

[Research]

Purification and characterization of antiviral protein from silkworm fecal matter

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ABSTRACT

Antiviral proteins (AVP), present in silkworm fecal matter, show activity against nuclear polyhedrosis virus (NPV) *in vitro* and *in vivo*. The extract of silkworm fecal matter prepared in phosphate buffer solution of pH 7.5 was subjected to 50% solid ammonium sulfate precipitation to enrich AVP, then which was dialyzed. The dialysate was applied to the column containing silica gel-G, the column elutes were purified by gelfiltration chromatography. The gelfiltration pattern gave three protein peaks A, B and C. The protein obtained from peak fractions of peak A is found to be active against NPV *in vitro*. Whereas the proteins were obtained from peak fractions of peaks C and B were not shown activity against NPV *in vitro*. The peak A fractions were collected and further purified by High Pressure Liquid Chromatography (HPLC) using C₄ column. Purified AVP of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) resulted in two protein bands with the molecular mass of 23 KD and 16 KD. Thymol sulphuric acid method of carbohydrate staining indicated that both of these protein bands are glycoproteins. AVP activity is determined *in vitro* by precipitation reaction. *In vivo* activity of the AVP is confirmed by conducting the bioassay in silkworms.

Keywords: Antiviral protein; Silkworm; *Bombyx mori*; Nuclear polyhedrosis virus.

INTRODUCTION

Silkworm excrement along with other substances contains glycoproteins and chlorophyll derivatives. So far, very few antiviral substances are isolated and purified from silkworm fecal matter. The earlier workers reported that the antiviral activity is due to the presence of chlorophyll like substance associated with a glycoprotein (Drazeniek *et al.*, 1971, Lewin *et al.*, 1980, Hirayama *et al.*, 1993, Hiraki *et al.*, 1996 and 1997, Akihiro Hiraki *et al.*, 2000). Suzuki (1936) and Aizawa (1962) investigated the inhibitory and antiviral properties of digestive juice of *Bombyx mori* larvae and reported that an unknown substance of high molecular weight in the gut juice could inactivate nuclear polyhedrosis virus *in vitro*.

Mukai *et al.*, (1969) extracted the red fluorescence protein (RFP) from the digestive juice of *B. mori* and reported that the RFP was able to inactivate the NPV of *B. mori*, *in vitro*. Hayashiya *et al.*, (1968, 1976, 1978) have reported the agglutination reaction of NPV and RFP, similar agglutination reaction was also found with flacherie virus (FV) of *B. mori*, but not between tobacco mosaic virus (TMV) and RFP.

Hirayama *et al.*, (1993) have also purified an antiviral protein from silkworm fecal matter and reported it to be a glycoprotein. AVP was observed to inactivate many types of cells in suspension (for instance, QMRSV cells) and the protein recognizes mannose on cell surface leading to aggregation. Hiraki *et al.*, (1996) obtained an antiviral substances

from silkworm fecal matter extract, which was shown to have marked antiviral effect on the growth of enveloped viruses, such as HVJ (Sendai virus), HSV (Herpes simplex virus type-1) and HIV (human immunodeficiency virus type-1). The review of literature highlights unity and diversity in antiviral substance investigations. The incidence of grasserie disease caused by the infection of NPV in silkworms is a common and serious disease, occurs in almost all seasons. The structure and biochemical composition of the NPV is reasonably well understood. Very few reports are available on the studies of AVP (an anti grasserie protein) present in the gut juice of silkworms. There are no reports on the AVP isolated from silkworm fecal matter that can inactivate NPV. In the present interdisciplinary investigation on purification and characterization of AVP present in silkworm *Bombyx mori* fecal matter was under taken in both *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Experimental conditions

The experiments were carried out in an aseptic laboratory maintained at 25 ± 2 °C, $65 \pm 10\%$ relative humidity and 12:12 hrs (Light: Dark) photoperiod. The 5th instar 3rd day larvae were used for collection of fecal matter and bioassay. Collected fecal matter were cleaned and stored at -20 °C until they were used for further analysis. The containers and the instruments were sterilized using 5% formaldehyde, washed and dried later before use. Ten larvae were used for each set of bioassay experiment and triplicate sets were maintained. The conventional rearing procedures and nuclearpolyhedrosis symptoms, cocoon weight, shell weight, were recorded as described by Nagaraja, *et al.*, (1993). The cocoon to shell ratio was calculated as follows: Cocoon to shell ratio is equal to shell weight/cocoon weight x 100.

