Biological and biochemical characterization of isolates of *Helicoverpa armigera Nucleopolyhedrovirus* [HaNPV] from different geographic locations of India

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Abstract

Six strains of HaNPV *collected from different places of India were compared for their biological and biochemical characteristics. Based on the bioassay tests against second and third instar larvae of* Helicoverpa armigera *the order of activity in increasing order is UASD-HaNPV< AK-HaNPV<TN-HaNPV<PAU-HaNPV<GAU-HaNPV<ICRISAT-HaNPV and no correlation was drawn between biological and biochemical characteristics. Electron microscopic observations of polyhedra, alkali disrupted polyhedra during purification and nucleocapsids are also presented.*

Key words: Nucleopolyhedrovirus, structural polypeptides, biological characterisation, *Helicoverpa armigera*

Introduction

The legume pod borer or cotton boll worm, *Helicoverpa armigera* (Hubner) is one of the most important constraints to crop production globally. It is polyphagous and attacks more than 182 plant species and is difficult to control as it has developed resistance to many common insecticides. Global crop losses due to *Helicoverpa* species exceed US$ 5 billion per annum, despite the use of US$ 1 billion worth of pesticides. Nucleopolyhedrovirus (NPV) (Family Baculoviridae) is considered to be an attractive alternative for control of this polyphagous pest. *Helicoverpa armigera nucleopolyhedrovirus* (HaNPV) is a naturally occurring pathogen of *H. armigera,* which has wide host distribution in Asia, Africa and Australia (Fauquet *et al.,* 2004; Grzywacz *et al*., 2005). In USA, NPV was first produced as viral insecticide against *Helicoverpa* species and registered by the Environmental Protection Agency (EPA) for agricultural use in the year 1973. Since then, several isolates and strains of NPV have been used to develop commercial biopesticides in America, Australia, India, China and Thailand. HaNPV has been shown to be highly effective in controlling *H. armigera* on a range of crops including legumes (Cherry *et al*., 2000), oil seeds (Rabindra *et al*., 1985), cotton (Jones, 1994) and vegetables (Jones *et al.*, 1998). Pathogenicity and virulence are known to vary considerably both between species and among geographic variants of the same HaNPV (Narang *et al.,* 2001). Presently, the information about the range of genetic variability within the HaNPV group is limited. Hence, the present investigation was carried out to know the genetic variability amongst the HaNPV isolates collected from different parts of India.

Materials and methods

HaNPV isolates used in this study were collected from different geographical locations and their abbreviations used in the text are as follows: ICRISAT-HaNPV (Hyderabad); GAU-HaNPV (Gujarat); PAU-HaNPV (Ludhiana); TN-HaNPV (Coimbatore); AK-HaNPV (Akola) and UASD-HaNPV (Dharwad). These virus isolates were multiplied in laboratory reared *H. armigera* larvae. Infection was attained by diet surface contamination method with the purified virus suspension @10^8 poyhedral occlusion bodies/ml. Larvae showing the typical symptoms of the disease were collected in separate jar containing distilled water.

Purification of polyhedra

After collecting all dead larvae in distilled water, the larvae were ground in a blender. Suspension containing POBs was collected and passed through the double-layered muslin cloth or plastic strainer to remove larval debris. Virus suspension centrifuged at 5000 rpm for 10 to 15 minutes. POBs were collected as sediment at the bottom of the tube,

which was dissolved in distilled water and stored at 4ºC for further studies.

Bioassay studies.

After assessing the concentration of stock solution, six concentrations of each strain were prepared by serial dilution. Concentrations from 1.8 x 10^7 to 1.8 x 10^2 were bioassayed against second instar and concentrations from 1.8 x 10^8 to 1.8 x 10^3 were bioassayed against third instar larvae of *H. armigera* by surface contamination method (Evans and Shapiro, 1997). For each treatment three replications were maintained each with ten larvae.

HaNPV purification

HaNPV was purified from infected larval extract as per the protocol given by Maskos and Miltenburger (1981) with slight modifications.

Preparation of samples for viral protein estimation by SDS-PAGE

Purified virus samples (about 10 µl) were assayed for proteins by separating them in polyacrylamide (PAGE) gel. The purified virus sample was mixed with equal volume of Laemmli buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 5% 2-amino-thioglycerpl, 10% glycerol, 0.05% bromophenol blue) and denatured by heat treatment in boiling water-bath for 3 min. Samples were loaded into 12% SDS-PAGE and electrophoresed at 100 volts for 2 h in Broviga ® apparatus. The gel was taken out from the apparatus and silver stained to visualize the proteins (Kumar *et al*., 2004).

