or not a signal was present on the film, designating hybridization to a specific fragment of either restriction digest. A determination was made as to which band was hybridized by referring to the photo of the original gel with the restriction digests next to a ruler.

Western Blot Analysis

Two discontinuous 7% SDS-polyacrylamide gels were prepared according to the method of Laemmli (1970), with the only modification being that the TEMED concentration was doubled to 0.05%. The gel apparatus was assembled according to Hames (1981). The reservoir buffer was also modified by the addition of 0.1 M sodium acetate (Christy *et al.*, 1989). The gels were prerun at 70 mA for 1.5 hr. The electrophoresis was carried out with a current of 70 mA until the bromophenol blue marker reached the bottom of the gels (about 5 hr).

Sample Preparation

For the AmEPV OB sample, previously frozen infected *Estigmene acrea* caterpillars were ground in Mitchell's buffer and the spheroids isolated as in the above description in grasshoppers. After washing, 1×10^8 spheroids were mixed with 25 μ l of sample buffer (Laemmli, 1970), boiled for 5 min to denature the protein, centrifuged 5 min, and 20 μ l of the supernatant was loaded onto the gel. The description of the isolation of MsEPV is found above ("Isolation of Spheroids from Grasshoppers"). A sample of 3×10^5 spheroids was treated like the AmEPV sample above before loading onto the gel. As a negative control, 1.4×10^9 *Nosema locustae* spores were isolated and prepared as described above. The Bio-Rad SDS-PAGE molecular weight standard for high range (45,000-200,000) was used. This standard was diluted 1:20 with sample buffer and the sample treated as above.

Protein Transfer

A western blot analysis was conducted using the methods described by Matsudaira (1987). Briefly, the gels were soaked in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), 10% methanol, pH 11.0, for 30 min, and two PVDF (polyvinylidene difluoride) membranes were presoaked in absolute methanol for 5 min. Both gels, each sandwiched between a

PVDF membrane and 2 sheets of presoaked 3M blotter paper, were assembled into a Hoefer Transphor® apparatus and electroeluted at 100 V for 1 hr.

Immunoblot

Polyclonal antiserum, prepared from rabbits immunized to purified AmEPV occlusion bodies obtained from cell cultures, was obtained from Dr. Richard Hall (Hall and Moyer, 1993). The western blot was blocked by soaking in TBS-T buffer (0.5 M NaCl, 0.02 M Tris, 0.05% Tween 20, pH 7.5) for one hr. The antiserum was diluted 1:500 with TBS-T and the blot was incubated with the diluted antiserum overnight. The blot was then washed 30 min with 3 changes of TBS-T. The secondary antibody, GAR-peroxidase conjugate diluted 1:10,000 in TBS-T, was then added. Following a 90 min incubation period, the blot was washed as above, and rinsed with TBS (Tween 20 omitted). The blot was then incubated with the substrate solution for 1 hr, rinsed 3 times in distilled water, and left in the dark, covered with water overnight. The blot was air dried in the dark on blotter paper. A picture was taken before the blot could fade, and any evidence of secondary antibody reactions and color development was noted.

Stained Blot

The second blot was stained by the method of Matsudaira (1987) to verify the correct protein concentrations and to correlate molecular weights with bands expressed in the immunoblot. The blot was washed with distilled water for 5 min, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min, and then destained in 50% methanol, 10% acetic acid for 10 min. After rinsing in distilled water, the blot was air dried overnight. The molecular weights of the major bands were determined, using the high molecular weight standard and the Gel™ program. The band to be sequenced (105 kDa) was noted.

CHAPTER 3

RESULTS

Selecting Peptide Fragments Based on AmEPV Homology

Several samples of the spheroidin protein were submitted to be sequenced, including band slices from an SDS-PAGE gel, a PVDF membrane to which the protein had been transferred, and purified occlusion bodies themselves. The last sample was finally used, since the presence of an amino terminal block prevented successful sequencing using the other sample preparations.

