Chapter - 4

Discussion
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The current fashion of adapting various strategies for preparing innovative drugs against serious infectious diseases and their continuous implementation will result in increasing rate of multidrug resistance of microorganisms. To overcome this crisis, we need to evolve an innovative investigation to develop a natural drug against various infectious diseases. In this investigation, purification of bioactive protein was achieved by applying biochemical techniques such as 50% ammonium sulphate precipitation, dialysis, adsorption chromatography and lyophilization etc., UV-Visible spectrum of purified protein gave two peaks at 430 and 663 nm (Fig-2). This could be due to the association of tetrapyrole pigment to bioactive protein. SDS-PAGE analysis of the partially purified protein was shown one major protein band along with some associated high molecular weight minor protein bands. The apparent molecular weight of the bioactive protein of major band was found to be 35 kDa (Fig-3).

Complete purification and characterization of the bioactive protein was accomplished by applying partially purified protein to gel filtration column followed by high performance liquid chromatography. The molecular weight of purified protein was observed to be 35 kDa by SDS-PAGE (Fig-7), and deep-rooted by performing MALDI-TOF-MS analysis were confirmed the molecular weight at 33437.3 Da (Fig-8) and LC-ESI-MS/MS analysis of same protein was resulted in molecular weight at 33287.9 Da (Fig-9). Purified protein fragments were generated by tryptic digestion and based on the m/z values obtained by MALDI-TOF-MS analysis. Peptide fragments showing succession homology with DEAD-box-ATP dependent RNA helicase 45, present in NCBI non-redundant database.
The antibacterial activity of the purified protein was determined by agar well diffusion method using ciprofloxacin as a standard (Table-3&4). The data of bioactive protein obtained during MIC were highly significant values compared to standard and control. The minimum inhibitory zone of 30 µg bioactive protein was observed for *Staphylococcus aureus*, *Streptococcus haemolyticus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* whereas, *Bacillus subtilis* and *Salmonella typhi* was shown a minimum inhibitory zone at 40 µg of protein (Table-5&6). Effect of temperature on antibacterial activity against clinical strains of bioactive protein was observed active at 10°C to 60°C for 30 min. However, there was rapid loss of activity observed, when purified bioactive protein incubated above the 70°C and below 10°C (Table-7). The pH on the antibacterial activity of bioactive protein was significant at wide pH range of 6.5 to 8.5, but the activity was observed to lost slowly below 6.5 and above 8.5 (Table-8).

This experimental result could suggest us to consider this purified bioactive protein was a strong broad spectrum antimicrobial. The antibacterial protein was isolated from Tasar silk worm gut juice and reported to compose of large number of peptides and proteins. Some of those proteins were analyzed and has been found to contain proline-rich peptides. Structural similarities of antimicrobial proteins isolated from Tasar silkworm (*Antheraea mylitta*), chicken lysozyme and α-lactoalbumins were also studied (Jain *et al.*, 2001). The antibacterial activity has been also reported in haemolymph of the silkworm, by the addition of formalin-treated *Escherichia coli* k-12 in anterior and posterior body part of the ligated larvae (Sumida *et al.*, 1992). It has been reported that the bacterial peptidoglycan elicits the production of antibacterial protein in the larvae of
the silkworm (Morishima et al., 1992; Maso et al., 1993) and configure the cecropins inducible antibacterial peptides (Morishima et al., 1990). Peptidoglycans of *Escherichia coli* and *Bacillus megaterium* are equally effective elicitor to induce antibacterial protein synthesis, but *Micrococcus luteus* peptidoglycan was poor elicitor even after being hydrolyzed and purified. *Escherichia coli* and *Bacillus megaterium* have common peptidoglycan of so-called ‘direct cross linkage’ structure, in which D-alanine at the C-terminal of peptide subunits is directly cross-linked to diaminopimelic acid of another adjacent peptide subunit (Morishima et al., 1992).

