Partial Purification and Biochemical Characterization of Antimicrobial and Analgesic Novel Bioactive Protein (Substances) from Silkworm (*Bombyx mori* Linn.) Fecal Matter

Ramappa Raghavendra • Shivayogeeswar Neelagund*

**ABSTRACT**

Silkworm fecal matter is considered to be one of the richest sources of antimicrobial and antiviral protein (substances), which was exploited by preparing fecal matter extract with 0.02 N phosphate buffer (pH 7.4). The transparent supernatant was subjected to 50% ammonium sulphate precipitation, dialyzed and lyophilized. The UV-visible spectrum of the partially purified protein indicated the association of tetrapyrrole pigment. This protein showed excellent antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Klebsiella pneumoniae*. Good analgesic activity was also assessed by conducting an acetic acid induced writhing test in mice.

**Keywords:** antimicrobial activity, analgesic activity, conjugated protein, tetrapyrrole

**INTRODUCTION**

Antibacterial substances, which originate from different plant and animal sources, are used for the treatment of various diseases caused by pathogenic bacteria. This inspired man to endeavor for a remedy from their sufferings (Zakir et al. 2002). Plenty of plants and animal extracts owe their potency to the presence of bioactive protein substances. As a result they have become very good sources for developing innovative antibacterial drugs for the pharmaceutical industry. Traditional medicine is a source of bioactive agents which can be applied for the preparation of medicine (Aboba et al. 2006) and these medicines are used as life-saving drugs. Various innovative drugs are really gaining resistance against various pathogenic microbes for example, Amoxicillin and Ciprofloxacin (Sundin et al. 1996; Sobhani et al. 2004; Amini et al. 2005).

Bacteria have evolved numerous defense mechanisms against antimicrobial agents, which are resistant to old and currently available drugs (Milton et al. 1997; Nizet 2006). Increasing failure of chemotherapeutic and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plant and animal extracts for their potent antimicrobial activity. There are several reports mentioned in the pertinent literature regarding the antimicrobial activity of crude extracts obtained from plant and animal species (Belboukhari et al. 2005; Yoga et al. 2007; Charu et al. 2008).

The increased prevalence of antibiotic-resistant bacteria, due to the extensive use of antibiotics, would render current antimicrobial agents insufficient to control bacterial diseases. Research on silkworm fecal matter is reported to have only few bioactive substances possessing antiviral activity against different viruses like HIV (Human Immunodeficiency Virus type-1), HJV (Sendai Virus), HSV (Herpes Simplex Virus Type-1) (Hiraki et al. 1997), NPV [Nuclear Polyhedrosis Virus] (Uchida et al. 1987; Neelagund et al. 2007). Antibacterial proteins, from non-mulberry silkworms, against *flacheria*-causing *Pseudomonas aeruginosa* were reported (Sharma et al. 2005). There was a clear study on structure of induced antibacterial protein from Tasar silkworm, *Antheraea mylitta* (Jain et al. 2001). Silkworm fecal matter is a natural substance and it is known to be one of the richest sources of antibacterial and antiviral substances (Akihito et al. 1997; Dae et al. 2002; Neelagund et al. 2007). Therefore, this investigation was conducted to examine the silkworm fecal matter for the presence of novel bioactive proteins.

**MATERIALS AND METHODS**

**Experimental conditions**

The experiments were carried out under sterile aseptic conditions at 25 ± 2°C. The containers and instruments were sterilized using 5% formaldehyde and 90% ethanol, washed and dried before use. Gram-positive and negative clinical isolates of bacterial strains were collected and stored in an incubator at 37°C for experimental use. Adult Swiss albino mice (25-30 g body weight) were obtained from the Viral Diagnostic Laboratory, Shivamogga. Institutional Animal Ethical Committee constituted by National College of Pharmacy Shivamogga, Karnataka, India (Reg. No.144/NCP/IAEC/CLEAR/P.Col.06/2007-08) was given permission to conduct analgesic activity experiments on mice. In each group six animals were housed individually in polypropylene cages with paddy husk beds. Animals were maintained at 25-27°C and 30-70% relative humidity.