After the trypsin digest on the spheroidin protein was performed, several of the internal peptides were sequenced. The amino acid sequences provided were 5, 7, 11, and 14 residues long. To check for homology on the NIH-DNA software (Prtaln), these peptide sequences were entered in total, as well as in smaller fragments. None of the longer fragments (over 8 residues) demonstrated homology with the AmEPV spheroidin gene amino acid sequence, and most of the other "homologous" sites had gaps of up to 40 residues. Based on the best evidence of homology with AmEPV, i.e., the greatest number of homologous residues in a six-residue span and a lack of degeneracy at the 3' end considering elimination of the 18th base, three of the peptides were selected:

Peptide #1: Met(assumed)-Gln-(Asn or Pro)-Gly-(Pro or Tyr)-Gln
 M Q N G P Q

Peptide #2: Tyr-Tyr-His-Asn-Ile-Val
 Y Y H N I V

Peptide #3: Trp-Lys-Asp-Tyr-Val-Ala
 W K D Y V A

The MsEPV spheroidin amino acid sequences, along with the corresponding AmEPV spheroidin sequence and position numbers are shown below:

Peptide #1

	3080	
AmEPV		atgagtaacgtaccttta
		M S N V P L
		: : :
MsEPV		M Q N G P Q

Peptide #2

	5549	
AmEPV		tattatagagaaatattt
		Y Y R E I F
		: : :
MsEPV		Y Y H N I V

Peptide #3

	4202	
AmEPV		aataaaaatttatgtagat
		N K I Y V D
		: : :
MsEPV		W K D Y V A

Primer Design

Primers 17 bases long were created for the positive and negative strands for each of these peptides. Where homology with AmEPV at the amino acid level existed, the same codon was used as in AmEPV.

Otherwise, degeneracy was used. The primers created (where r represents a or g, y represents c or t, and x can be any of the four bases) were as follows:

Pr1:	atg car aac ggx cct ca
Pr1-comp:	tg agg xcc gtt ytg cat
Pr2:	tat tat cay aay ata gt
Pr2-comp:	ac tat rtt rtg ata ata
Pr3:	tgg aar gay tay gta gc
Pr3-comp:	gc tac rta rtc ytt cca

The various permutations for the DNA sequence for each of these peptides were then checked against AmEPV for homology. Several sites for nucleic acid homology were selected, and these, in turn, led to various predicted PCR product sizes that could ostensibly be created from the above primers on the thermal cycler (Table 1).

The various nucleic acid sequences based on the MsEPV peptide sequences that showed homology with the AmEPV spheroidin gene are shown below along with the location of the homologous site on the AmEPV spheroidin gene:

	Peptide #1:	Met Gln Asn Gly Pro Gln
	Pr1:	atg car aac ggx cct ca
3080-3096	Am1A:	atg agt aac gta cct tt
6044-6058	Am1B:	att gaa aat tgt tat ca
5798-5814	Am1C:	aca aga aat ggt gaa ca
4955-4971	Am1D:	gaa tat cct gga tat gt
	Peptide #2:	Tyr Tyr His Asn Ile Val
	Pr2:	tat tat cay aay ata gt
5549-5565	Am2A:	tat tat aga gaa ata tt
3407-3423	Am2B:	gta tca cat aat gac gt
	Peptide #3:	Trp Lys Asp Tyr Val Ala
	Pr3:	tgg aar gay tay gta gc
4202-4218	Am3A:	aat aaa att tat gta ga
5894-5910	Am3B:	tgg aat gac tat aga aa

The relative location of each of these prospective primer sites on AmEPV and the resulting predicted PCR products were mapped as follows:

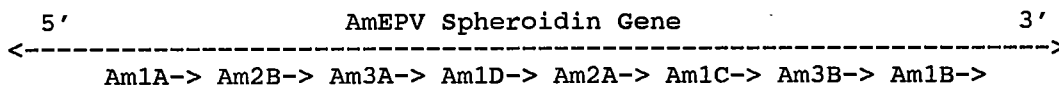


Table 1. Predicted PCR fragment sizes in base pairs.

Positive strand:Am1A Am2B Am3A Am1D Am2A Am1C Am3B							
Negative strand:							
Am2B	344	X	X	X	X	X	X
Am3A	1139	812	X	X	X	X	X
Am1D	1892	1565	770	X	X	X	X
Am2A	2486	2159	1364	611	X	X	X
Am1C	2735	2408	1613	860	266	X	X
Am3B	2831	2504	1709	956	362	113	X
Am1B	2979	2652	1857	1102	510	261	164

PCR Products

Upon running the thermal cycler using all primer and primer complement combinations, only two of the products were in sufficient concentration and within 10% of the predicted molecular weight (Fig. 1).

