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BIOCHEMICAL CHARACTERIZATION OF ANTIVIRAL PROTEIN FROM SILK WORM FECAL MATTER *BOMBYX MORI* (L)

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Abstract

Introduction: Silkworm fecal matter is considered as one of the important medicinal sources for many infectious diseases. Therefore, we have explored the antiviral protein (AVP) by exploiting the silkworm fecal matter. Present investigation display the biochemical characterization of a novel anti nuclear polyhedrosis virus (NPV) protein from silkworm fecal matter.
Material and Methods: Purified AVP was found to be associated with Tetrapyrrole pigments. The associated Tetrapyrrole was separated and analyzed using TLC (Thin layer chromatography) Spectrophotometry and Spectrofluorometry. The antiviral activity determination in both separated components of AVP was done separately.
Results: The AVP associated Tetrapyrrole analyzed was found to be *monovinyle pheophytine a*; the derivative of *chlorophyllide a*, in which Mg was absent. The antiviral activity was observed only in protein associated with tetrapyrole.
Conclusion: The antiviral protein associated tetrapyrole part was found to be *monovinyle pheophytine a*, is essential for its antiviral activity.

Keywords: Antiviral protein, Tetrapyrrole, Silkworm fecal matter, Spectrofluorometric screening, *Monovinyl pheophytin a*

Introduction

The decomposition products of chlorophyll, the derivatives of tetrapyrrole are reported to have photosensitivity in animals and photo oxidation of foods. [22] The earlier workers isolated many antiviral proteins from sources containing chlorophyll and reported that the antiviral activity is due to the presence of chlorophyll like substance associated with a glycoprotein. [5, 8, 9, 10, 14] Very few studies on viruses have been carried out with porphyrins as photosensitizers, although most viruses might be expected to be quite sensitive because they are composed of proteins, nucleic acids, and in some cases lipids.[14] Enveloped viruses such as HSV (Herpes Simplex Virus type-1) and HIV (Human immuno deficiency virus type-1) were effectively inactivated by the photodynamic effect of hemetoporphyrin derivative (HpD) but naked viruses were resistant to photo inactivation.[15] HpD is known to react with the lipid portion of cytoplasmic membranes of HSV and HIV.[4,12,] The antiviral substances from silkworm fecal matter were found to contain a chlorophyll-like substance. These substances shown clear antiviral activity against HVJ and HSV. Their antiviral activity was dependent on light intensity and temperature. Furthermore, it was also found to possess a strong hemolytic activity under light. [9] The detection of monovinyl magnesium porphyrin monoester and other monovinyl magnesium porphyrin in higher plants by spectrophotometry and spectrofluorometry was reported.[3]

Chlorophyll biosynthetic heterogeneity was rooted mainly in parallel divinyl and monovinyl biosynthetic roots interconnected by- vinyl reductase that convert divinyl tetrapyrroles to monovinyl tetrapyrroles by converting vinyl group was shown by spectrofluorometry.[13]. Degradation products of silkworm excrement from crude chlorophyll mixture was analyzed using HPLC, this work was led them to conclude that the chlorophyll undergo metabolism in the body of silkworm to form pyrochlorphyll derivatives. [11]

In our previous study, we have reported that, purified AVP present in silkworm fecal matter was shown effective antiviral activity against NPV. The grasserie disease caused by NPV in silkworms is common and serious, that occurs in almost all the seasons. The activity of AVP against NPV was determined by precipitation reaction. The same was confirmed by conducting bioassay in silkworms. This AVP shows presence of tetrapyrrole pigments. The pigment / tetrapyrrole part is found to be essential for the activity of the AVP. [16] Hence, in continuation of previous work, the extraction and characterization of tetrapyrrole pigment associated with AVP has been

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achieved using TLC, spectrophotometry and spectrofluorometry.

Material and Methods

Chemicals

All the chemicals used for the experiments were of A. R grade. They were obtained from Sigma Aldrich Chemical Company (St. Louis, MO. USA).