**Chemicals**

All chemicals used for the experiment were of analytical grade, obtained from Sigma Chemical Co. (St. Louis, Mo., USA) except for silica gel mesh size 60/120 which was obtained from Acme’s Laboratory Chemicals.
Isolation and partial purification

Fifth instar 3rd day larvae fecal matter of Bombyx mori L. were collected from a farmer’s silkworm rearing house during bed cleaning at 8 am in the Kachinkattay area, Shivamogga District, Karnataka State, India. Then, it was thoroughly air dried at room temperature and the leaves, dust and other contaminants of the silkworm fecal matter were manually separated. The above cleaned silkworm fecal matter was stored at 4°C in a closed polythene container. The stored silkworm fecal matter was used for further experiments. Partially purified bioactive protein was isolated and purified from silkworm fecal matter as described in Neelagund et al. (2007) with slight modifications. Silkworm fecal matter (60 g) was powdered using a mortar. Then it was, mixed with 0.02 N phosphate buffer (pH 7.4), by continuous stirring for ~40 hrs at 60°C. This preparation was filtered, using Whatman filter paper no. 1 (11.0 cm diameter) and the filtrate was centrifuged for 30 min at 5000 rpm using a refrigerator centrifuge at 4°C. The supernatant was used for further purification by subjecting it to 50% ammonium sulphate precipitation. Then, it was centrifuged at 5000 rpm for 10 min at 4°C. The precipitate obtained from above preparation was dissolved in 10 ml of 0.02 N phosphate buffer (pH 7.4). Then it was dialyzed against 0.02 N phosphate buffer using a dialysis membrane having pore size of 110, average flat width 32-34 mm, average diameter 5-21 mm and flow rate 3.63 ml/cm (Sigma). The dialysate was subjected to lyophilization and lyophilized protein was applied to column chromatography using silica gel 'G' as a matrix [Mesh number 60/120; column 0.5 x 40 cm and matrix obtained from M/s Acme’s Laboratory Chemicals]. The bioactive protein fractions were eluted using phosphate buffer. Protein fraction (2 ml size) peaks were detected by monitoring absorbance at 280 nm on a Systronics-119 UV-Visible spectrophotometer. The A and B peaks of protein fractions were separately pooled, lyophilized and stored at 4°C.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using a 6% polyacrylamide gel by passing a constant current of 40 mA (Laemmli 1970). The proteins in the gel were stained with Coomassie brilliant blue and destained with solution containing 5% methanol and 7% acetic acid.

Antibacterial activity

The antibacterial activity of purified bioactive protein was screened by agar well diffusion method against Gram-positive (Staphylococcus aureus, Bacillus subtilis, Streptococcus haemolytus) and Gram-negative (Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae) bacterial strains. The bacterial strains used for screening antimicrobial activity were collected from different infectious patients who had not administered any antibacterial drugs for at least two weeks with suggestions of an authorized physician, in the Kiran diagnostic health centre of Chitradurga, Karnataka State, India. The plates were incubated for 24 h at 37°C. Ciprofloxacin (Sigma) was used as the standard (50 ng in 100 μL) in phosphate buffer solution (pH 7.4) and later loaded into corresponding wells. The standard drug ciprofloxacin was used (50 μg in 100 μL). The plates were incubated for 24 hrs at 37°C and the diameter of the zone of complete inhibition of bacteria was measured around each well and readings were recorded in mm. The results of these experiments were expressed as mean ± SE of six replicates in each test. The data were evaluated by one-way ANOVA followed by Tukey’s Pair-wise comparison test and results were considered significant when P < 0.05.