Chemicals and virus material

All the chemicals used for the experiments were of A.R grade, obtained from Sigma chemical company (St.Louis, MO. USA) except Silica gel -G and sephadex-G 75, were obtained from Pharmacia Company. The virus material used for experiment was purified and stored as suggested by Khosaka

et al., (1971). It was in the form of purified suspension of polyhedral inclusion bodies (PIBs), stored at -4 °C in the light proof bottles.

Purification of antiviral protein

Fifth instar 3rd day larvae fecal matter of *B. mori* were collected and extracted using 0.02 M phosphate buffer solution (PBS) of pH 7.4 at 60 °C for a period of 40 hrs with constant stirring. The mixture was then filtered, and the filtrate was centrifuged at 7,000 g for 30 min. The supernatant was brought to 50% solid ammonium sulfate saturation and allowed to stand for 90 min at 4 °C. It was then centrifuged at 10,000 g for 10 min at 4 °C. The precipitate was dissolved in suitable volume of PBS and dialyzed against PBS.

The dialysate was applied to the column chromatography of column (2x12cm) containing silica gel-G, the column elutes were further purified by performing, gel filtration chromatography using sephadex G-75 as the matrix at 4 °C. Separation was accomplished by passing through gelfiltration column (1.65 X 43 cm) at a flow rate of 10 ml/hr. Protein peaks were detected by monitoring absorbance at 280 nm on a Hitachi UV-visible spectrophotometer model U-2001. Fractions of the peaks A, B and C were separately pooled, concentrated by lyophilization and kept at 4 °C. Then peaks A, B and C fractions are separately used to check the activity against NPV *in vitro*. Fractions obtained from peak A shown marked activity against NPV where as B and C fractions were not shown activity against NPV. Therefore Peak fractions obtained from peak A was further purified by HPLC using C₄ column (250 x 4.6mm) at a flow rate of 1ml/ min at 25 °C. Peaks were detected by using Water's 2487 dual λ absorbance detector at 280 nm (Water's HPLC 510 pump attached with automated gradient controller and Breeze software.).

Estimation and Molecular weight determination of AVP

The protein concentration in each active fraction was measured by the method of Lowry *et al.*, (1951). The molecular weight of the protein was estimated using sephadex G-75 column. The gelfiltration chromatographic column was pre-calibrated using the

standard molecular weight marker proteins according to Andrews (1964) method.

Electrophoretic methods

Native PAGE was carried out in 12.5 % polyacrylamide gel by passing a constant current of 20 mA (Davies, 1964), and gel was silver stained as per the method of Wray *et al.*, (1981). SDS-PAGE was carried out by using Laemmli method (1970) on 12.5 % polyacrylamide gel by passing a constant current of 20 mA. The proteins in the gel were stained with Coomassie brilliant blue R 250 and destained with solution containing 5 % methanol and 7 % acetic acid.

2.6. Carbohydrate staining and total carbohydrate estimation of AVP

Carbohydrate staining was performed by the method of Racuson-(1990). And total carbohydrate was estimated by the method of Dubois *et al.*, (1956).

Isolation of NPV

The NPV was isolated according to the procedure of Khosaka *et al.*, (1971). The turbid haemolymph of silkworm *B. mori* larvae infected with NPV, was collected and filtered. The filtrate was then centrifuged at 5000 g for 10 min at 4 °C to remove the fat body and other materials. The samples were washed with distilled water and 50 mM NaCl, then treated with 50 mM Na₂CO₃ solution containing 50 mM NaCl for 2 hr at 0 °C. The solution was centrifuged at 1500 g at 5 °C and then the supernatant was centrifuged at 40,000 g for 90 min at 5 °C. The resulting pellet was suspended in 40 mM phosphate buffer pH 7.5 and again centrifuged at 3000 g for 20 min at 5 °C. The supernatant containing virions isolated from polyhedral bodies was used as the source of NPV.