Purification of AVP from Silkworm fecal matter was performed as described in Neelagund et al., (2007),

Antiviral protein activity against NPV by precipitation method was assayed by serial two fold dilution techniques.

50µl of AVP is taken serially in micro titer plate. Then add 25µl of buffer solution serially to the same wells, again add 25µl of NPV is add serially to same wells. Then it was keep for incubation at 25°C as described in Uchida et al; 1984.

Extraction and characterization of the pigment part associated with AVP

Isolation of pigment

All steps were carried out at 4°C under subdued condition. Purified AVP (200 µg) was mixed with 6.7 ml of acetone / 0.1 NH_4OH (9:1 v/v). The mixture was centrifuged at 39,000 g for 10 min and the pellet was redissolved with 2 ml cold 80% acetone. The combined 80 % acetone extracts were extracted with hexane to remove the fully etherified pigments. The monocarboxylic and dicarboxylic tetrapyrroles remained in the hexane-extracted acetone fraction.

Pigments in the remaining hexane extracted acetone fraction were then transferred to 1.5 ml peroxide free diethyl ether after addition of 1/17 volume saturated aqueous NaCl and 1/70 volume 0.5 M KH_2PO_4. [18] The diethyl ether layer was collected and the aqueous residue was re-extracted 2-3 times with 1 ml of diethyl ether until the extract was colorless. The hexane layer and ether layer (containing pigment derivatives) obtained from above extraction were separately subjected to the spectral and chromatographic analysis.

Spectrophotometry

UV-Visible spectra was taken on Hitachi model U-2001 UV-Visible spectrophotometer attached with Hitachi data processor operated in the split beam mode of band pass 2 nm with photometric accuracy ± 0.002 OD checked with NBS 930 filter at room temperature (Parham and Rebeiz, 1992) .

Spectrofluorometry

The samples were maintained in dark condition and they were used to record the fluorescence spectra at room temperature. The excitation and emission spectra were recorded with Hitachi model F 2000 fluorescence spectrophotometer attached with Hitachi 2001 microcomputer model. Emission spectra were recorded with an excitation slit width of 6 nm and an emission slit width of 3 nm while excitation spectra were recorded with an excitation slit width of 3 nm and an emission slit width of 6 nm. Under these conditions, the spectral accuracy of the reported maxima is about 1 nm. [16]

Detection of Mg and Zn metals in the extracted tetrapyrrole pigment from the purified AVP by using atomic absorption spectrophotometer

The Mg and Zn were detected in the pigment in double beam Atomic absorption spectrophotometer model GBC 902 using GBC hallow cathode Mg & Zn lamps made in Australia. [20]

Demetallation of the Mg-porphyrins

The demetallation studies are carried out as described by Belanger FC, Parham, Rebeiz, (1992).

Thin-layer chromatography

The diethyl ether extract containing pigment derivatives was purified on thin layer of silica gel H. The diethyl ether solution was concentrated under Nitrogen (N_2) gas to a small volume, usually less than 1 ml of a turbid aqueous residue. The pigments in this residue were removed by repeated extraction with 0.5 ml volumes of diethyl ether, and the combined diethyl ether extract was dehydrated over 0.5 g/ml granular NaCl. The dehydrated diethyl ether extract was removed, and the pigments absorbed on the NaCl granules were removed by washing twice with 2 ml volumes of diethyl ether. The diethyl ether extract and washings were combined, concentrated under N_2 gas to about 0.3 ml, then applied quantitatively to a thin-layer chromatographic plate of silica gel H which was developed in toluene/ethyl acetate/ethanol (8:2:1, v/v) at 4°C in darkness. Thorough dehydration of the diethyl extract as described above greatly improved the chromatographic resolution. [6] The R_f values were calculated as a ratio of the distance traveled by the solute to solvent.