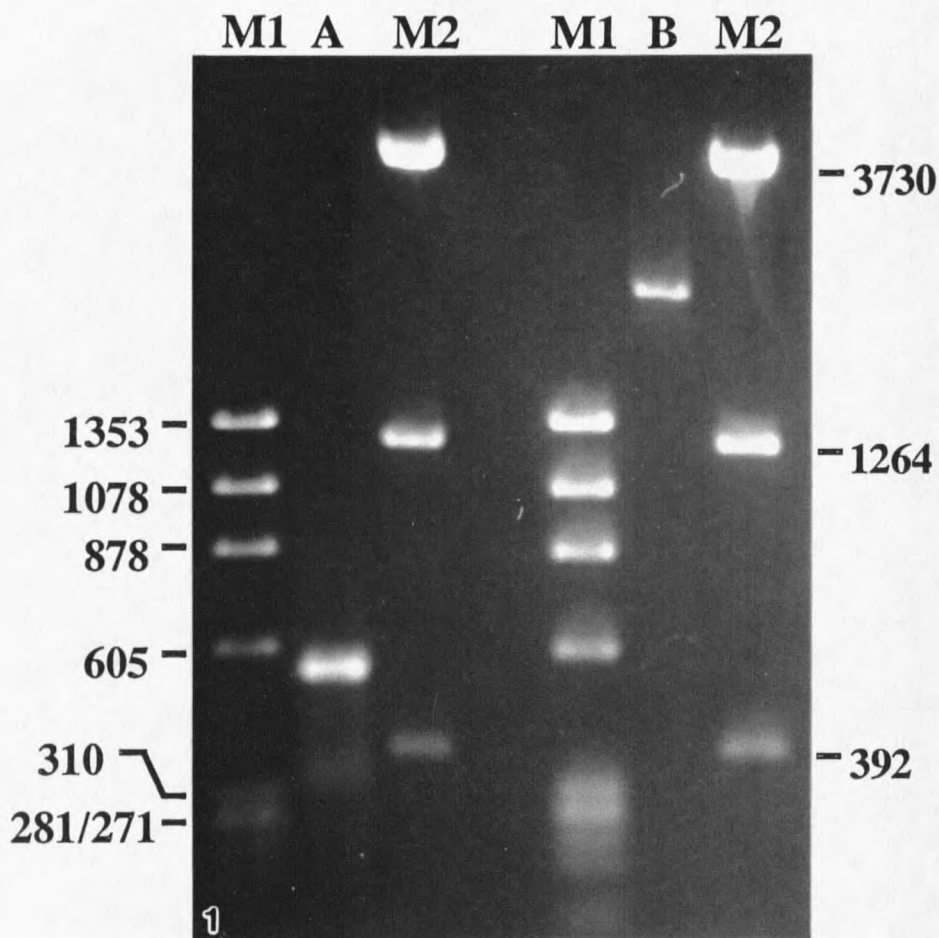


Fig.1. Ethidium bromide-stained agarose gel showing PCR products derived from primers based on the amino acid sequence of the MsePV spheroidin gene. The template (60ng/100ul reaction mixture) was MsePV DNA. Lane M1 shows a ϕ x174/*Hae*III digest, while the M2 lane shows a ϕ x174/*Hpa*I digest. These are used as a molecular weight standards. The band in Lane A represents a PCR product resulting from using primers Pr2 and Pr1-comp. Lane B contains a product resulting from the use of primers Pr2 and Pr3-comp.

The molecular weights for PCR products A and B were determined and compared to the predicted fragment sizes on the AmEPV genome (Table 2).

Table 2. Characteristics of PCR Products used for probes.

	Primers	Molecular Weight (bp)	Predicted Size (bp)	%Variance
PCR Product A	Pr2 Pr1-comp	535	510	4.7
PCR Product B	Pr2 Pr3-comp	2365	2504	5.6

Restriction Digests

The choice of restriction enzymes for the digests used on the Southern blots was based on the clear separation of the fragments, better enabling a determination of the hybridized band.

The restriction fragment sizes (Fig. 2) in kilobase pairs for the two restriction digests were determined using the GelTM program and are noted below (Table 3). Possible smaller fragments of MsePV may exist, but without further studies, this information is not known.