Analgesic activity

The analgesic activity was performed by the acetic acid-induced writhing test in mice (Satyanarayana and Rao 1993; Bagavant et al. 1994; Vagdevi et al. 2001), and approved by the animal ethics committee (Institutional Animal Ethical Committee, IAEC) of the National College of Pharmacy, Shimoga. Five groups of six male Swiss albino mice, each 25-35 g of body weight, were used. 0.6% acetic acid (dose = 10 ml/Kg of body weight) was injected intraperitoneally. The numbers of writhes were counted for 20 min, after 5 min of injection of acetic acid into each mice. This reading was taken as a control. Next day, same groups of mice were used for evaluating analgesic activity. Each group was administered orally with the suspension of partially purified novel bioactive protein from silkworm fecal matter. The dose of 100 mg/kg body weight of animal was given 1 hour before injection of acetic acid. After 5 min of acetic acid injection, mice were observed for the number of writthes for the duration of 20 min. The mean value for each group was calculated and compared with control. Acetyl salicylic acid was used as a standard for comparison of analgesic activity. Percent protection was calculated using following formula:

\[(1-V/V_c) \times 100\]

where \(V_c = \text{Mean number of writhing in test animals}\) and \(V_c = \text{Mean number of writhing in control}\).

RESULTS

Partial purification of novel bioactive protein

Novel bioactive protein partial purification was achieved by applying conventional biochemical techniques, such as ammonium sulphate precipitation, dialysis, and column chromatography. Two peaks were obtained: A and B (Fig. 1). Peak A fraction was active against bacterial strains and also showed very good analgesic activity. Antibacterial and analgesic activity was not found in peak B fraction. Protein obtained from peak A fraction was used for further experiments.

Minimum Inhibitory Concentrations (MIC)

The MIC of purified bioactive protein isolated from silkworm fecal matter was determined by a micro dilution method in nutrient broth (Harish et al. 2007). The inoculates were prepared using the above nutrient broth at a density adjusted to a 0.5 McFarland turbidity standard colony forming units and diluted to 1:10. The plates were incubated at 37°C and MIC was determined after 24 hrs of incubation period. Sterilized nutrient medium was poured into sterile Petri dishes. Nutrient broth containing 100 μL of 24 hrs incubated cultures and optical density (OD) was read at 660 nm. This respective clinical strain was spread separately on the agar medium. The wells were created using a stainless steel sterilized cork borer under aseptic conditions. The purified bioactive protein at different concentrations viz. 10, 20, 30, 40 and 50 μg was dissolved respectively in 25, 50, 75, 100 and 125 μL phosphate buffer

![Fig. 1 Adsorption chromatography was performed using silica gel mesh 60/120. Novel Bioactive Protein was eluted using phosphate buffer, 0.02 N (pH 7.4). Two peaks, A and B, were obtained. Antimicrobial and analgesic activity was observed in the peak A fraction.](image)
Biochemical characterization

UV-Visible spectrum of the purified bioactive protein showed absorbance peaks at 430 and 663 nm (Fig. 2). The SDS-PAGE analysis revealed one major protein band at 35 kDa. Some high molecular weight minor bands were also observed (Fig. 3).

Antibacterial activity

Bioactive protein showed significant antibacterial activity against Gram-positive and -negative bacteria. The highly significant values of antibacterial activity of bioactive protein was obtained and compared with standard ciprofloxacin (50 μg in 100 μL) and control (Table 1).

Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations were obtained for the following clinical strains: Staphylococcus aureus, Streptococcus haemolyticus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia) which showed a minimum inhibitory zone at 30 μg of partially purified protein, whereas Bacillus subtilis, and Salmonella typhi showed a minimum inhibitory zone at 40 μg of protein (Table 2).

Analgesic activity

Analgesic activity of bioactive protein was carried out using the acetic acid-induced writhing test. This experimental result yielded highly significant values for bioactive protein compared to standard and control values (Table 3).