Elution of the pigment derivatives from thin layer chromatogram

Two clear spots were observed under UV-light on the developed chromatogram. One of them was red fluorescent and the other one is greenish with different R_f value. Both the pigment spots were separately scraped into a beaker containing organic solvent ether before allowing the chromatogram to dry, and silica gel pellet was washed twice with 1 ml of diethyl ether and it was removed by centrifugation. The supernatant liquid was evaporated to dryness under nitrogen gas and the residue was dissolved in 1 ml of 80% v/v acetone. Both the elutes were separately used to record the

absorbance spectra, fluorescence spectra and the detection of metals. Both the elutes were demetallated separately and subjected to spectral analysis and also for metal detection.

Isolation of authentic monovinyl pheophytin a

The authentic monovinyl pheophytin *a* was isolated using the procedure of Belanger and Rebeiz (1982) and Shadbalkar *et al.,* (1992). All the steps were carried out at 4°C in dark condition. The UV visible spectrum and fluorescent spectrum were recorded at room temperature.

Activity of tetrapyrrole Isolated from AVP

Isolated tetrapyrrole activity against NPV by precipitation reaction method was assayed by two fold dilution technique.

50µl of pigment is taken serially in micro titer plate. Then add 25µl of buffer solution serially. Then add 25µl of NPV serially to same wells. Then keep this for incubation at different temperature. Precipitation activity was recorded. (Uchida et al; 1984)

Activity of protein separated from associated Tetrapyroles

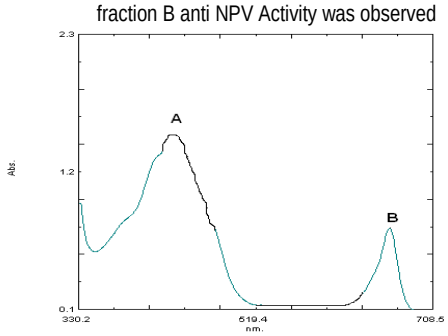
The separated protein from associated tetrapyrrole of silk worm fecal matter is used to check the activity against NPV by using above precipitation method of Uchida *et al;* 1984

Results

Purification and Determination antiviral activity of protein against NPV invitro

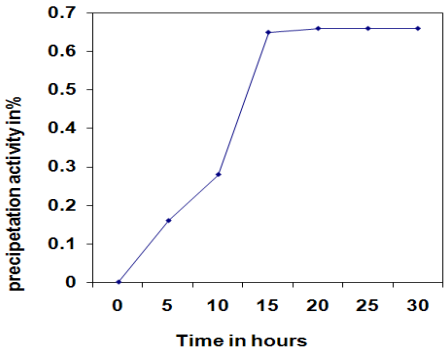
The partially purified antiviral protein isolated from silkworm fecal matter is further purified by performing Gel filtration chromatography. Using gel sephadex G-75 as a matrix at room temperature. Separation was accomplished by passing through gelfilteration column [1.2x 38cm] at a flow rate of 10ml/ hour. Protein peaks were detected by monitoring absorbance at 280nm on UV-visible spectrophotometer model-119 systronics. Fraction of the peaks were separately collected, the collected fractions are separately used to check the activity against NPV in vitro. Fraction obtained from the 2nd peak shown activity against NPV. (Neelagund *et, al*; 2007) (Fig-1).

Fig -1. Gel filtration pattern showing two peaks A and B. In peak fraction B anti NPV Activity was observed



The purified antiviral protein from silkworm fecal matter was evaluated for its activity. The antiviral activity of AVP against NPV optimum precipitation activity was obtained after 11 hours complete incubation at 25°C. Then it is found to be in stable precipitation state (Fig 2).

Fig. 2: The precipitation reaction of the AVP against NPV in vitro at 25ºC. Optimum activity was observed after 11 hour incubation



Isolation of the pigment from the purified AVP

The ether and hexane layer samples obtained during isolation of pigment associated with AVP were separately used to record the visible and fluorescence spectra and for TLC analysis. The visible spectrum of the ether sample recorded has show absorbance at 407 nm and 672 nm, at room temperature. Whereas fluorescence spectrum of the ether layer gave excitation maximum at 407 nm and emission maximum at 672 nm, but the hexane layer did not show any visible or fluorescence absorbance maxima corresponding to tetrapyrrole derivatives. Mg and Zn metals were detected in the ether layer obtained during the pigment extraction. The fluorescence spectral records obtained from demetallated solution were similar to those of ether extract spectral results. Both Zn and Mg metals were not detected in demetallated solution.

