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Characterization of the Nuclear Polyhedrosis Virus of the Cotton Bollworm, *Heliothis armigera*

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ABSTRACT

The nuclear polyhedrosis virus (NPV) was isolated from the cotton bollworm, *Heliothis armigera* found in Thailand. Ultrastructural studies revealed the virus of single embedded type with the characteristic symptoms of the NPV infection. The virus inclusion bodies (polyhedra) varied considerably in shape ranging from spherical to cuboidal with the average sizes of 0.98 μm. The virions and nucleocapsids were rod-shaped with the mean width and length of 72.79 × 354.79 nm. and 30.13 × 276.87 nm. respectively. Virus propagation was achieved by *per os* inoculation of the third instar larvae with the preparation of polyhedra. The polyhedra were extracted from infected larvae and purified by low speed centrifugation. Virus particles were released from polyhedra by sodium bicarbonate dissolution. The viral nucleic acid, extracted by phenol: chloroform treatment and precipitated by absolute ethyl alcohol, had a 260/280-nm. ratio of 2.01. Nucleic acid type was confirmed by DNase and RNase treatments. Digestion of the DNA genome with EcoRI, Hind II, PstI, SacII, HpaI and ScaI showed specific restriction endonuclease cleavage patterns. Preliminary analysis of restriction endonuclease profiles indicated a molecular weight of approximately 65×10^6 for this viral DNA.

INTRODUCTION

The American bollworm, *Heliothis armigera* is a major worldwide pest which attack several different food and fiber crops. One of these crops, cotton is of major economic importance in Thailand. Chemical insecticides provided an effective control measure until resistance to all available insecticides began to develop in this insect species. To obtain reasonable level of control, the amount and frequency of insecticide applications progressively increased leading to the serious development of several problems especially the environmental pollution. Thus the alternative methods of control were called for, with the emphasis on natural systems. Biological insecticides received prime consideration to be developed as pesticide agents. Baculovirus, in particular nuclear polyhedrosis viruses offer an excellent potential candidate for this purpose. They possess unique features of host specificity, stability, safety, producibility and efficacy against major insect pests that make them attractive as safe biological control agents.

Nuclear polyhedrosis viruses (NPVs) are insect viruses belonging to the family Baculoviridae which is characterized by the following basic features: an enveloped, rod-shaped virion (approximately 50 × 250 nm.) containing a single molecule of double-stranded, circular supercoiled DNA (Burgess, 1977; Kelly, 1977 and Summers, 1977). The molecular weight is 58 to 110 million daltons (G + C content 28 to 59%), and the DNA

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content of the infective particle is 8 to 10% (Fenner, 1976; Matthews, 1982). NPV of *H. armigera* has been isolated from field collected larvae in Thailand. Its efficacy for bollworm control has been recognized since 1976, (Israngul Na-Ayuthaya, 1976). However this virus has not been fully exploited due to the lack of basic information especially the molecular biological data of the virus. Therefore basic characterization studies are required to provide data necessary for improving the efficacy and broadening host range of the virus.

NPVs of *Heliothis* spp. isolated from different locations in Thailand have not been reliably identified. The recent application of restriction endonuclease analysis has proven useful in determining the genotypic relationship among various NPV isolates. Lee and Miller (1978) revealed the presence of many closely related variants of *Autographa californica* nuclear polyhedrosis virus differing in the number and size of the DNA fragments produced by restriction endonuclease digestion. The similar method has been applied to determine the genotypic variation among isolates of *Heliothis* spp. nuclear polyhedrosis viruses from different geographical regions (Getting and McCarthy, 1982). We are attempting to identify various virus isolates found in Thailand utilizing restriction endonuclease analysis. Proper identification will provide an opportunity to select better strains or variants of the viruses suitable for pest control program.

The objective of this study was to characterize the *H. armigera* NPV found in Thailand and to determine its restriction endonuclease cleavage pattern. The methodology and informations obtained will be further used for identification and improving the efficacy of this virus.

MATERIALS AND METHOD

Virus source. The nuclear polyhedrosis virus was obtained from diseased larvae of *H. armigera* collected from the cotton field at Utong district, Supanburi province, Thailand. The virus was propagated in host larvae which are fed on artificial diet. Third instar larvae were infected *per os* with virus suspension. Diseased larvae were collected and kept frozen until adequate materials were obtained for purification.