DISCUSSION

The current fashion of adapting various strategies for preparing innovative drugs against serious infectious diseases and their continuous implementation will result in increasing rates of multidrug resistance of microorganisms. To overcome this crisis, we need to evolve an innovative investigation to develop a natural drug against infectious diseases. In this investigation partial purification of novel bioactive protein was achieved by applying biochemical techniques such as 50% ammonium sulphate precipitation, dialysis, adsorption chromatography and lyophilization. UV-Visible spectrum revealed two peaks for bioactive protein at 430 and 663 nm, which can register the association of tetrapyrole pigment to bioactive protein. SDS-PAGE analysis of the protein showed one major protein band along with some associated high molecular weight minor protein bands. The molecular weight of the bioactive protein was found to be around 35 KDa.

The antibacterial activity of the purified protein was determined by the agar well diffusion method using Ciprofloxacin as a standard. The data of bioactive protein obtained during MIC were highly significant values compared to the standard and control (Table 2). This experimental result allows us to consider this purified bioactive protein as a strong candidate of broad spectrum antimicrobial activity.

The antibacterial protein was isolated from Tasar silk worm gut juice and was composed of a large number of peptides and proteins. Some of these proteins were analyzed to contain proline-rich peptides. Structural similarities of antimicrobial proteins isolated from Tasar silkworm (Antheraea mylitta), chicken lysozyme and α-lactalbumins were also studied (Jain et al. 2001). All three were comparably similar in their structure and functions. Literature on antibacterial proteins isolated from silkworm fecal matter (Bombyx mori) is lacking. Therefore, a detailed study pertaining to this protein and comparing it with other standard antimicrobial agents such as lysozyme would be beneficial.

| Table 1: Antibacterial activity of partially purified Novel Bioactive Protein. |
|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Compound            | Streptococcus haemolyticus | Staphylococcus aureus | Salmonella typhi | Pseudomonas aeruginosa | Escherichia coli | Bacillus subtilis | Klebsiella pneumonia |
| Protein             | 25.44 ± 0.06      | 25.29 ± 0.04     | 28.17 ± 0.03    | 20.24 ± 0.05      | 18.37 ± 0.07    | 26.32 ± 0.05    | 25.22 ± 0.03     |
| Control (PBS)       | 0.19 ± 0.04       | 0.24 ± 0.01      | 0.18 ± 0.01     | 0.23 ± 0.01       | 0.25 ± 0.01     | 0.28 ± 0.02      | 0.24 ± 0.01      |

The value of each constituent consisted of Mean ± SE of 3 replicates. Value is significantly different when p<0.05. Standard drug used: ciprofloxacin (50 μg in 100 μL). Protein used: (40 μg in 100 μL). Control: Phosphate buffer solution (PBS) of 0.02 N of pH 7.4.
Apart from this, purified protein was also investigated for its analgesic activity. We demonstrated the effect of purified bioactive protein by conducting an acetic acid induced writhing test on mice. This acetic acid induced writhing response was evident for peripheral activity whereas the formalin test was conducted for both peripheral and central activity. Acetic acid caused algia by producing endogenous substances such as serotonin, histamine, and prostaglandins, stimulating pain in nerve cells (Ghule et al. 2006). This in turn causes abdominal writhing responses (Bentley et al. 1983). Therefore, in general this experimental method is high for the bioactive protein group compared to experiments it can be observed that the percentage of protection is high for the bioactive protein group compared to standard and control groups. The numerical findings of analgesic activity, experiments showed significant values when compared to the standard and control values (Table 3). Therefore, this experimental evidence enables us to make strong confirmation of the analgesic activity of bioactive protein. From the results of this study, it can be suggested that in the future purified bioactive protein can be successfully used as an antibiotic as well as a potent natural analgesic drug. Further vision of our research is on the complete purification and characterization of the bioactive protein.