Analysis of the tetrapyrrole pigment present AVP

Two spots were observed when ether layer sample is subjected to TLC; one was a red fluorescent spot and the other was non-fluorescent greenish spot with the R_f values of 0.71 and 0.44 respectively.

Elution of the pigment derivatives from the thin layer chromatogram

The visible absorbance spectrum of the elute of red fluorescent spot has shown the absorbance at 407 nm and 672 nm (Fig.3) while, the greenish spot elute gave absorbance maximum at 585 nm. The fluorescence spectrum of the red fluorescent spot eluate has displayed a spectrum with excitation maximum at 407 nm and emission at 672 nm (Fig-4).

Whereas the green spot did not show any fluorescence peaks at 585 nm. The red fluorescent spot eluate was found to contain Zn but no Mg while, the greenish spot eluate showed the presence of both Mg and Zn metals. The authentic monovinyl pheophytin *a* is displayed UV visible absorbance maximum at 408 and 672 nm and fluorescent spectrum of excitation at 408 and emission at 672 nm. The overlay of UV visible spectra and fluorescent spectra of red fluorescent spot elute from TLC of the isolated pigment in acetone and authentic monovinyl pheophytin *a* in acetone were matched each other (Fig 5 and 6).

The precipitation activity was not observed in both individual components separated from AVP (Fig 6 and 7), where as good precipitation activity was observed when protein is in intact form (Fig-2).

Fig. 3: The UV- Visible absorbance spectrum of the red fluorescent spot elute from TLC of the isolated pigment in acetone at room temperature. Absorbance maximum is observed at 407 and 672nm

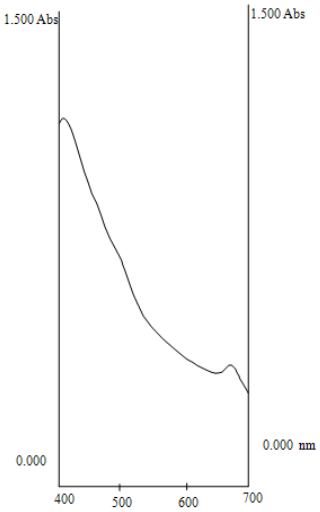


Fig. 4: The fluorescence spectrum of the red fluorescent spot elute from TLC of the isolated pigment in acetone at room temperature. Excitation maximum at 407nm (fig-a) and emission at 672 nm (fig-b)

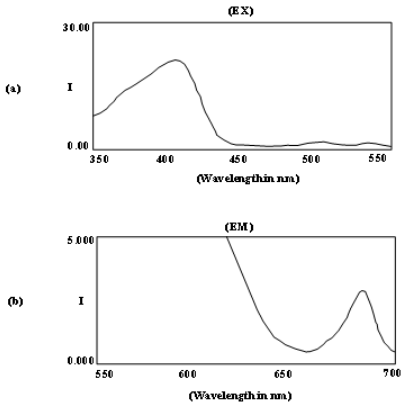


Fig. 5: The overlay of UV- Visible absorbance spectrum of the red fluorescent spot elutes from TLC of the isolated pigment and isolated authentic monovinyl pheophytin a in acetone at room temperature. Both displayed absorbance at 407 and 672 nm

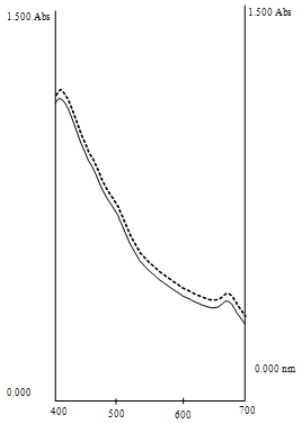


Fig. 6: The overlay of fluorescence spectrum of the red fluorescent spot elutes from TLC of the isolated pigment and isolated authentic monovinyl pheophytin *a* in acetone at room temperature. Both displayed excitation maximum at 407nm (fig-a) and emission maximum at 672 nm (fig-b). -------- Authentic monovinyl pheophytin *a*