Electron microscopy. Investigations of the diseased tissues and purified virus preparation were performed to determine the morphological characters of the virus. Pieces of tissue from diseased larvae were fixed for 3 hr. in 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate, postfixed for 1 hr. in buffered 1% osmium tetroxide. The tissues were then dehydrated through an ethanol series to propylene oxide, and embedded in an Epon-Araldite mixture (Mollenhauer, 1963). Ultrathin sections were made and stained with uranyl acetate and lead citrate. Purified virus particles were observed by negative staining with 2% uranyl acetate. All viewing was done with a JEOL-JEM-100 S electron microscope operating at 80 kv.

Virus purification. Infected larval cadavers were homogenized in distilled water and filtered through 3 layers of cheesecloth. The filtrate was then purified on 30% (w/w) sucrose solution at 10,000 rpm. for 30 min. in the JA-14 rotor on a Beckman J21-C centrifuge. After dialysing overnight, the polyhedra were pelleted by low speed centrifugation. The virus particles were released by dissolving the polyhedra in 0.1 M Na_2CO_3 pH 11.2 for 20 min. at room temperature. The reaction was stopped by lowering the pH of the mixture solution to 8.0 with 0.005 M tris buffer. Undissolved polyhedra and debris were removed from the solution by centrifugation at 2,500 rpm. for 10 min. in JA-14 rotor. The supernatant containing virus particles was subsequently centrifuged into a pellet at 16,000 rpm. for 90 min. The virus was resuspended in 0.005 M tris buffer pH 8.0 and kept frozen.

Purification of viral DNA. A modification of the method of McIntosh and Ignoffo (1986)

was used. The purified virus particles were disrupted using SDS (final concentration 0.5%) and incubated at 37°C for 60 min. The DNA was extracted three times with an equal volume of phenol: chloroform. The DNA was then precipitated from the aqueous phase by adding two volume of absolute ethanol and 0.3 M sodium acetate pH 4.8 and kept at -20°C for 1 hr. After centrifugation at 10,000 rpm. for 15 min. at 4°C, washed the pellet with 70% ethanol and reconcentrated by low speed centrifugation. The pellet was then vacuum dry for 30 min and resuspend in TE buffer (0.01 M tris and 0.001 M EDTA pH 7.4) and stored at-20°C. The 260/280 nm ratio of the DNA obtained was determined by Hitachi Model 200-20 Spectrophotometer. The DNA was quantitated using the relationship 1 OD_{260} = 50 μg DNA.

Restriction endonuclease digestion. Purified viral DNA was digested with EcoRI, Hind III, PstI, SacII, HpaI and ScaI restriction enzymes. Each digestion mixture (total of 20 μl) contained 15 μl of viral DNA (0.034 mg/ml in TE buffer), TBE buffer (0.089 M tris base, 0.089 M Boric acid and 0.002 M EDTA, pH 8) and 5 units of enzyme (1-2 μl). These mixtures were incubated for 1 hr. at 37°C. Enzyme activity was stopped by adding 5 μl of 0.1% bromophenol blue, 40% Ficoll and 5 mM EDTA. Samples were then layering on the agarose gel. Phage DNA digested with Hind III and double digested with Hind III and EcoRI were used to ensure the digestion procedure and to provide molecular weight standard.

Agarose electrophoresis. Horizontal 0.7% agarose gels dissolved in TBE buffer were prepared on electrophoresis apparatus. The gels of 99 × 175 cm had 9 wells with the capacity of 2 μl per well. The digested DNA was electrophoresed at a constant voltage of 90 V for 2 hr at room temperature. Gels were statimed in a solution of electrode buffer (TBE buffer) containing 0.1% μg/ml of ethidium bromide. DNA fragments were visualized and photographed using a UV transilluminator.