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RESEARCH ARTICLE

Protective effect of partially purified 35 kDa protein from silk worm (Bombyx mori) fecal matter against carbon tetrachloride induced hepatotoxicity and in vitro anti-viral properties

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Abstract

Context: It has been found that many proteins from silkworm (Bombyx mori L.) fecal matter have been active against human immunodeficiency virus, Sendai virus, herpes simplex virus type-1, and nuclear polyhedrosis virus.

Objective: A partially purified 35 kDa protein from silkworm was screened for its hepatoprotective activity, and in vitro antioxidant, and antiviral properties against camelpox and goatpox viruses.

Materials and methods: The study investigated the efficiency of the partially purified 35 kDa protein from silkworm fecal matter against CCl₄-induced liver damage measured in terms of enzyme levels such as aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and total bilirubin, which maintain liver integrity. In vitro antioxidant potential of this protein was determined based on its ability to scavenge 2,2-diphenylpicrylhydrazyl (DPPH) and superoxide anions scavenging activity. Further, in vitro cytotoxic effect on Vero cells and antiviral activity against camelpox and goatpox viruses were also studied.

Results: The protein had significant hepatoprotection against CCl₄-induced liver damage and scavenging of DPPH radical and superoxide anion activity. However, the protein did not inhibit the multiplication of either virus tested at its maximum non-toxic concentration (MNTC) in vitro.

Discussion and conclusion: The partially purified 35 kDa protein from silkworm Bombyx mori L. fecal matter possessed protective effect against CCl₄-induced oxidative stress in rat model. The protein was found to be ineffective against camelpox and goatpox viruses at its MNTC in vitro.

Keywords: Bombyx mori, 35 kOa protein; hepatotoxicity, antioxidant, cytotoxicity, antiviral activity

Introduction

Silkworm Bombyx mori L. fecal matter is identified as having beneficial effects against different pathophysiological states. The protein purified from silkworm Bombyx mori fecal matter was reported to act as an antiviral against viruses such as human immunodeficiency virus (HIV), Sendai virus (HVI), herpes simplex virus type-1 (HSV) (Hiraiki et al., 1997) and nuclear polyhedrosis virus (NPV) (Uchida et al., 1983; Neelagund et al., 2007) and also antibacterial against infectious clinical
strains such as Staphylococcus aureus, Bacillus subtilis, Streptococcus haemolyticus, Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae (Raghavendra & Neelagund, 2009). In addition the silkworm fecal matter was also explored in the field of medicine against various traditional infections in China and this implies that the silkworm fecal matter has high medicinal value (Zhang et al., 2008).

The high medicinal value of the silkworm fecal matter can be exploited to examine its efficacy on various diseases including toxicity of chemicals on liver. The liver is a key organ for metabolic, excretory and detoxification activities; thus it will be constantly exposed to xenobiotics (Wolf, 1999). The toxins absorbed from the intestinal tract pass through the liver resulting in a variety of liver ailments, which is one of the most serious health problems. Hence, certain plant extracts are applied to treat liver disorders (Karan et al., 1999; Chaudhari et al., 2009). Despite tremendous strides in modern medicine, there are few drugs which stimulate liver functions or regenerate hepatic cells (Satyapal et al., 2008). Natural drugs are considered less toxic and free from side effects to liver than synthetic drugs (Satyapal et al., 2008). So far, no studies have been reported regarding the beneficial property of a natural protein obtained from silkworm fecal matter. Therefore, the present study was undertaken to investigate the protective efficacy of the partially purified 35kDa protein from silkworm fecal matter against carbon tetrachloride (CCl₄)-induced liver damage, in vitro antioxidant potential and antiviral activity against two DNA viruses such as camelpox and goatpox viruses.

Materials and methods

Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), phenazine methosulfate (PMS), disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Merck Chemicals, Mumbai, India. Eagle’s minimum essential medium (EMEM) from Gibco Invitrogen, Grand Island, New York, USA, Fetal bovine serum (FBS) from Hyclone, Logan, UT, and crystal violet from Qualigen, GlaxoSmithKline, Mumbai, India.