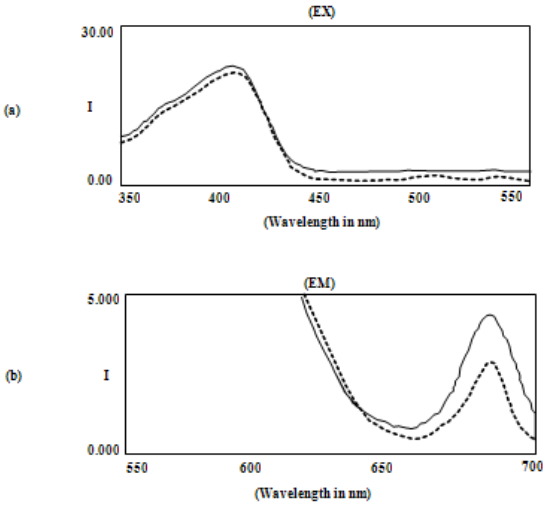


Fig. 7: The precipitation reaction of the tetrapyrole activity against NPV invitro at 25^{0}C. No activity was observed even after 30 hours of incubation

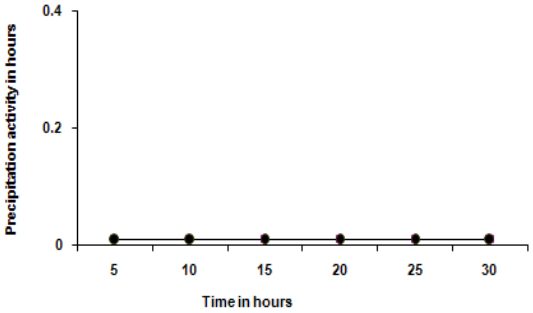
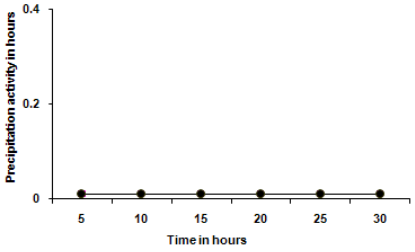


Fig. 8: The precipitation reaction of the separated protein from associated tetrapyrole activity against NPV in vitro at 25^{0}C. No activity was observed even after 30 hours of incubation



Discussion

The ether and hexane layers obtained during the extraction were collected and used to determine tetrapyrrole pigments present in AVP. The hexane layer did not display visible or fluorescence spectral peaks indicating the absence of etherified tetrapyrrole derivatives. The ether layer yielded a visible spectrum of maximum absorbance at 407 nm and 672 nm and, fluorescence spectrum of excitation maximum at 407 nm and emission at 672 nm indicating the presence of mono / dicarboxylic tetrapyrrole pigment derivatives. The visible and fluorescence spectroscopic results of AVP have revealed that the purified antiviral protein has a porphyrin derivative, related to chlorophyll or its metabolic intermediate. Since, mulberry leaves were the sole sources food for silkworms, their fecal matter may contain metabolic intermediates of chlorophyll [11] have studied the separation and characterization of crude chlorophyll mixture of the acid degradation products of silkworm excrement using HPLC. They have suggested that the chlorophylls undergo metabolism in the body of silkworm to give pyrochlorophyll derivatives. From these results, it may be reasonable to think that the pigment bound to AVP may be one such metabolic product of chlorophyll. The substances having antiviral towards HSV, HVJ and HIV type-1, were purified from silkworm fecal matter and, were examined for their chemical and biological properties. The active substance was found to be a type of porphyrin, having absorbance at 408 nm and 663 nm and fluorescence spectra of excitation maximum at 408 and emission at 663 nm. The results of visible and florescence spectra were similar to those of chlorophyll *a* derivatives. [21, 23] On considering the visible and florescence spectral analyses, AVP bound pigment seems to be one of the chlorophyll *a* derivatives. The substances obtained from silkworm fecal matter were known to possess hemolytic activity. It was chemically reported to be phyropheophorbide-*a*, a degradation product of chlorophyll *a.* [11, 22] The considerable point is that AVP is also derived from silkworm fecal matter but it may not be one of the phyropheophorbide-a, because Mg was detected in AVP bound pigment (Zn and Mg metals were detected in the ether layer before applying it to TLC) unlike phyropheophorbide-*a*.