Estimation of molecular weights. The molecular weights of the protein bands were estimated by comparing with the protein molecular weight standards (MBI Fermentas Cat# SM0441). Standard graph was prepared by plotting the distance migrated by protein standards on X-axis and molecular weights on Y-axis. The molecular weights of viral proteins were calculated from the standard graph by plotting the distance migrated by the viral proteins. Standard graph was prepared for each PAGE and average molecular weight calculated from three graphs was taken as molecular weight of the viral protein. The purified virus preparations were observed under an electron microscope using the negative staining procedure (Summers and Paschke, 1970).

Results and discussion

Bioassays with different isolates of HaNPV against third instar *H. armigera* larvae showed that the ICRISAT isolate is more virulent with the lowest LC_{50} value of 1.5x10^3 POB/ml, which was followed by GAU-HaNPV (1.9x10^3 POB/ml), PAU-HaNPV (2.26x10^3 POB/ml), TN-HaNPV (2.3x10^3 POB/ml), AK-HaNPV (3.18x10^3 POB/ml) and UASD-HaNPV (3.7x10^3 POB/ml). The highest slope value was recorded for UASD-HaNPV (0.410) followed by GAU-

Table 1. Log concentration-probit mortality regression relationship for different isolates of HaNPV against third instar *H. armigera* (9 days after treatment)

NPV isolate	Regression equation		Heterogeneity	LC_{50}(POB/ml)	Fiducial limits		Chi-square
	Intercept	Slope			Lower	Upper	
ICRISAT-HANPV	3.83	0.36	0.465	1.50X10^3	4.75	1.1X10^4	1.40
GAU-HaNPV	3.76	0.37	0.252	1.90x10^3	97.55	9.0x10^3	0.75
PAU-HaNPV	3.80	0.35	0.552	2.26x10^3	2.88	1.9x10^4	1.65
TN-HaNPV	3.78	0.35	0.416	2.30x10^3	97.80	1.2x10^4	1.66
AK-HaNPV	3.68	0.37	0.756	3.18x10^3	0.85	2.9x10^4	2.26
UASD-HaNPV	3.50	0.41	0.551	3.70x10^3	44.50	2.2x10^4	1.65

Table 2. LT_{50} (h) values of NPV strains against third instar *H. armigera*

Concentration (POB/ml)	LT_{50} (h) values of NPV strains against third instar *H. armigera*					
	ICRISAT	GAU	PAU	TN	AKOLA	UASD
1.8x10^8	121.68	125.52	126.48	128.16	132.48	130.32
1.8x10^7	122.88	138.96	132.72	136.56	139.44	138.48
1.8x10^6	139.20	142.56	150.24	151.68	153.12	150.00
1.8x10^5	154.32	168.24	160.32	167.04	167.04	161.04
1.8x10^4	162.96	176.64	174.00	179.04	181.20	184.08
1.8x10^3	207.12	228.96	238.80	221.28	247.68	236.16

HaNPV (0.37), AK-HaNPV (0.37), ICRISAT-HaNPV (0.36), PAU-HaNPV (0.35) and TN-HaNPV (0.35) (Table 1). However, all the isolates have over lapping fiducial limits, which indicated that there was no significant difference in virulence of different HaNPV isolates tested.

The LT_{50} was calculated at each concentration. At the highest concentration ($1.8x10^8$ POB/ml) ICRISAT-HaNPV recorded the lowest LT_{50} value (121.68 h), which was followed by GAU-HaNPV (125.52 h), PAU-HaNPV (126.48 h), TN-HaNPV (128.16 h), UASD-HaNPV (130.32 h), AK-HaNPV (132.48 h). At the concentration of $1.8x10^6$ POB/ml ICRISAT isolate recorded LT_{50} value of 139.20 h and the same value was recorded for AKOLA isolate at the concentration of $1.8x10^7$ POB/ml. For all the other isolates concentration between $1.8x10^7$ and $1.8x10^6$ was required to get the same LT_{50} value. At the lowest concentration of $1.8x10^3$ POB/ml ICRISAT-HaNPV recorded the lowest LT_{50} (207.12 h), which was followed by TN-HaNPV (221.28 h), GAU-HaNPV (228.96 h), UASD-HaNPV (236.16 h), PAU-HaNPV (238.80 h), and AK-HaNPV (247.68 h) (Table 2).