Dzierski (1991) had been reported the peptidoglycan and Lipo-polysaccharide binds to the same binding protein on mouse B lymphocytes. Further he suggested that the binding is specific for the backbone of peptidoglycan and the part of lipo-polysaccharide. This would not be the case in the induction of antibacterial protein synthesis in *Bombyx mori*. Since not only sugar backbone but peptide subunit of some definite structure is required for the induction. *Bombyx mori* have two separate recognition system or receptors for peptidoglycan and lipo-polysaccharides (Morishima et al., 1992). An induced antibacterial protein of 15.5 kDa has activity against both Gram-negative and Gram-positive bacterial strains which have been detected in the female reproductive accessory glands of medfly, *Ceratitis capitata* (Marchini et al., 1991). Xylander and neverman (1990) have reported the presence of an antibacterial substance other than lysozyme which was active against *M. luteus* and *Enterobacter cloacae* in the hemolymph of myriapods. There were different types of proteins isolated from silk worm, which have been already established to have antibacterial activity viz. 5 kDa, effective against both Gram+/- bacteria at a concentration of 0.1 μg / ml (Okada and Natori, 1983) and 18-24
kDa proteins effective against *Pseudomonas aeruginosa* AC-3 (Sharma *et al.*, 2005). Likewise, a few proteins from gut juice of silkworm larvae found to inhibit nuclear polyhedrosis virus (NPV) (Mukai and Hara, 1969; Uchida *et al.*, 1984).

Literature on antibacterial proteins isolated from silkworm fecal matter (*Bombyx mori*) is lacking. Therefore, a detailed study pertaining to this purified protein and comparing it with other standard antimicrobial agents such as lysozyme would be beneficial. Apart from this, bioactive protein was also investigated for its analgesic, antioxidant, hepatoprotective and *in vitro* antiviral activity. The analgesic activity of purified bioactive protein was assessed by conducting an acetic acid induced writhing response for peripheral activity. Analgesic drug operate in different behavior mode on the peripheral and central nervous systems; acetic acid caused algesia by stimulating endogenous substances such as serotonin, histamine, and prostaglandins. (Ghule *et al.*, 2006). This in turn causes abdominal writhing responses (Bentley *et al.*, 1983). Therefore, in general this experimental method involved association of increased level of prostaglandins (PGE2 and PGE2α) in peritoneal fluids and lipoxygenase metabolites (Dhara *et al.*, 2000). In acetic acid-induced writhing response, the visceral pain model, the analgesic mechanism of abdominal writhing was induced, which involves the release of arachidonic acid (AA) via cyclooxygenases (COX) and prostaglandin (PG) biosynthesis (Koo *et al.*, 2006) in peritoneal fluids and lipoxygenase metabolites (Dhara *et al.*, 2000). From the above facts and experiments it could be observed that the percentage of protection value was high for the bioactive protein 57.30 % group compared to standard value 56.24 % and control groups 0.5 %. Hence, the purified
bioactive protein could suppress the creation or liberation of the above stated endogenous substances, which could be the basis for its analgesic property.

Purified bioactive protein from silk worm *Bombyx mori* fecal matter was also investigated for its antioxidant and hepatoprotective activities. This investigation confirms the purified bioactive protein could able to control the hepatic disorders induced by CCl₄. Further, the protein was found to have a major defense mechanism involving free radical scavenging potential. Antioxidant reacts with DPPH, which was a stable free radical, and converts DPPH into 1, 1-diphenyl-2-picrylhydrazine. (Chidambara et al., 2002). DPPH was known to abstract labile hydrogen (Constantin et al., 1990; Matsubara et al., 1991; Jain et al., 2008). The protein showed good scavenging activity was enhanced with increasing concentration. The calculated IC₅₀ value of DPPH and superoxide anion scavenging activity of partially purified protein was found to be 52 μg/ml and 111 μg/ml. The inhibition concentration (IC₅₀) of DPPH and superoxide anion scavenging activity of purified protein was found to be 112.2 μg/ml and 55.2 μg/ml. It was clear from the experimental observation that the dose-dependent decrease in the concentration of DPPH radical was due to scavenging ability of the protein.