The pigment extracts containing tetrapyrrole pigments were further separated on thin layer chromatography. This TLC analysis results in two spots; one had a red fluorescence with R_f value 0.71 and second one was greenish spot having R_f value 0.44. Red fluorescent elute displayed fluorescence spectrum of excitation maximum at 407 nm and emission maximum at 672 nm. Greenish spot elute showed visible spectrum absorbance at 585 nm but it did not shown any fluorescence peaks.[9] have obtained purified form of antiviral substances from silkworm Fecal matter against HIV-1 and separated on TLC (chloroform: methanol: water, 65: 25: 4 v/v) yielded 3 spots. One of them was red fluorescent with R_f value 0.68. Further it was characterized by spectral studies and metal detection using atomic absorption spectrophotometer as chlorophyll like substance containing Mg metal. But TLC analysis of the AVP bound pigment yielded the red fluorescent spot Rf = 0.71 that differs from the red fluorescent spot (R_f = 0.68) obtained by Hiraki. Red fluorescent spot eluate (R_f = 0.71) did not show the presence of Mg but only Zn was detected, whereas greenish spot eluate (R_f 0.44) has shown the presence of both the metals.

Rebeiz *et al.,* (1971) have carried out thin layer chromatographic analysis of vegetable dyes using silica gel H as the adsorbent and, the mobile phase was toluene : ethyl acetate : ethanol (8:2:1 v/v). They have obtained R_f value of 0.70 – 0.75 for monovinyl pheophytin *a.* TLC analysis of the purified pigment from AVP yielded a R_f value of 0.71.

The fluorescence characteristics of chlorophyll *a,* cholorophyllides, pheophytins, etc are documented in literature. [2, 18, 20] The authentic monovinyl pheophytin *a* was shown to have the excitation and emission maxima of 408 and 672 nm, respectively. [2] On TLC, the red fluorescent spot of the pigment extract of AVP has exhibited the fluorescence maxima corresponding to the monovinyl pheophytin *a* (Table1). The overlay of UV visible and fluorescence spectra of tetrapyrrole isolated from AVP and authentic monovinyl pheophytin *a* were confirm that pigment associated with AVP is monovinyl pheophytin *a.* Comparable chromatographic and spectroscopic characteristics (Table-1) of the authentic monovinyl pheophytin *a* and the tetrapyrrole of purified AVP would led us to characterize the part AVP bound pigment as monovinyl pheophytin *a*. The antinuclear polyhedrosis activity was not observed in both individual components separated from AVP, where as good precipitation activity was observed when protein is in intact form. Hence, monovinyl pheophytin *a* could be the essential component for the antiviral activity of the protein.

Table 1. Properties of the AVP bound tetrapyrrole

Pigment	R_f value	Fluorescence properties	
		Emission maximum (nm)	Excitation maximum (nm)
Tetrapyrrole isolated from AVP	0.71	672	407
Monovinyl pheophytin a (authentic)	0.70 - 0.71	672	408

