Chapter - 5

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Silkworm fecal matter is a natural substance and it was known to be one of the prominent medicinal sources for many conventional infectious diseases (Zhang et al., 2008). Modern investigations on innovative drug design against infectious diseases visualize the rigorous studies towards more successful antimicrobial drugs. In particular, antimicrobial proteins discovered as a component of imprecise innate mechanism of infections in humans and animals (Mak et al., 2001). To prevail over, there is a need to evolve an innovative research to develop a natural drug against various infectious diseases. In this context the present study was projected to; purify a bioactive protein from silk worm fecal matter having various biological activities. Initially, the protein was purified by applying various biochemical techniques such as 50% ammonium sulphate precipitation, dialysis, adsorption chromatography and lyophilization. After purifying the protein, it was subjected to UV-visible spectral analysis, which yielded two peaks at 430 and 663 nm (Fig-2), indicating the association of tetrapyrrrole pigment to bioactive protein. SDS-PAGE analysis of the partially purified protein showed 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 mark 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 HPLC. The molecular weight of purified protein was observed to be 35 kDa by SDS-PAGE (Fig-7). MALDI-TOF-MS analysis of the protein gave a molecular weight of 33437.3 Da (Fig-8), and similarly, the LCMS analysis of same protein was resulted in
molecular weight of 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 for bioactive protein obtained during MIC were highly significant values compared to standard and control. The minimum inhibitory zone at 30 μg of bioactive protein, whereas Bacillus subtilis and Salmonella typhi showed a minimum inhibitory zone at 40 μg of protein (Table-5&6). Effect of temperature on antibacterial activity against different clinical strains of bioactive protein was observed; protein activity was observed in the range of 10°C to 60°C for 30 min. However, there was rapid loss of activity observed, when purified bioactive protein was incubated above 70°C and below 10°C (Table-7). The effect of pH on the antibacterial activity of bioactive protein was observed to be significant at wide pH range of 6.5 to 8.5, but the activity was lost slowly below 6.5 and above 8.5 (Table-8). These experimental results could suggest us to consider the purified bioactive protein which was a broad spectrum antibacterial. In future it could be productively used as a successful innate antibacterial and analgesic drug.

Purified bioactive protein from silk worm Bombyx mori fecal matter was also investigated for its antiviral, antioxidant and hepatoprotective activities. Purified protein did not inhibit the two viruses; DNA viruses such a. camelpox and goatpox virus at its MNTC in vitro. On the other hand, no literature could be found pertaining to the effect of natural hepatoprotective protein isolated from silkworm fecal matter. The purified 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 also support the hepatoprotective potential of the bioactive protein. It is to be inferred that the bioactive protein purified from silk worm *Bombyx mori* fecal matter possess preventive as well as curative roles against CCl₄ induced oxidative stress in liver due to its antioxidant property.

Bioactive protein was shown UV-Visible spectrum peak at 415 and 655 nm, which could implies the presence of associated tetrapyrrole pigment. Analysis of this associated tetrapyrrole was done using ammonical-acetone method (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 yielded a 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-2) 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. The pigment separated on
TLC was further characterized for its structural analysis using atomic absorption chromatography, $^1$H-NMR, and IR. Data obtained from the above technique could be considered that the bound tetra-annular may correspond to monovinyl pheophytine $a$; the derivative of chlorophyllide $a$. The antibacterial and analgesic activity was not observed in tetra-annular components separated from bioactive protein whereas good antibacterial and analgesic activity was observed when protein was in intact form. Hence, it could be confirmed that monovinyl pheophytin $a$ was one of the essential components for the bioactivity of the protein. Therefore, the biochemical characteristic features and pigment properties of bioactive protein isolated from silkworm fecal matter sustain to validate that protein is distinctive and novel. In future this investigation results could be helpful for conducting research in the various disciplines such as Pharmacology, Physiology, Pathology, Medical Biochemistry, Immunology etc.,. The combination of antibacterial and analgesic activity having protein could be used as an effective natural antibiotic. It would be possible to give promotion to purified bioactive protein; for its expression and cloning through Biotechnology.