RESULT AND DISCUSSION

Ultrastructure studies. Electron microscopic investigation of the diseased tissue demonstrated the virus isolated from *H. armigera* was nuclear polyhedrosis virus of single-embedded type. The virus replicated in the nuclei of the infected cells (Figure 1 a and b). The diffuse mass of chromatin so called the virogenic stroma where the virus particles were assembled, was clearly developed in the central area of the nucleus. The infected nuclei was hypertrophied and at the late stage of infection, almost filled the cell leaving cytoplasm in loose mass at the periphery of the cell. Large number of virus particles were produced in the nuclei, however only small number were occluded in the polyhedra (Figure 1 c and d). Since the virus particle itself, not the polyhedra, is the infectious unit, therefore, in comparison to other NPVs, higher concentration of polyhedra suspension may be necessary to produce infection in *H. armigera*. There are morphological distinct between this virus and the isolate of *Bombyx mori* NPV found in Thailand. They were both considered the single-embedded type of NPV. In *H. armigera* NPV, all virions contained only one nucleocapsids but in *B. mori* NPV, a significant proportion of the occluded virion possess two or three nucleocapsids per envelope (Attathom and Sinchaisri, 1987). There were more virions embedded in the polyhedra of *B. mori* NPV than that occurred in *H. armigera* NPV.

Ultrastructural studies revealed that the polyhedra inclusion bodies of *H. armigera* NPV varied greatly in shape. They were cuboidal tetrahedra, dodecahedra and irregular (Figure 1 and 2). It was reported that in any one virus, the shape of the mature polyhedra is usually the same and many NPVs known to be differently produced polyhedra of the same shape (Federici, 1986). The *B. mori* NPV has mature polyhedra of cuboidal shape which can be recognized easily.