Assay of the precipitation activity between AVP and NPV

Precipitation activity between purified AVP and NPV was assayed by serial two-fold dilution technique of Liner and Hill (1953) and Uchida *et al.*, (1984).

a. Effect of temperature on the stability of AVP

AVP (50 µg) in 0.02 M sodium phosphate buffer pH 7.4 taken in different eppendorff

tubes and incubated at desired temperatures in a thermostat water bath for about 30 min. Then the incubated protein solutions were cooled to room temperature and the precipitation activity was determined and the AVP (50 µg) was incubated at room temperature, which served as a control.

b. Effect of pH on the stability of AVP

The purified antiviral protein was incubated with an equal volume of 0.02 M buffers of desired pH (2 to 11.5) for 6 hr at 4 °C. The 50 µl of this AVP was used for determining the precipitation activity by standard assay procedure as described in section 2.8. A control was maintained to compare the effect of pH on the protein.

Various buffer systems used for obtaining the desired pH are as follows:

Gly-Tris HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-7.0), Tris -HCl (pH 8.0-9.0) and sodium carbonate (pH 10.0-11.5).

The bioassay for antiviral activity was carried out as described by Nagaraja, *et al.*, (1993) The following solutions were prepared and used

- i) The NPV solution (O.D 260 = 1.00) was mixed with an equal volume of the purified AVP solution (O.D 280 = 2.6) (AVP +NPV).
- ii) The NPV solution (O.D.260 = 1.00) was mixed with an equal volume of BSA (Bovine serum albumin) (O.D280 = 2.6) served as negative control. (NPV+BSA).
- iii) The NPV solution (O.D 260 = 1.00) was mixed with an equal volume of 40 mM sodium phosphate buffer at pH 7.5 which served as negative control. (NPV+ PB)
- iv) The AVP solution (O.D 280 = 2.6) was mixed with an equal volume 40 mM phosphate buffer (PB) at pH 7.5, to check how AVP alone acts. (AVP+PB)
- v) The BSA solution (O.D 280 = 2.6) was mixed with equal volume of 40 mM phosphate buffer at pH 7.5 to examine whether the protein affects the normal worms or not. (BSA+PB)
- vi) The 40 mM phosphate buffer solution at pH 7.5, which served as the positive control. (PB)
- vii) Uninjected larvae were also kept along with above sets to serve as untreated control. (No injection set).

All the solutions were incubated at 25 °C for 60 min. Each mixture was injected into

silkworm *B. mori* 5th instar 3rd day larvae (5 μ l /larva).

RESULTE

Purification of AVP from silkworm fecal matter

AVP was purified from the silkworm fecal matter. Fecal matter extract prepared in PBS, was subjected to 50% ammonium sulfate precipitation, then dialysis. Further purification was achieved by applying to silica gel-G column and gelfiltration chromatography using sephadex G-75 column. The gelfiltration pattern gave three protein peaks A, B and C (Fig-1).

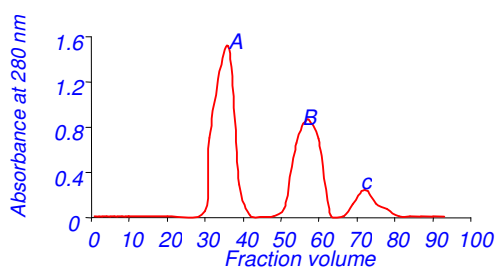


Fig 1. Gelfiltration pattern of antiviral protein

Gelfiltration chromatography was performed using sephadex G-75 column (1.65 cm x 43 cm) at a flow rate of 10 ml/hr at 4 °C. Protein was eluted with PBS and three peaks A, B and C were obtained. Protein pigment complex present in peak A fractions were found to have anti NPV activity.