Similar trend was observed against second instar *H.armigera* also where ICRISAT-HaNPV recorded the lowest LC_{50} of $3.25x10^2$ POB/ml followed by GAU-HaNPV ($3.43x10^2$ POB/ml), PAU-HaNPV ($5.28x10^2$ POB/ml), TN-HaNPV ($7.33x10^2$ POB/ml), AK-HaNPV ($8.18x10^2$ POB/ml) and UASD-HaNPV ($9.99x10^2$ POB/ml). The highest slope value was recorded for ICRISAT-HaNPV (0.400), which was followed by TN-HaNPV (0.395), PAU-HaNPV (0.385), AK-HaNPV (0.355), GAU-HaNPV (0.349), UASD-HaNPV (0.349). All the isolates have over lapping fiducial limits (Table 3). Results showed that the highest concentration ($1.8x10^7$ POB/ml) ICRISAT-HaNPV recorded the lowest LT_{50} of 118.80 h followed by PAU-HaNPV with 121.68 h, GAU-HaNPV 123.12 h, TN-HaNPV 126.24 h, UASD-HaNPV 131.76 h and AK-HaNPV 133.92 h. At higher concentrations, there was no much difference between LT_{50} values, which increased with decrease in the concentrations. At $1.8x10^5$ concentration ICRISAT-HaNPV recorded the lowest LT_{50} (148.32 h) followed by GAU-HaNPV (149.76 h), PAU-HaNPV, TN-HaNPV (150.72 h), AK-HaNPV and USAD-HaNPV (154.32 h). At the lowest concentration of $1.8x10^2$ the increasing order of LT_{50} values were ICRISAT-HaNPV followed by GAU-HaNPV, PAU-HaNPV, TN-HaNPV, AK- HaNPV, UASD- HaNPV (Table 4).

Bio-assay studies against second and third instar larvae of *H. armigera* showed subtle variation in virulence. Based on the LC_{50} values on increasing order of activity was UASD-HaNPV<AK-HaNPV<TN-HaNPV<PAU-HaNPV<GAU-HaNPV<ICRISAT-HaNPV. The time required to cause 50 per cent mortality (LT_{50}) of larvae of *H. armigera* fed on various concentrations of different

Table 3. Log concentration-probit mortality regression relationship for different isolates of HaNPV against second instar *H. armigera* larvae (9 days after treatment)

NPV isolate	Regression equation		Heterogeneity	LC_{50}(POB/ml)	Fiducial limits		Chi-square
	Intercept	Slope			Lower	Upper	
ICRISAT-HANPV	3.990	0.400	0.417	$3.25X10^2$	7.83	$1.73X10^3$	1.25
GAU-HaNPV	4.114	0.349	0.266	$3.43x10^2$	16.09	$1.60x10^3$	0.80
PAU-HaNPV	3.950	0.385	0.155	$5.28x10^2$	99.98	$1.50x10^3$	0.46
TN-HaNPV	3.860	0.395	0.287	$7.33x10^2$	124.56	$2.30x10^3$	1.14
AK-HaNPV	3.965	0.355	0.350	$8.18x10^2$	86.09	$3.20x10^3$	1.40
UASD-HaNPV	3.960	0.349	0.244	$9.99x10^2$	171.07	$3.29x10^3$	0.97

Table 4. LT_{50} (h) values of NPV strains against second instar *H. armigera*

Concentration (POB/ml)	LT_{50} (h) values of NPV strains against second instar *H. armigera*					
	ICRISAT	GAU	PAU	TN	AKOLA	UASD
$1.8x10^7$	118.80	123.12	121.68	126.24	133.92	131.76
$1.8x10^6$	131.28	136.32	134.64	141.12	142.80	145.68
$1.8x10^5$	148.32	149.76	150.72	150.72	154.32	154.32
$1.8x10^4$	157.20	162.72	165.60	167.04	165.84	167.28
$1.8x10^3$	175.68	180.72	178.32	181.20	179.04	189.84
$1.8x10^2$	205.20	210.96	214.80	234.24	246.96	254.64