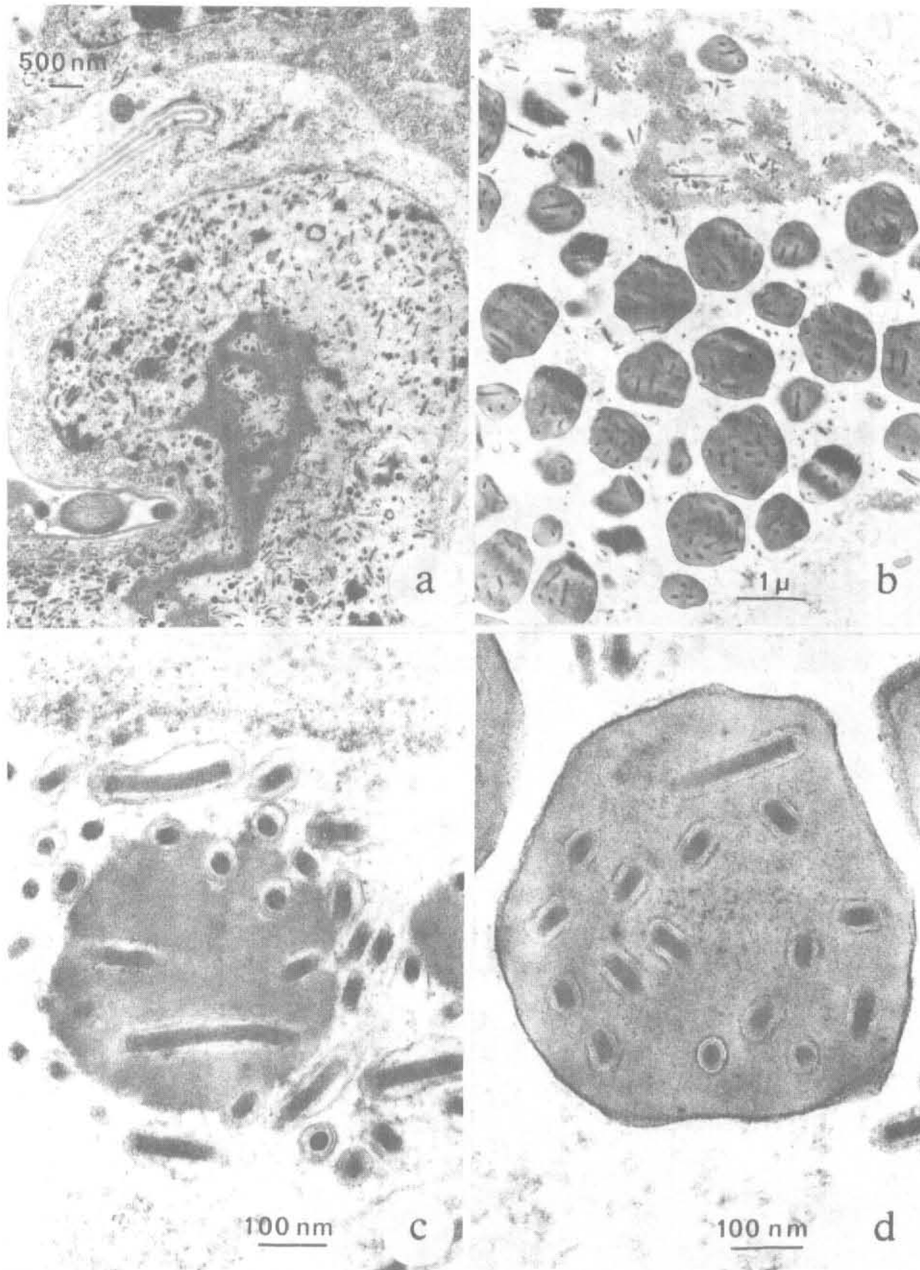

Figure 1 Electron micrographs of *Heliothis armigera* NPV. a) Virus particles replicate and assembly in the hypertrophied nucleus. b) Polyhedra inclusion bodies in the disintegrated nucleus. c) Virions are singly embedded in the polyhedra inclusion body. d) Mature polyhedra inclusion body.

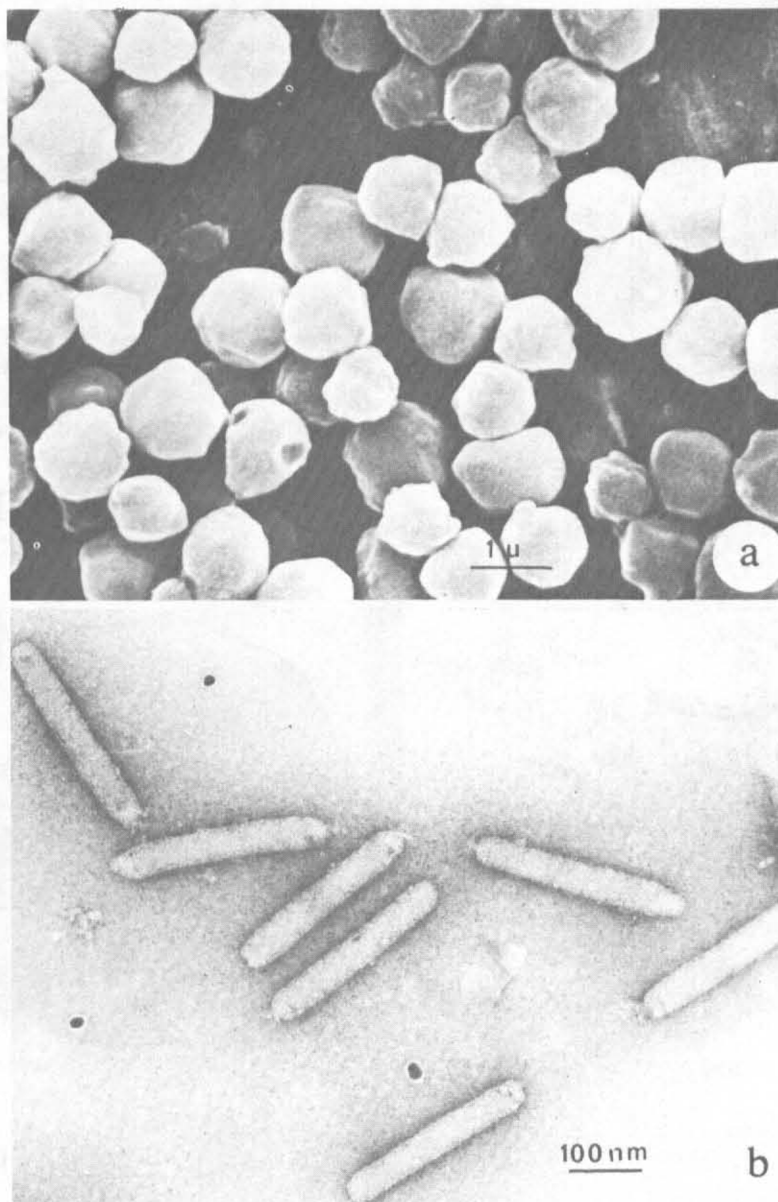

Figure 2 Purified preparation of *Heliothis armigera* NPV. a) Scanning electron micrograph of purified polyhedra inclusion bodies. b) Negatively stained preparation of purified nucleocapsids after alkaline dissolution of the polyhedra.