These peaks were separately collected, concentrated and used to determine the activity *in vitro*. The protein obtained from peak A fractions is found to be active against NPV *in vitro*. Whereas the proteins obtained from peaks B and C were not shown activity against NPV *in vitro*. Therefore, the peak fractions collected from Peak A were applied to HPLC and purification of AVP was accomplished (Fig-2).

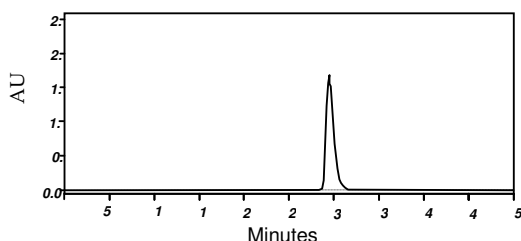


Fig 2. HPLC profile of AVP

HPLC (Water's HPLC 510 pump attached with automated gradient controller and Breeze software.) was performed using C₄ column (250 x 4.6mm) at a flow rate of 1ml/min at 25 °C. Peaks were detected by using Water's 2487 dual λ absorbance detector at 280 nm.

Biochemical characterization of AVP

The determination of molecular weight of the purified protein was performed by gelfiltration chromatography using sephadex-G75 column calibrated with standard molecular weight markers. From the calibration curve, molecular weight was found to be about 39.9 KD. The native PAGE analysis of purified AVP has shown only one protein band, whereas SDS-PAGE on nonreducing condition resulted in two protein bands with the molecular mass of about 23 KD and 16 KD. The SDS-PAGE analysis under reducing condition of purified AVP shown the same result as in SDS-PAGE analysis in non-reducing condition. (Fig-3). Glycoprotein staining of the gel after SDS-PAGE analysis indicated that both the protein bands were glycoproteins (Fig-4). The total carbohydrate content in AVP was about 13% w/w.

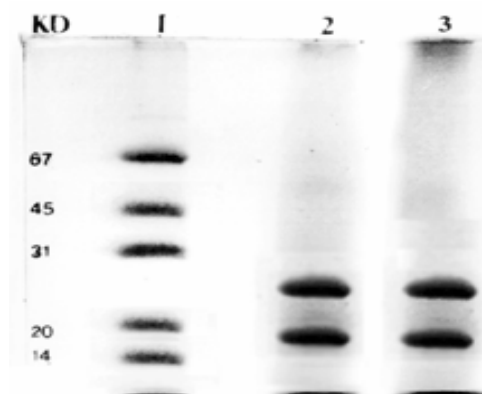


Fig 3. SDS-PAGE was carried out on 12.5% polyacrylamide gel and stained with coomassie brilliant blue.

Lane-1. Standard molecular weight protein markers. Lanes-2 and 3. Purified AVP was treated under reducing and non-reducing conditions, respectively.

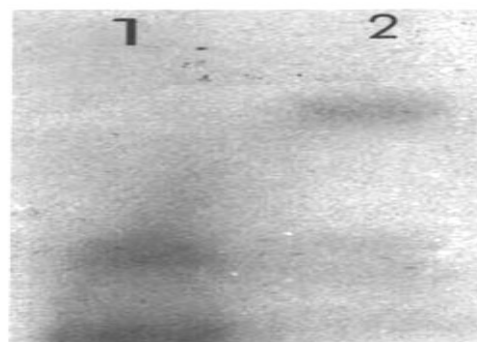


Fig 4. Carbohydrate staining.

AVP was applied to SDS-PAGE on 12.5% polyacrylamide gel and electrophoresed gel was stained for glycoproteins

Lane-1. Antiviral protein.

Lane-2. Standard Ovalbumin.

The precipitation activity between AVP and NPV

The precipitation activity between AVP and NPV *in vitro* was carried out; the complete precipitation activity was observed after 8 hrs 26 min of incubation (Fig.5). The effect of pH and temperature on the observed precipitation activity of the AVP was also investigated. The AVP activity against NPV was stable over a wide pH range of 5.5 to 9.5 (Fig.7) but the activity was lost slowly below pH 5.5 and above 9.5. The 100 % activity was retained even incubated at 65 °C for 30 min. However, there was a rapid loss of the precipitation activity was observed when AVP was incubated beyond 70 °C (Fig.6).