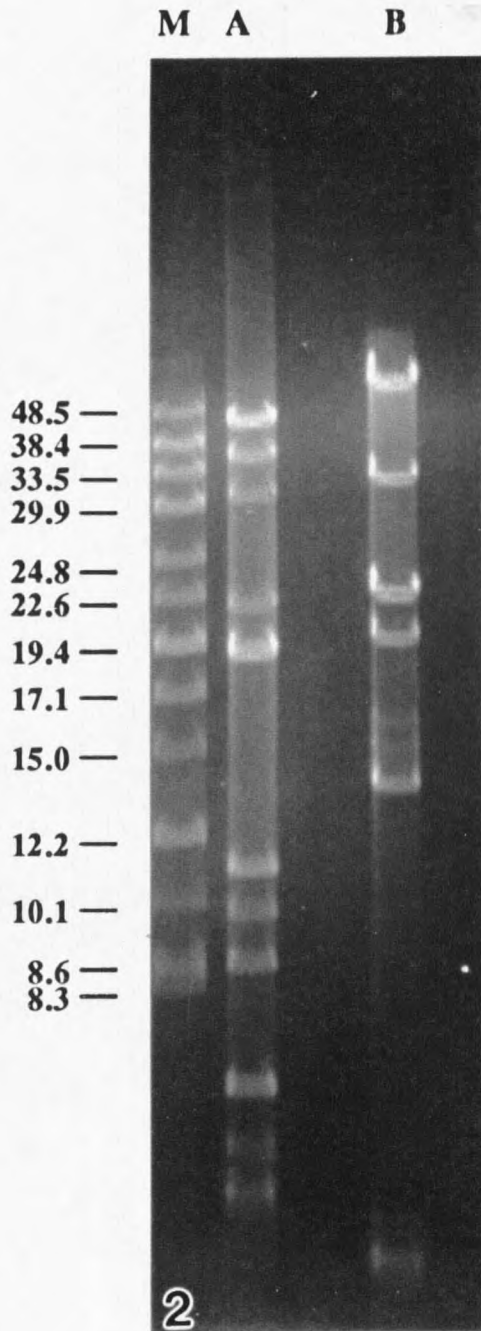


Fig.2. Ethidium bromide-stained agarose gel showing restriction digests used in the Southern blot. Lane A contains a restriction digest of AmEPV with *EcoRI*. Lane M is a high molecular weight standard by BRL. Lane B represents MsEPV digested with *BsiCI*. Gel electrophoresis (0.3%) was carried out at 50V for 26 hours, after an initial 10 min boost at 100V.

Table 3. Restriction fragment sizes in kilobase pairs.

Fragment No.	M _s EPV/ <i>Bsi</i> CI	A _m EPV/ <i>Eco</i> RI
1	52.5	43.8
2	33.9	37.3
3	22.6	31.7
4	19.4	22.6
5	16.1	19.4
6	15.0	11.3
7	13.4	10.1
8	5.4	9.1
9	4.6	7.0
10		6.8
11		5.9
12		5.4
13		4.2
14		3.1
15		2.9

DNA Hybridization

Radiolabeled PCR products and M_sEPV DNA were used as probes against Southern blots of restriction digests of A_mEPV and M_sEPV. Hybridization occurred between the "A" probe and fragment #2 on M_sEPV, and fragment #3 on A_mEPV. Probe "B" hybridized with fragment #1 of M_sEPV and did not hybridize with A_mEPV (Fig. 3). Based on the mapped A_mEPV genome by Hall and Hink (1989), the fragment that hybridized with A_mEPV did not contain the spheroidin gene. The control dot blot hybridization assay revealed that "A" does not hybridize with "B" and vice versa, indicating exclusivity of the two probes.