The polyhedra shape of *H. armigera* NPV, therefore is of little value for virus identification.

The polyhedra of *H. armigera* NPV also varied enormously in size (Figure 1 and 2). However their range in size may be depended on their stage of development. From ultrathin section electronmicrographs the mean size of 0.98 μm was measured for the polyhedra of this virus isolate. The large sharp edges cuboidal inclusion bodies of 2.1 μm were also found. This structure had been previously reported in polyhedra preparation of several Lepidopterous NPVs (Attathom, 1978). It was suggested that this large inclusion devoid of virions are probably aggregations of polyhedral protein (Federici, 1986).

The virions of *H. armigera* NPV were rod-shaped measuring 354.79 $\times$ 73.44 nm. and consisted of only one rod-shaped nucleocapsid. The mean length and width of the nucleocapsid were 276.87 $\times$ 30.13 nm. Teakle (1973) reported the virions and nucleocapsids of *H. armigera* NPV measured 325 $\pm$ 4.9 $\times$ 76 $\pm$ 1.1 nm. and 268 $\pm$ 18.8 $\times$ 54.5 $\pm$ 1.5 nm. respectively. Eventhough this virus isolate is of single-embedded type, the different in size between the virion and the nucleocapsid is remarkably noticed. This suggested an amorphous difinite layer existed between the nucleocapsid and the envelope. This layer may extend due to the chemical treatments during the process of tissue preparation.

Virus purification and nucleic acid extraction. Polyhedra were extracted from diseased larvae and purified by low speed centrifugation. This purified polyhedra were air-dried, gold coated and observed with JEOL-JSM-35 CF scanning electron microscope. The result indicated that the partially purified polyhedra suspensions obtained were satisfactory clean enough for further study (Figure 2 a). Virus particles were released from the polyhedra by alkaline dissolution. It was suggested more vigorous treatment may be necessary for complete polyhedra dissolution. When using 0.1 M Na_2CO_3 at pH 11.2, the dissolution time as long as 20 min. was required while 5-10 min. was sufficient for dissolving other NPVs polyhedra. Nonidet P40 detergent was previously used for removing the envelope of the virions, however in this present study the alkaline liberated virions readily loss their envelope. Intact nucleocapsids were observed after the alkaline treatment (Figure 2 b) but distorted or degraded nucleocapsids were also presented. This is the first report of obtaining purified nucleocapsids of this virus isolate. Modified purification procedures are currently being investigated in order to achieve high yield of purified intact nucleocapsids.

Sufficient viral DNA for restriction endonuclease analysis was obtained from the DNA extraction procedure described. The DNA had the highest ultraviolet absorption at 260 nm. which is characteristic of the nucleic acid and had a 260/280 nm. ratio of 2.01 (Figure 3 a.) The presented purification procedure is precise, reproducible and result in considerable good preparation of DNA in both quality and quantity.

DNase and RNase digestions were used to ensure type of nucleic acid obtained. The nucleic acid was digested by DNase, RNase and double digested by both enzymes. Digestion mixture of nucleic acid sample and enzyme (1:20) was incubated for 2 hr. at 37°C. Enzyme activity was stopped and the samples were analyzed by agarose gel electrophoresis according to the method described above. Only one distinct band was observed when electrophoresed undigested nucleic acid and that digested by RNase (Figure 3 b). The nucleic acid was completely digested by DNase. The result indicated the nucleic acid extracted is DNA which was homogeneous in respect to the forms of DNA obtained. McCarthy *et. al* (1979) reported that DNA preparation of *Lymantria dispar* NPV contained 10-25% linear duplex DNA and 75-79% open circular and supercoiled DNA. They suggested baculovirus DNAs exist as supercoiled molecules and that open cir-