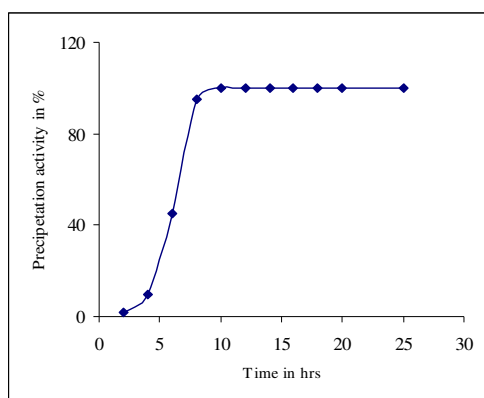


Fig 5. Precipitation activity of the purified AVP against NPV.

Assay of the precipitation activity between AVP and NPV was assayed by the serial two-fold dilution technique. Plates containing mixture of AVP and NPV were incubated at room temperature. The complete precipitation activity was observed after 8 hrs 26 min of incubation

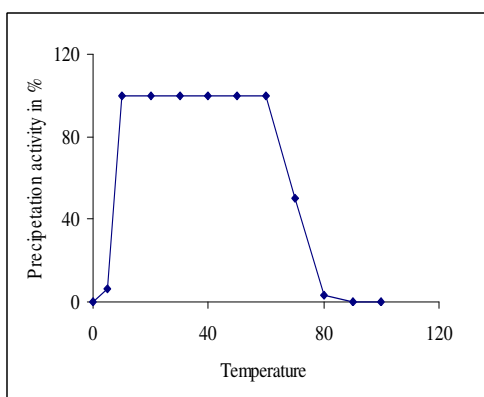


Fig 6. Effect of temperature on the purified AVP.

The AVP activity against NPV was retained incubated at 60 °C for 30 min. However, their was rapid loss of activity was observed when AVP was incubated above 70 °C.

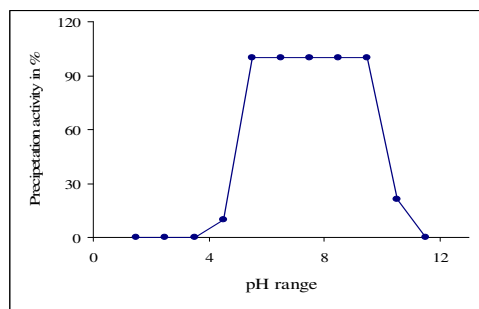


Fig 7. Effect of pH on the purified AVP.

The effect of pH on the AVP activity against NPV was stable over a wide pH range of 5.5 to 9.5 but activity was observed to lost slowly below 5.5 and above 9.5.

Bioassay of AVP against NPV in silkworms

When larvae were treated with NPV + phosphate buffer and BSA+NPV 100% mortality was observed. However, when larvae treated with AVP+NPV shown 80% survival. Whereas in all positive control sets (AVP+ phosphate buffer, BSA+phosphate buffer, and phosphate buffer,) and untreated control (no injection set) 100 % survival was observed. The treatment group (table, 1,) having BSA in place of AVP served as a negative control. The 100 % mortality of the ten larvae in all the two sets treated with BSA + NPV and NPV + phosphate buffer (table 1) clearly demonstrated that the observed antinuclear polyhedrosis activity was only due to AVP. The statistical analysis of the data was done by conducting "Z" test. The Z value of 10.9 was obtained indicating 99 % confidence limit (table-2).

Cocoon characters

The bioassay experiment was continued up to cocoon formation and the cocoon characters were studied. The cocoon weight, shell weights were recorded, and cocoon to shell ratio was calculated. The AVP+NPV treated silkworms yielded the cocoon weight, shell weight and cocoon to shell ratio values of 9.50, 1.85 and 19.47 respectively. The BSA + NPV, and NPV + PB treated sets resulted in 100% mortality of the larvae. The AVP+PB, BSA+PB treatment sets yielded the cocoon weight, shell weight and cocoon to shell ratio values are comparable (close) to the untreated values of 12.5, 2.58 and 21.06, respectively. The slightly higher values for the three parameters for the uncontrolled set were obtained compared to the positive controls (Table-3).