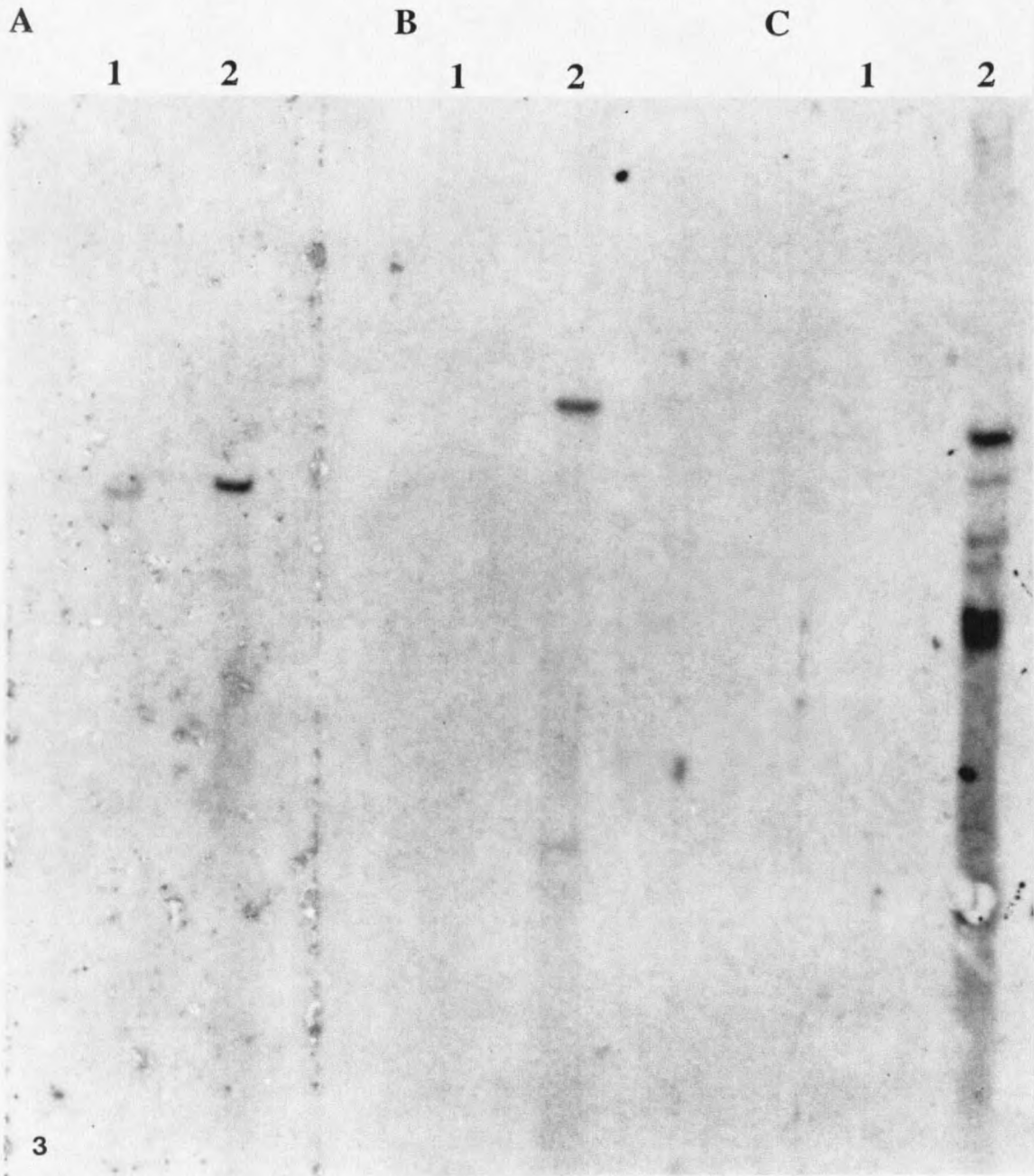


Fig.3. An autoradiogram of the blots probed with either (A) probe "A", (B) probe "B", or (C) MsEPV DNA. Lane (1) indicates the location of the AmEPV digest; Lane (2) represents the position of the MsEPV restriction digest on the blot.

Immunoblot

A cross-reaction between the polyclonal AmEPV spheroidin antibodies and the 105 kDa spheroidin band of MsEPV was noted (Fig. 4). On a scale of 1+ to 4+, with 4+ indicating a large reaction, MsEPV gave a 2+ reaction, AmEPV a 4+ reaction, and the negative control showed a trace reaction. The smaller MsEPV spheroidin band (approx. 49 kDa) showed no reaction.