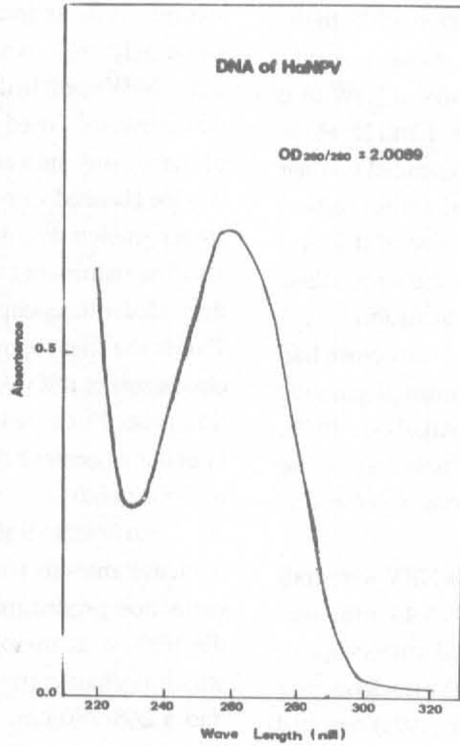

Figure 3 Ultraviolet absorption spectrum of DNA of *Heliothis armigera* NPV. The DNA had a 260/280 nm. ratio of 2.01.

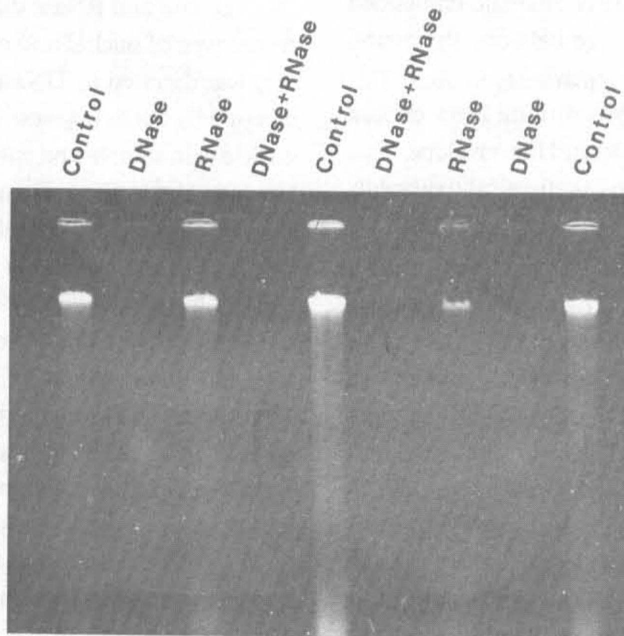

Figure 4 DNase and RNase analysis of the nucleic acid extracted from the purified nucleocapsids of *Heliothis armigera* NPV.