Table 1. Anti NPV activity of the AVP

Set numbers	Treatment	Sets and number of larvae	Number of larvae severely infected and died (average of three sets)	No. of larvae not infected and survived (average of three sets)
1	AVP + NPV	3 X 10	2	8
2	BSA + NPV	3 X 10	10	0
3	NPV+ Phosphate buffer	3 X 10	10	0
4	AVP + Phosphate buffer	3 X 10	0	10
5	BSA + Phosphate buffer	3 X 10	0	10
6	Phosphate buffer	3 X 10	0	10
7	No injection	3 X 10	0	10

(Values are mean of three replicates) The various combinations of solutions prepared for treating silkworms were as shown in the table.

(Set 2 & 3 were negative controls. Sets 4, 5 and 6 were positive controls. Set 7 was untreated control).

Table 2. Statistical analysis of "Antiviral activity of AVP on the infectivity of NPV".

Treatment	Set and number of Larvae	Survival Percentage	Z
NPV + Phosphate Buffer	3 X 10	00.00	
AVP+ NPV	3 X 10	80	10.9**
BSA+ NPV	3 X 10	0.00	

Optical density of NPV solution at 260 nm is 1
 Optical density of BSA solution at 280 nm is 2.6
 Optical density of AVP solution at 280 nm is 2.6
 40 mM Phosphate buffer at pH 7.4

** Significant at 99 % confidence limit

Table 3. Cocoon character studies.

Set numbers	Treatment	Sets and number. of larvae	Cocoon weight in gm	Shell weight in gm	Cocoon to shell ratio yield
1	AVP + NPV	3 X 10	9.50 ± 0.06	1.85 ± 0.01	19.47 ± 0.06
2	BSA + NPV	3 X 10	0.0 ± 0.0* †‡	0.0 ± 0.0* †‡	0.0 ± 0.0* †‡
3	NPV+ Phosphate buffer	3 X 10	0.0 ± 0.0* †‡	0.0 ± 0.0* †‡	0.0 ± 0.0* †‡
4	AVP + Phosphate buffer	3 X 10	9.26 ± 0.06*	1.82 ± 0.0* †	19.71 ± 0.00
5	BSA + Phosphate buffer	3 X 10	11.50 ± 0.06	2.31 ± 0.01	20.00 ± 0.06
6	Phosphate buffer	3 X 10	11.96 ± 0.02	2.41 ± 2.02	20.66 ± 0.01
7	No injection	3 X 10	12.25 ± 0.02	2.58 ± 0.06	21.06 ± 0.03

The Cocoon character were studied by taking the average weight of five cocoons and shell weights, the values are the mean of three replications and standard mean ± errors. Statistical significance of one-way anova and Students "t" test at level 0.05 was calculated using SPSS windows software

* P < 0.05 Compared to no injection control set (7)

† P < 0.05 Compared to Phosphate buffer set (6)

‡ P < 0.05 Compared to BSA + Phosphate buffer set (5)

DISCUSSION

The silkworm fecal matter is one of the rich sources for antiviral proteins. The anti NPV protein present in silkworm fecal matter was extracted by series of three steps, namely precipitation with 50% ammonium sulfate, and then dialyzed. Silica gel -G column and by gel filtration chromatography further purified by HPLC. The AVP obtained from the HPLC was found to be pure by electrophoretic analysis. The AVP was eluted from the calibrated sephadex G-75 gelfiltration chromatographic column. Native PAGE of the purified AVP resulted in a single protein band. Both the reducing and non reducing SDS-PAGE (in the presence and absence of β -mercaptoethanol,) of the purified AVP yielded two protein bands suggesting that the native protein is a dimer consisting of two non-identical subunits. The electrophoresed gel was subjected to thymol sulfuric acid staining to identify the presence of carbohydrate moieties attached to the proteins; two red bands on the pale yellow background indicated that both the subunits of AVP are glycoproteins.