geographic isolates of HaNPV showed that at the highest concentration ICRISAT-HaNPV isolate recorded the lowest LT_{50} value against the second and third instar *H. armigera* which was closely followed by GAU-HaNPV, PAU-HaNPV and TN-HaNPV. Similar observations were made against the second instar *H. armigera* larvae at the lowest concentration also. Hughes *et al.* (1983) compared the time mortality response of *H. zea* to 14 isolates of HzNPV and identified six activity classes. Shapiro *et al.* (1984) tested 19 NPV isolates of *L. dispar* and reported nearly 1000-fold difference in activity. Rabindra *et al.,* (1992) demonstrated the tremendous variation in virulence among the three HaNPV isolates and recorded the lowest LC_{50} value of $3.47X10^4$ POBs/ml for the HaNPV isolate from Nilgiris. Somasekhar *et al.* (1993) on characterizing five Indian isolates of HaNPV found that the most virulent isolate was that from Ooty with the lowest LC_{50} value of $2.54X10^3$ POBs/ml, followed by the isolate from Coimbatore ($2.97X10^3$ POBs/ml) where as the Rajasthan isolate was least effective with LC_{50} value of $13.08X10^3$ POBs/ml. Geetha and Rabindra (1999) found that among 11 HaNPV isolates collected from different regions in India, Negamum and Ooty isolates from Tamil Nadu were significantly more virulent with LC_{50} values of 83.807 and 93.926 POBs/cm^2 respectively and the Rajasthan isolate was the least potent with LC_{50} value of 111.778 POBs/cm^2.

In the present study the LC_{50} values ranged from $1.5x10^3$ to $3.7x10^3$ with overlapping fiducial limits which indicated that the differences in activity among the HaNPV isolates were not significant. All these studies indicated that there is a significant variation in LC_{50} values with overlapping fiducial limits and suggests the use of locally produced NPV appeared to be more useful for managing the respective insect pests than commercially available NPV from other parts of the country. The variation in the activity of different isolates may be due to different reasons. Inherent genetically controlled factors may be an important reason. The other reason may be that the different isolates had different number of passages in the host either under natural conditions or in the laboratory (Geetha and Rabindra, 1999).

Log concentration-probit mortality relationship indicated Lower slope values for all the isolates which show greater variability (Table 3). Arora *et al.,* (1997) reported slope values varying from 0.58 to 0.96 for the five HaNPV isolates evaluated against second instar larvae of *H. armigera.* The low slope of dosage-mortality curves for insect pathogens often indicates a more stable host-pathogen relationship.

HaNPV purification and analysis of the viral proteins and DNA

Purification of *H. armigera* NPV was achieved by differential centrifugation, alkali dissolution of polyhedral occlusion bodies using a high pH buffer (10.9) and treatment with detergent (NP 40), followed by centrifugation of partially purified preparations through 25-60% linear sucrose gradients, for all the isolates 2-3 diffused light scattering zones (named as Zone-1 and Zone-2) were observed. Light scattering fractions were collected and analysed separately in PAGE gels for viral proteins. This revealed that both the Zone-1 and Zone-2 contained the HaNPV nucleoprotein of size ca. 32 kDa. However, the Zone-2 contained few additional proteins of higher and lower molecular weights. It appeared that the formation of multiple zones depended on the virus concentration, degradation of the proteins during the purification process and state of the virus culture at the time of harvest. Analysis of sample layer revealed that it also contained the polyhedrin of size ca. 32 kDa. The PAGE analysis of various samples clearly showed that the purified virus preparations of HaNPV contained ca. 32 kDa protein and a few minor proteins of variable molecular weights.

Electron microscopy

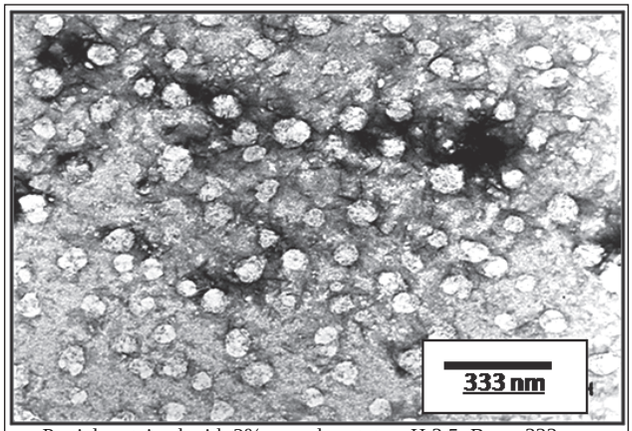
At various stages of purification the presence of nucleocapsids and purity of preparations were tested under electron microscope, which confirmed the presence of nucleocapsids.