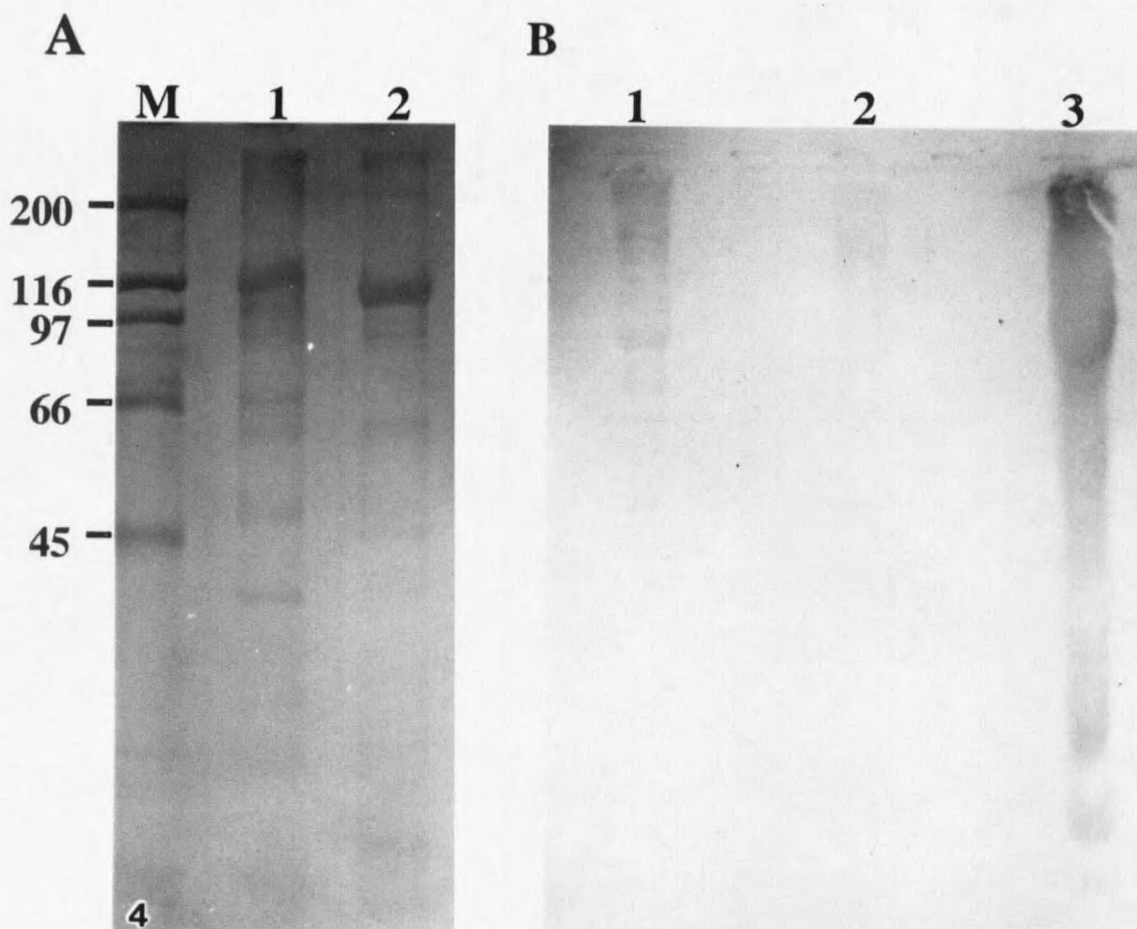


Fig.4. The reaction of polyclonal antiserum created against AmEPV spheroidin with various protein samples. (A) The Coomassie blue-stained blot. Lane M = molecular weight standards; Lane 1 = MsEPV spheroidin protein. Lane 2 = AmEPV spheroidin protein; (B) Immunoblot analysis. Lane 1 = MsEPV spheroidin; Lane 2 = *Nosema locustae* protein; Lane 3 = AmEPV spheroidin.

CHAPTER 4

DISCUSSION

This study was intended to prove the existence of homology between the spheroidin genes of entomopoxviruses which infect different insect orders. The results indicated a degree of homology between MsEPV and AmEPV genomes, without conclusive evidence of homology between the actual spheroidin genes.

Southern Blot

The process chosen to determine homology with the Southern blot was based on the fact that when primers are designed that correspond to conserved domains in related proteins, the distance between the priming sites should be conserved. By designing primers based on the amino acid sequence of MsEPV spheroidin and using them in a thermal cycler with a MsEPV DNA template, the PCR product should consist of MsEPV DNA located within the spheroidin gene. This DNA fragment, in turn, should hybridize to the restriction fragment of MsEPV, and, hopefully, AmEPV which contains the same gene.

While definite homology was indicated with probe A against AmEPV, the fact that the probe hybridized with an AmEPV fragment which did not contain the spheroidin gene was puzzling. The primers may have annealed to a portion of the MsEPV genome outside the spheroidin gene because of primer degeneracy and shortness of length. Another conserved gene, with common regions in both viruses, may have contained the homologous segment. An alternate explanation could be that the actual location of the AmEPV spheroidin gene may differ from the conclusions of Hall and Moyer (1991).