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Reference

1. Belanger FC, Rebeiz CA. Plant Sciences Letters. 1980; 18:343-350
2. Belanger FC, Rebeiz CA. Chloroplast Biogenesis. Detection of monovinyl megnesium-protoporphyrin monoester and other monovinyl megnesium-porphyrins in higher plants. Journal of Biological Chemistry. 1982;257:1360-1371
3. Belanger FC, Duggan, JX, Rebeiz, CA. Chloroplast Biogenesis: Identification chlorophyllide *a* (E458 F674) As a divinely chlorophyllide *a*. J. Biol. Chem. 1981; 257: 4849-4858
4. Berenbaum MC, Bonnett R, Scourides PA. In vivo biological activity of the components of hematoporphyrin derivarive. Br J. Cancer. 1982; 45:571
5. Drzenick R, Rott R, Eggers HJ. Photodynamic inactivation of lipid containing RNA viruses by chlorophyllin. Boll. Chem. Farm. 1971;110:20-23
6. Duggan JX, Rebeiz CA. Chloroplast biogenesis conversion of divinyl chlorophyllide *a* to monovinyl chlorophyllide a in vivo and in vitro. Plant Science Letters. 1982; 27:137-145
7. Duggan JX, Rebeiz CA. Quantitative detection of a chlorophyllide *b* pool in higher plants. Biochemica et Biophysica Acta. 1982;714:248-260
8. Hiraki A.. Ikuno Y. Kim J. and Ueda S. Suppression of enveloped virus production with a substance from silk worm Fecal matter. Cell Structure and Function.. 1996;21: 501-514
9. Hiraki A, Yukawa M, Kim J, Ueda S. Antiviral substance form silkworm Fecal matter: purification and its chemical characterization. Biological and Pharmaceutical Bulletin. 1997; 20 (5): 547-555
10. Hirayama, E. Maekawa, H. Hiraki, A. and Kim, J. Biological activities of novel lectin derived from silkworm Fecal matter: Characteristic changes of mouse peritoneal macrophages by the lectin. Cell Structure and Function.. 1998;23:303-313
11. Hu Longqin, XU Deyu. Separation and characterization of chlorine derivatives with intact ring V from acid degradation products of silkworm excrement crude chlorophyll mixture. Biomedical Chromatography. 1989;3:2
12. Kessel, D. Effects of photoactivated porphyrins at the cell surface of leukemia L1210 cells. Biochemistry. 1977;16:3443-34490
13. Kolssova VL, Bohnert HJ, Rebeiz CA. Chloroplast biogenisis 92: In situ screening for divinyl chlorophyll A reductase mutants by spectrofluorometry. 2005; 348:192-197
14. Lewin AA, Schnipper LE, Crumpacker CS. Photodynamic inactivation of HSV by hematoporphyrin derivatives. Proceedings of the Society for Experimental Biology and Medicine. 1980; 163:81-90
15. Matthews JL, Newman JT, Sogandares-bemal F, Judy MM, Skiles H, Leveson JE, Marengo-Rowe A.J, Chan TC. Photodynamic therapy of viral contaminants with potential for blood banking applications. Transfusion. 1988;28: 81-83
16. Neelgund SE, Ingalhalli SS, Savanurmath CJ, Hinchigeri SB. Purification and characterization of antiviral protein from Fecal matter of silkworm. Caspian journal of Environmental Sciences. 2007; 5:77-85
17. Parham R, Rebeiz CA, Chloroplast Biogenesis: [4-Vinyl] Chlorophyllide *a* reductase is a divinyl chloroophyllide *a*-specific, NADPH-dependent enzyme. Biochemistry. 1992;31:8460-8464
18. Rebeiz CA, Castelfranco PA. Plant Physiology. 1971; 47:24-32

19. Rebeiz CA, Mattheis JR, Smith BB, Rebeiz CC, Dayton DF. Archives of Biochemistry and Biophysics. 1975;166:446-465
20. Shadbalkar VP. and Rebeiz CA. Chloroplast biogenesis: Determination of the molar extinction coefficients of divinyl chlorophyll *a* and *b* their pheophytins. Analytical Biochemistry. 1992; 207: 261-266
21. Smith, J. H.C. Beniter A. Moderm methods of plant analysis ed. By pach K, Traccy, M. V., Springer –verlag. Berlin. 1955;142-196
22. Takeda Y, Toyoda M, Yoshihira K. Effect of chlorophyll-related compounds on the hemolysis of Erythrocytes. J. Food Hyg. Soc. Japan. 1989; 30: 223-227
23. Yshitake Y, Tamino M, Morshige K, Shigematsu T, Nishikawa Y. Bunseki Kagaku. 1989; 38:182-187.