Simultaneously hepatoprotective effects of partially purified and purified bioactive protein were assessed in biochemical studies by measuring the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin in the serum under experimental conditions. The purified bioactive protein on CCl₄ intoxicated rats were observed and explained in (Table 10 &11). In the CCl₄ intoxicated group (II), serum AST, ALT, ALP and total bilirubin values were increased to 478.6, 660.8, 111.2, 456.8% and 507.01, 62.55, 89.15, 500 %
respectively, when compared to the control group. The elevated levels of serum AST, ALT, ALP, and total bilirubin values were significantly reduced to 110.2, 97, 63.4, 53.1% and 312.4, 349.2, 78.1, along with 108.3%. 122.2, 95.9, 52.9, 60% and 223.4, 291.2, 57.3, 106.9 %, respectively, in the animal groups treated with 50 and 100 mg dose of bioactive protein. Rats treated with bioactive protein 100 mg/kg of bw was shown highly significant hepatoprotective activity.

The protein also protected the liver against histopathological changes produced by CCl₄ such as necrosis, fatty changes, ballooning and degeneration. Carbon tetrachloride damages the liver that the changes resemble histologically the changes induced during viral hepatitis (James and Pickering, 1976). The aforementioned enzymes normally remain in the cytosol of hepatocytes. But, when the hepatocyte membrane was damaged, these enzymes were released into the blood stream. Estimation of these enzymes in the serum was a quantitative marker for the extent of liver damage. (Ansari et al., 1991). Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures (Recnagal, 1983). The toxic metabolite CCl₃ radical was produced which further reacts with oxygen to give trichloromethyl peroxy radical. Cytochrome P450 2E1 was the enzyme responsible for this conversion. This radical binds covalently to the macromolecule and causes peroxidative degradation of lipid membrane of the adipose tissue. In this view, the reduction levels of AST and ALT by the protein was an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl₄. These findings were in agreement with the fact that the serum levels of transaminases return to
normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew et al., 1987).

The CCl₄ was biotransformed by the cytochrome P-450 in the liver endoplasmic reticulum to the highly reactive trichloromethyl free radical. This free radical in turn reacts with oxygen to form a trichloromethylperoxy radical, which may attack lipids on the membrane of endoplasmic reticulum more readily than the trichloromethyl free radical. The trichloromethylperoxy radical leads to elicit lipid peroxidation, the disruption of Ca²⁺ homeostasis, elevation of hepatic enzymes and finally results in cell death (Shanmugasundaram and Venkataraman 2006. Clawson, 1989). The body weight of experimental animals decrease as a result of CCl₄ injection was considered to be the result of direct toxicity of CCl₄ and/or indirect toxicity related to the liver damage. Changes in the body weight after CCl₄ dosing have been used as a valuable index of CCl₄-related organ damage (Reham et al., 2009 Bruckner, et al., 1986, Pradeep, et al., 2005). On the other hand, no literature could be found pertaining to the effect of natural hepatoprotective protein isolated from silkworm fecal matter. The protein exhibited hepatoprotective effect against CCl₄-induced liver damage in a dose-dependent manner by normalizing the elevated levels of the hepatic enzymes. Histopathological observations were also support the hepatoprotective potential of the protein.

Some reports (Tsoi & Fong, 2005; Oh et al., 2006; Lee et al., 2006) revealed that protein molecules from various plant sources possess antioxidant, hepatoprotective and antiviral activities. Some of the proteins from silk worm fecal matter have been found to have marked antiviral activity on enveloped viruses, but not on non-enveloped viruses (Hiraki et al., 1997). An enveloped animal virus, vesicular stomatitis virus (VSV, an
RNA virus), was inhibited by chlorophyll derivatives (CPD) from silk worm excreta (Lim et al., 2002) and some of the virus deactivating protein was isolated from digestive juice of the silkworm Bombyx mori (Uchida et al., 1983). Few of the proteins from silkworm fecal matter have been found to have marked antiviral activity against viruses such as human immunodeficiency virus (HIV), Sendai virus (HVJ), herpes simplex virus type-1 (HSV) (Hiraki et al., 1997) and nuclear polyhedrosis virus (NPV) (Uchida et al., 1983; Neelagund et al., 2007). Antiviral activity on enveloped viruses, was reported but not on nonenveloped viruses (Hiraki et al., 1997). The virus deactivating protein was isolated from digestive juice of the silkworm Bombyx mori (Uchida et al., 1983). However, these reports have not been described the mechanism of action of those proteins. In this investigation, the purified protein did not inhibit the two viruses; DNA viruses such as camelpox and goatpox virus at its MNTC in vitro.