The precipitation was observed when AVP and NPV were mixed and incubated. This recognition and subsequent binding of AVP to NPV is probably due to surface interaction between AVP and NPV. AVP was maintained at 70 °C or above it lost its precipitation activity rapidly against NPV. The AVP activity was stable over a wide pH range of 5.5 to 9.5 and the activity was lost slowly on either side of this pH range. Uchida *et al.*, (1984) have isolated about 60 KD molecular weight protein from gut juice of silkworm larvae, which can precipitate NPV *in vitro*. Hiraki *et al.*, (1986) observed the antiviral substances about 25 KD and 14 KD, which suppress the production of enveloped viruses, like HVJ, HSV, HIV. Some high molecular weight substances in the gut juice could inactivate nuclear polyhedrosis virus *in vitro*. Mukai *et al.*, (1969) extracted about 60 KD protein having red fluorescence (RFP) from the digestive juice of *B. mori* and was able to inactivate the NPV of *B. mori*, *in vitro*. Looking at the characters of the various antiviral proteins purified from gut juice and fecal matter of the silk worm. The AVP purified by us is different and the first protein isolated from

fecal matter which can inhibits the grasserie disease causing virus NPV in silkworms.

As the AVP is produced in the silkworm body, it was thought to be of interest to investigate its biological role in controlling the NPV infection in silkworms and its activity. The data in table-1 suggests that NPV infection to silkworm can be inhibited to the extent of 80%, when the silkworm larvae were injected with NPV+AVP. Injection of only NPV caused the total loss of larvae after third day of the treatment, while the silkworm injected with NPV+AVP showed that 20% of the silkworms get mild infection on 4th day, which becomes severe on 7th day of the experiment.

A Comparison of the survival rates of the larvae in the presence of AVP, the delayed onset of the infection symptoms compared to positive and negative controls, makes it very clear that AVP inhibits the NPV infection significantly in silkworms. The statistical data obtained in table-2, indicate high significant value suggesting 99% confidence limit which strongly support marked antiviral activity of AVP against NPV. To our literature understanding, it is the first report of the AVP purified from fecal matter and *in vitro* and *in vivo* AVP activity against NPV; hence it deserves immediate attention of researchers in the area.

These bioassay studies were extended till the cocoon formation stage to examine the effect of purified AVP on silk production. The cocoon weight, shell weight and values of the cocoon to shell ratio were determined (table-3); the AVP+NPV treated silkworms yielded the cocoon weight, shell weight and cocoon to shell ratio values, the BSA + NPV and, NPV + PB treatments resulted in 100% mortality of the larvae and the AVP+PB and, BSA+PB treatment sets yielded the cocoon weight, shell weight and cocoon to shell ratio values are comparable to the untreated values.

The slightly higher values for the three parameters for the untreated sets were obtained compared to the positive and negative controls. All these observations support the fact that AVP does not affect silk yield, and it effectively reduces the NPV infection and it significantly increases the survival rate by inhibiting the NPV in silkworms.

The antiviral substances are obtained from a variety of sources. The biochemical composition and their roles in inhibiting different types of viruses are different (Spikes *et al.*, 1984, Willem 1997, Faith *et al.*, 2001). The antiviral activity of these proteins may be due to one or more of the following mechanisms; 1) Blocking the synthesis of viral specific gene (Hiraki *et al.*, 1996, Pollack *et al.*, 1969, Lewin *et al.*, 1980). 2) The disruption of the viral envelope by formation of amorphous material on the viral envelope (Uchida *et al.*, 1984, Tinsley 1979, Hirayama *et al.*, 1993).

Enveloped viruses were effectively inactivated by photodynamic effect (Lewin *et al.*, 1980, Munson 1977, Matthews *et al.*, 1988 Akihiro Hiraki *et al.*, 2000). We are now focusing our attention to establish the biochemical composition of the AVP and its mechanism of action in inactivation of virus lifecycle in silkworms.

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