The Hall and Moyer study on the location of the spheroidin gene left little room to doubt their conclusion. A degenerate probe derived from microsequencing of the spheroidin protein was used to select a *Bgl*III clone from a *Bgl*III library of AmEPV DNA. This clone was then radiolabeled and hybridized back to various AmEPV genomic digests. Complete genomic mapping of various digests was done, and the labeled probe hybridized fragments between 138 - 225 kb in 4 out of 5 digests. The fragment hybridized by the PCR-created MsePV probe was located between 95 - 124 kb, out of the spheroidin gene range. Even just considering the *Eco*RI map, the distance between the Hall-Moyer AmEPV spheroidin gene-containing fragment and the fragment hybridized by the PCR-created probe is 17.6 kb, well above the size of the 3 kb AmEPV spheroidin gene. This overrules the possibility of a gene overlap, giving greater credence to the possibility of the primers annealing to a portion of the MsePV genome outside the spheroidin gene.

Working with entomopoxvirus DNA presented a special set of problems. Optimal primers were difficult to achieve, due to the low G+C content of MPV DNA (18.6%) (Arif, 1984). According to Xu and Larzul (1991), optimal G+C content of primers should be 40-60%. Because of the degeneracy of the primers, the G+C content of the actual annealing primer could have been anywhere from 12-59%, with the lower percentages being more likely given the G+C content of the whole genome. The length of the primers was another problem area. While a length of 17 bases was the lower limit of acceptable, a 20mer would typically have been selected because of its stability. However, 17mers were selected due to the necessity of eliminating 3' end degeneracy, and the fact that the 18th base was inevitably degenerate.

Western Blot

The western blot provided evidence of peptide structure similarities between the two spheroidin proteins. The cross-reaction

between the AmEPV spheroidin antibodies and the 105 kDa spheroidin band of MsEPV clearly indicated epitope commonality. The fact that the anti-serum directed against the 115 kDa spheroidin protein from AmEPV was polyclonal probably accounted for the very faint reaction with *Nosema locustae* spore proteins. However, the reaction against the MsEPV spheroidin band was more pronounced (Fig. 4), and clearly indicated a cross-reaction and the presence of similar antigenic determinants on the 105-115 kDa polypeptide subunits.

Based on both the presence of cross-reactivity between anti-AmEPV spheroidin serum and MsEPV, and the presence of limited homology between the AmEPV and MsEPV genomes, this study further supports the premise that the 115 kDa peptide from CbEPV, versus the 50 kDa peptide, is the correct spheroidin subunit. The anti-AmEPV serum reacted against the larger fragment of both virus genomes, versus the 50 kDa fragment proposed by Yuen *et al.* (1990). The fact that homology existed between the two larger peptide fragments of AmEPV and MsEPV at the genomic level supports Hall and Moyer's finding (1993) that the larger fragments of AmEPV and CbEPV show homology, while the AmEPV 115 kDa fragment shows no homology with the 50 kDa fragment of CbEPV.

Because the polyhedrin of baculovirus is functionally analogous to the spheroidin of EPV, parallels are often made between them. Smith and Summers (1982) observed that, in the case of baculoviruses, whether or not a virus replicates in the same host appears to play little role in the degree of homology observed. Zanotto *et al.* (1993) observed that baculovirus classification based on hosts may not be entirely accurate since it does not reflect the genetic relatedness of the viruses. In the case of EPVs, while morphological and biochemical similarities exist between EPVs infecting different insect orders, homology, while evident to a minor degree, decreases radically when observed between viruses from different host orders.

Although previous studies (Streett *et al.*, 1986) (Langridge *et al.*, 1983) indicated that there was no homology between the entire genomes of MsEPV and AmEPV, evidence of conserved structural organization, microscopically, biochemically, and immunologically, indicated otherwise, especially with respect to the spheroidin gene. However, further tests would be needed to determine the degree of homology that exists.

Future investigation of this topic could include sequencing of the MsEPV PCR probe which hybridized to the AmEPV fragment. Sequencing of the target AmEPV fragment might also assist in determining the degree of homology, and whether or not there is spheroidin gene sequences within this fragment. It would be interesting to note if the probe represented spheroidin gene sequences or not, as one would expect given the method by which this probe was derived.