Silkworm fecal matter may contain the enzymatically digested part of chlorophyll. Because silkworm exclusively feed on mulberry leaves, the undigested part of silkworm fecal matter could be the witness for the lot of proteins associated with the metabolic intermediates of the chlorophyll. Those metabolic intermediate of chlorophyll were found to be red florescent in nature. Few authors have published about Red Fluorescent Protein (RFP) in the fecal matter and gut juice of the silkworm but the molecular weights of all the reported RFPs were distinctly different from each other (Sethuraman et al., 1993, Neelagund and Hinchigiri 2011). All the reported RFPs were also analyzed for associated pigment and found to be the presence of mono / dicarboxylic tetrapyrrole pigment derivatives. Therefore in our study it was necessary to analyze the pigment associated with bioactive protein for its characterization. Bioactive protein was
shown UV-Visible spectrum peak at 415 and 655 nm, which could implies the presence of associated tetrapyrrole pigment of this protein. Separations of this associated tetrapyrrole were done using ammonical-acetone method (Chapter-2, page 5-7) (Rebeiz et al., 1975). During separation of pigment; ether and hexane layers were obtained. The hexane layer did not exhibit visible or fluorescence spectral peaks representing the absence of etherified tetrapyrrole derivatives. The ether layer was shown visible absorbance at 415 nm and 655 nm (Fig-11) indicating the presence of mono/dicarboxylic terapyrrole pigment derivatives.

Further analysis of pigment present in ether layer was subjected to thin layer chromatography (TLC); one red florescent spot was observed with Rf value of 0.72 (Table-1) in solvent system-toluene: ethyl acetate: ethanol (8:2:1 v/v). The red fluorescent spot was taken and dissolved in 1 ml 80 % v/v acetone. Processed red florescent spot containing pigment derivative was shown UV- spectrum at 415 nm and 655 nm (Fig-12). Fluorescence spectra of excitation maximum at 415.2 (Fig-13) and emission maximum at 655 nm (Fig-14) which could indicate the presence of mono/dicarboxylic terapyrrole pigment derivatives. The visible and fluorescence spectroscopic results of bioactive protein have revealed that the purified protein containing pigment was porphyrin derivative, related to chlorophyll or its metabolic intermediate. Longqin and Deyu (1989) have reported the separation and characterization of crude chlorophyll mixture of the acid degradation products of silkworm excrement using HPLC. They have recommended that the chlorophylls endure metabolism in the body of silkworm gave pyrochlorophyll derivatives. From this outcome, it may be rational to think that the pigment bound to bioactive protein may be one such metabolic product of chlorophyll.
The substances having antiviral activity towards HSV, HVJ and HIV type-1 found to be 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 fluorescence spectra were similar to those of chlorophyll \(a\) derivatives (Smith and Beniter 1955; Yshitake, 1989). Kalyankumar et al., (2009) reported unique SE-RFP of silkworm associated with two photochromic moieties namely tetrapyrrole-I (TP-I), tetrapyrrole-II (TP-II), its UV-Visible and fluorescence spectral analyses shown absorbance at 280 nm (red) and 410 nm (green). Santosh et al., (2008) observed that Photochromic Pigments in RFPs (A, B and C) found to be heterogeneous with respect to their components and its absorption extinction coefficients of fluorescence quantum yielded three RFPs namely monovinyl chlorophyllide \(a\), divinyl protochlorophyllide \(a\) and monovinyl pheophytin \(a\).