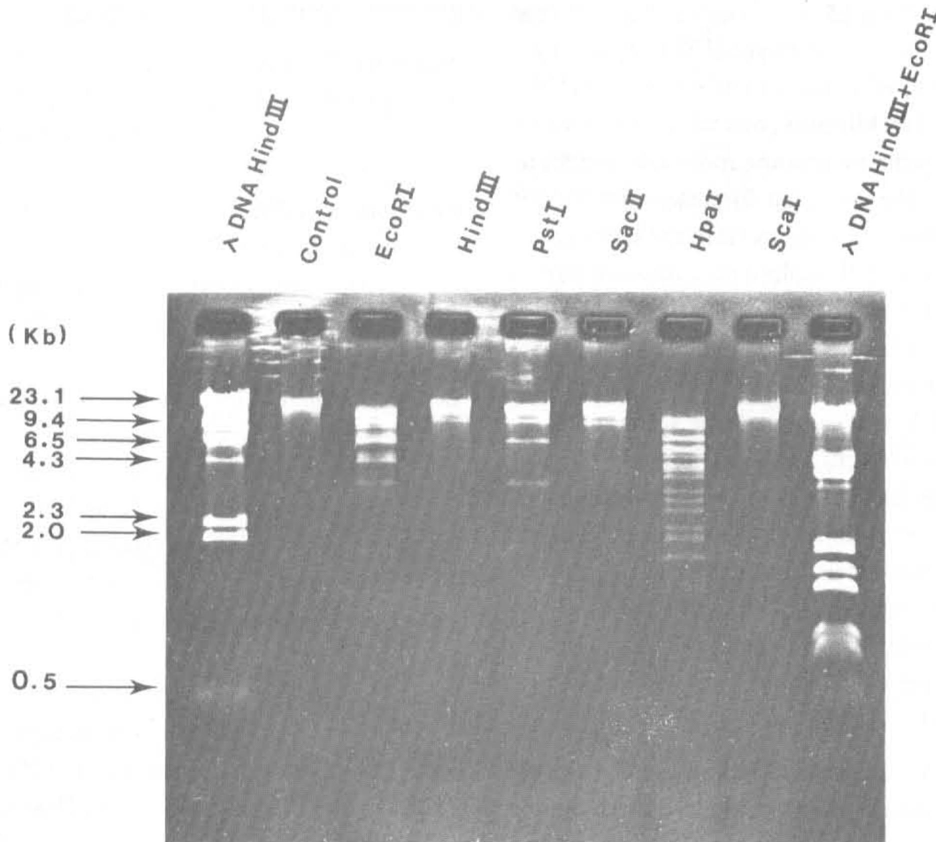

Figure 5 Restriction endonuclease analysis of the DNA of single-embedded *Heliothis armigera* NPV. The scale illustrates the sizes of DNA fragments in kilobase pairs.

cular and linear duplex DNAs are a result of degradation. We assumed from the result of a single band on agarose gel that only one form of DNA was obtained in this present study.

Restriction endonuclease. The fragmentation profiles resulting from the digestion of the *H. armigera* NPV DNA with EcoRI, Hind III, PstI, Sac II, HpaI and ScaI are shown in Figure 5. It is obvious that specific cleavage patterns were obtained when the viral genome was digested with different restriction enzymes. There was no reaction when ScaI enzyme was used. Hind III and Sac II generated only a few relatively large fragments and Pst I produced 4 distinct bands. The Hind III profile does not resemble those of wild isolates of *Heliothis* spp. NPV from different geographical regions reported by Gettig and McCarthy (1982). Digestion with HpaI gave more resolution and revealed many smaller distinct restriction fragments which were little different in size. The profile of EcoRI although similar, was not identical to that reported by Williams and Payne(1984). In our study, EcoRI produced bands which were more discrete and one additional band of 1.5 kilobase pair was observed. Based on the above restriction endonuclease analysis, the size of the DNA fragments of this virus isolate ranged from 23 kilobase pairs to as low as 1.0 kilobase pairs. The sum of molecular weights of DNA fragments was 98 kilobase pairs which was similar for most of restriction endonuclease used and indicated a molecular

weight of about 65 $\times$ 10^6 daltons for this viral DNA. Williams and Payne (1984) reported genome size of *H. armigera* single-embedded NPV DNA was 109 kilobase pairs which is consistent with approximate genome molecular weight of 73 $\times$ 10^6. The significant differences in molecular weight values obtained by different laboratories for the same NPV isolate may possible due to errors in technique or heterogeneous DNA preparations. McCarthy *et. al.* (1979) estimated the molecular weight of the DNA from *Lymantria dispar* NPV and suggested the value obtained by sucrose gradient sedimentation and electron microscopy tended to be higher when compared to the values obtained by reassociation kinetics and restriction endonuclease digestion. We attempt to further analyse *H. armigera* NPV DNA by the above-mentioned techniques and the molecular weight value of this viral genome can be confirmed.

Restriction endonuclease analysis, however is more practical since it offers a relatively simple and defined method with acceptable accuracy. This method has been used to determine the genotypic variations between viruses. The genomic heterogeneity can be detected not only among viruses of different hosts but also viruses isolated from the same host in different geographical regions (Getting and McCarthy, 1982). Moreover alterations in the viral genome upon continuous passage in cell culture can be detected by observing the restriction profiles (Burand and Summers, 1982). In view of the usefulness of this method, we are continuing identify different virus isolates found in Thailand utilizing restriction endonuclease analysis.

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