Infected larval extract, samples after detergent [1% (v/v) NP40] treatment and purified HaNPV preparations were

Table 5. Molecular weights (kDa) of the major polypeptides recorded from six HaNPV isolates

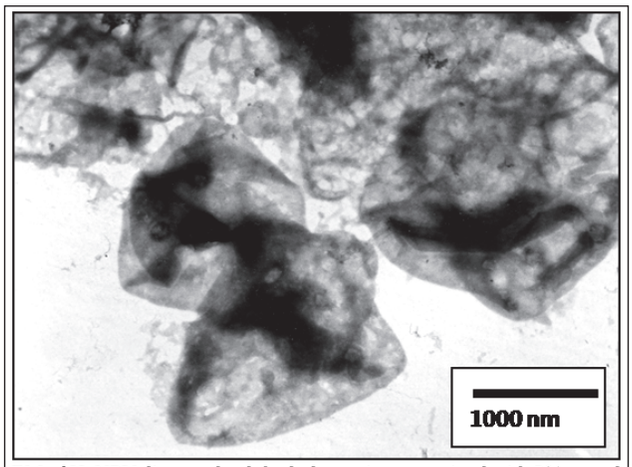
Major polypeptides	ICRISAT	UASD	TN	AK	PAU	GAU	Average size
	MEAN ± SD	*MEAN± SD*	*MEAN± SD*	*MEAN± SD*	*MEAN ± SD*	*MEAN ± SD*	
1	42.66 (±2.30)	41.66 (±2.51)	41.66 (±2.51)	41.66 (±2.51)	42.33 (±2.08)	44.00*	42.32 (±0.92)
2	35.50 (±0.70)	34.66 (±1.52)	34.33 (±2.08)	34.33 (±1.52)	34.66 (±1.52)	35.00*	34.74 (±0.27)
3	32.33 (±1.52)	31.66 (±1.52)	31.66 (±1.52)	31.66 (±1.52)	31.66 (±1.52)	31.66 (±1.52)	31.77 (±0.44)
3.1	nd	30.33 (±0.57)	31.00 (±1.73)	30.66 (±1.15)	30.66 (±1.15)	nd	30.66 (±0.27)
4	nd	nd	nd	nd	nd	19 (±1.41)	

nd = not detected; values from three experiments; * = detected in one of the three preparations



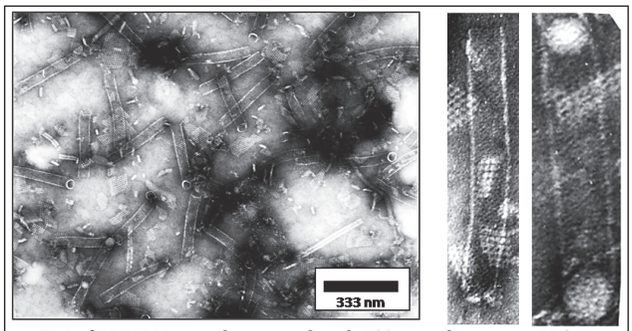
Particles stained with 2% uranyl acetate, pH 3.5. Bar = 333 nm

Figure 1. Electron micrograph of negatively stained HaNPV polyhedral particles



EM of HaNPV disrupted polyhedral protein sacs stained with 2% uranyl acetate, pH3.5. Bar = 1000 nm.

Figure 2. Electron micrograph of negatively stained HaNPV polyhedral protein sacs



EM of HaNPV particles stained with 2% uranyl acetate, pH 3.5. Bar = 333 nm. Single particles were magnified and placed at far right

Figure 3. Electron micrograph of negatively stained HaNPV purified particles after NP40 treatment

observed under electron microscope for polyhedral particles. Initial studies on the extract of the diseased larvae showed the presence of large polyhedral particles of diameter ca. 78 nm at 10,000x magnification (Fig. 1). After the dissolution with alkali, empty polyhedral sacs of 2.31 µm length and 2.05 µm width were observed (Fig. 2). Observations on final purified sample revealed bacilliform to cylindrical rod shaped particles with 282 x 49 nm (Fig. 3). Similarly, Tuan *et al*., (1999) reported that the occlusion bodies of HaNPV isolated in Taiwan were irregular shape with size ranged from 0.79 ± 0.22 µm and the nucleocapsids were bacilliform to cylindrical tubular shaped structures with dimensions of 319 ± 7.80 ×44.45 ± 4.54 nm.