The substances obtained from silkworm fecal matter were known to possess hemolytic activity. It was chemically reported to be phyropeophorbide-\(a\), a degradation product of chlorophyll \(a\) (Longqin and Deyu 1989; Takeda, 1989). Thin layer chromatographic study 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 Rf value of 0.70 – 0.75 for monovinyl pheophytin \(a\) (Rebeiz et al., 1971). The authentic monovinyl pheophytin \(a\) (Table-2) was shown to have the excitation and emission maxima of 408 and 672 nm, respectively (Belanger and Rebeiz, 1982). Hence most of the chlorophyll \(a\) in greening and green tissue was made up of monovinyl chlorophyllide \(a\) (Duggan and Rebeiz 1982; Freyssinet et al., 1980). The reason behind the synthesis of MV Chl \(a\) Via MV and DV pathway that was via MV pchlde\(\rightarrow\)MV Chlide \(a\) \(\rightarrow\)MV Chl \(a\) and Via DV Pchlde\(\rightarrow\)DV Chlide \(a\)\(\rightarrow\)MV Chlde \(a\) \(\rightarrow\)Chl \(a\), molecules in vivo was extremely
important for the proper operation of photosynthesis (Duggan and Rebeiz, 1982 and Becker et al., 1976). The above all chromatographic and spectroscopic characteristic of monovinyl pheophytin a, the pigment separated from bioactive protein posses the red fluorescent property would led us to identify as monovinyl pheophytin a the derivative of chlorophyllide a.

The pigment separated on TLC was further characterized for its structural analysis using atomic absorption chromatography, $^1$H-NMR, and IR. $^1$H-NMR the separated pigment shown $\delta$ ppm = 0.8 (Singlet 15-H CH$_3$), 1.5 (Multipiplate 39-H Aliphatic CH$_2$), 1.7 (Multipiplate 4-H Aromatic-H), 1.9 (Triplet 6-H CH$_2$), 3 (Singlet 3-H CH$_3$), 3.8 (Singlet 2-H CH$_2$), 5.5 (Singlet 2-H Aromatic-H), 5.6 (Singlet 1-H Aromatic-H). Hence these peaks could be corresponds to monovinyle pheophytine a; the derivative of chlorophyllide a, (Fig-16) and IR of red florescent spot elute shown $\sqrt{\text{Max}}$ (m$^{-1}$) = 2918.8 peak corresponds to [C$_{20}$H$_{39}$ Aliphatic chain], 2849.7 peak corresponds to [C-H], 2719.5 peak corresponds to [CH$_2$ Aromatic], 1685.5 peak corresponds to [C=O], 1593.1 peak corresponds to [C=C] and 1542.2 peak corresponds to [C=N]. Therefore, these peaks could be evidences for predicting the structure monovinyle pheophytine a; the derivative of chlorophyllide a, (Fig-17) in which Mg was absent. Vinay P et al., 1992 determined the molar excitation co-efficient of divinyl chlorophyll a and b and their pheophytins; Dv Chl a NMR spectrum peaks at about 3.98 ppm was due to methanol contamination of CD$_3$OD unidentified peaks between 3.0 and 4.5 ppm represent contamination by colorless lipids. The sample was free of detectable water contamination as evidenced by lack of water peak at 2.80 ppm. Longqin and Deyu 1989 characterized chlorine derivative with intact ring V from acid degradation products of silkworm.
Excrement crude chlorophyll mixture proton NMR spectra of carbon-10 methyle group in pheophorbide \( \alpha \) absorb at \( \delta = 3.84 \) and the carbon-10 at \( \delta = 6.23 \). These two kinds of protons in pheophorbide \( \alpha \) absorb at \( \delta = 3.80 \) and \( \delta = 6.17 \), both a little downfield as compared with those in pheophorbide \( \alpha \). The two carbon-10 protons in pyropheophorbide \( \alpha \), which were not equivalent, absorb at 5.24 and 5.09, respectively. Each carbon-10 proton signal splits into two peaks (one was small and other was large) with a gem-Coupling constant \( |J| = 19.8 \) Hz. Resolved NMR chemical shifts of various functional groups of the metalated pigments were very similar to those reported earlier by Ramin and Constantin (1992). Comparable (Table-2) chromatographic and spectroscopic characteristics of the tetrapyrrole associated with the purified bioactive protein would led us to identify as monovinyl pheophytin \( \alpha \) the derivative of chlorophyllide \( \alpha \). The antibacterial and analgesic activity was not observed in components separated from bioactive protein, whereas good antibacterial and analgesic activity was observed when protein was in intact form. Hence, monovinyl pheophytin \( \alpha \) could be the essential component for the bioactivity of the protein. Therefore the characteristic features and pigment properties of bioactive protein isolated from silkworm sources support to confirm that protein is unique and novel.