Comparative analysis of viral proteins of different isolates

Purified samples of HaNPV isolates from ICRISAT, UASD, TN, AK, PAU and GAU were analyzed in 12% SDS-PAGE gels for proteins (Fig. 4). This has revealed that all the isolates have 4 to 5 major polypeptides of 42.32 (± 0.92) kDa, 34.74 (± 0.27) kDa, 31.77 (± 0.44) kDa, 30.66 (± 0.27) kDa and 19 (± 1.41) kDa, and several minor peptides (Fig. 4). Three major proteins were present in all except in GAU isolate. The molecular weights of the major proteins were nearly similar, but not identical (Table 5). GAU HaNPV sample was unique in that it is devoid of the ca. 42 and ca. 34 k Da protein.

Several minor proteins were also seen in the gel (indicated with arrows). GAU isolate recorded one extra protein of 19 (± 1.41) k Da (Lane 7 in Fig 4; Table 5). It was also noticed in other isolates but it was not as conspicuous as in case of GAU. Hence the purified preparation after sucrose gradients was observed under electron microscope. As in ICRISAT isolate, rod shaped nucleocapsids, with 317 x 45 nm were observed in GAU isolate also.

Summers and Smith (1978) studied the structural polypeptides of eight insect baculoviruses which revealed a complex but unique composition of 15 to 25 bands with molecular weights ranging from 15,000 to1, 60,000 Daltons. *A. californica* MNPV capsids contained two major polypeptides VP18.5 and VP37, *R. ou* MNPV capsids contained VP16, VP18, VP30 and VP36, *A. gemmatalis* MNPV contained one major capsid protein VP29 and major capsid proteins of *H. zea* SNPV were VP16,VP28 and VP63. Kelly *et al.,* (1980) observed high degree of similarity between the polypeptides of two SNPVs of *H. armigera and H. zea.* Monroe and Mc Carthy (1984) characterized the structural polypeptides of *H. armigera* Nucleopolyhedrovirus from India, China and USSR. For Indian isolate the molecular weights of polypeptides ranged from 14.2 to 90.0 kDa.

Lane 1, Protein molecular weight markers
Lane 2, ICRISAT
Lane 3, UASD
Lane 4, TN
Lane 5, AK
Lane 6, PAU
Lane 7, GAU

Resolution of purified HaNPV protein preparations from various locations (Lanes 2 – 7) in 12% SDS-PAGE and gels were silver stained.

Four major proteins (indicated as 1, 2, 3 and 4) are present in all isolates. The molecular weights of the major proteins are nearly similar, but not identical (also see Table 5). The GAU sample (lane 7) is unique in that it lacks the ca. 34 (2) and ca. 42 (1) kDa proteins. Several minor proteins are also seen in the gel (indicated with arrows). Note that dashed lines overlaid on figure for visualizing difference in migration of protein bands.

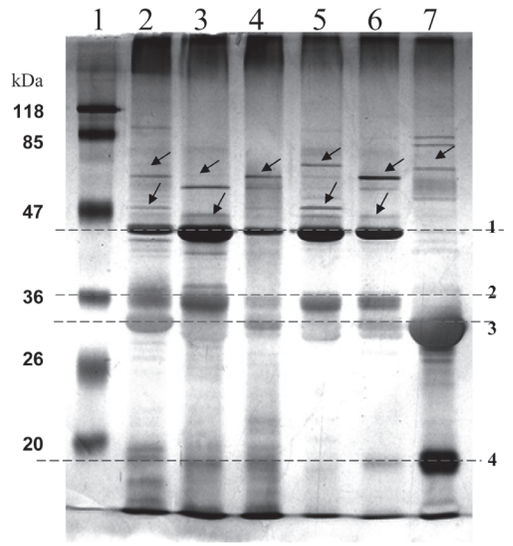


Figure 4. Purified HaNPV protein profiles of 6 isolates from various locations in India in 12% SDS-PAGE

Bioassay studies against second and third instar larvae of *H. armigera* with the six HaNPV isolates collected from six geographic locations of India revealed that the ICRISAT-HaNPV was superior among all. The PAGE analysis of various samples clearly showed that the purified virus preparations of HaNPV contained ca. 32 k Da protein and a few minor proteins of variable molecular weights. Electron microscopy observations of final purified sample revealed the presence of bacilliform to cylindrical rod shaped virus particles with 282 x 49 nm size. Comparative analysis of viral proteins of different isolates revealed that the presence of 4 to 5 major polypeptides in all the isolates except GAU-HaNPV. GAU HaNPV sample was unique and devoid of the ca. 42 and ca. 34 k Da protein.

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