Experimental animals

Wistar albino male rats weighing 240–250 g were procured from the Animal Experimental Laboratory of the National College of Pharmacy, Shivamogga, Karnataka, India and used throughout the study. They were housed in microcon boxes in a controlled environment (temperature 25°±2°C and 12 h dark/light cycle) with standard laboratory diet (M/s Sai Durga Feeds and Foods, Bangalore, Karnataka, India) and water ad libitum. The study was conducted after obtaining Institutional Animal Ethics Committee’s clearance (144/1999/CPCSEA/SMG under the Certification Reference No. NCP/IAEC/CLEAR/06/2007-08). As per the standard practice, the rats were segregated based on their gender and quarantined for 15 days before the commencement of the experiment.

CCl₄ induced hepatotoxicity

Four groups of rats, each having 6 animals, were used in the experiment as detailed below.

Group I: Untreated control

Group II: Negative control treated with CCl₄ (carbon tetrachloride)

Group III: 50 mg/kg bw of lyophilized partially purified protein and on day 7 CCl₄ was administered orally.

Group IV: 100 mg/kg bw of lyophilized partially purified protein and on day 7, CCl₄ was administered orally.

Group I and II rats were given a single dose of 0.5 mL double distilled water (vehicle) once daily for 7 days. Group III and IV were simultaneously treated with 0.5 mL of lyophilized protein dissolved in double distilled water (50 and 100 mg/kg bw), respectively, for 7 days. On day 7, 6 h after the last treatment, group II, III and IV rats were given a single dose of CCl₄ in olive oil (1:1) at a dose of 2 mL/kg bw. After 24 h of CCl₄ administration, the rats were killed by cardiac puncture after being anesthetized lightly with diethyl ether, blood samples were collected and serum was separated by centrifuging at 800 g for 15 min, and the serum samples were subjected to biochemical investigations. Liver from each rat was dissected out for studying histopathological changes.

Serum enzymes

The biochemical parameters of serum enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Reitman & Frankel, 1957), alkaline phosphatase (ALP) (King, 1965) and total bilirubin (Malloy & Evelyn, 1937) levels were investigated.

Preparation of partially purified 35 kDa protein from silkworm (Bombyx mori) fecal matter

Isolation of partially purified 35 kDa protein and the yield of protein were reported in our previous study (Raghavendra & Neelagund, 2009).

In vitro antioxidant activity

DPPH scavenging activity

The method described by Chidambara Murthy et al. (2002) and Singh et al. (2002), and was used for determining the antioxidant activity of partially purified 35 kDa protein on scavenging DPPH radical. Various concentrations (20, 40, 60, 80 and 100 μg) of partially purified protein were taken in 100 μL of methyl alcohol in separate test tubes and to each test tube 5 mL of 0.1 mM methanol solution of DPPH was added and shaken vigorously and allowed to stand at 27°C for 20 min. The blank was prepared without the sample and methyl alcohol was used for baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was calculated and expressed in terms of percentage inhibition.
% radical scavenging activity = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100

Superoxide anion scavenging activity
Superoxide anion scavenging capacity of the partially purified protein was assessed as per Nishimiki et al. (1972). Reaction mixture containing 156 μM nitroblue tetrazolium, 468 μM NADH in 100 mM phosphate buffer (pH 7.4), with 0.1 ml. of partially purified protein (25–125 μg) in methanol were mixed and the reaction was initiated by adding 100 μL of 60 μM phenazine methosulfate in 100 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm against blank.

Histopathological studies
The liver tissue of treated rats was dissected out and fixed in 10% formalin, dehydrated in graded ethanol (50–100%), cleared in xylene and finally embedded in paraffin. Sections of tissues were prepared and then stained with hematoxylin and eosin (H&E) dye for photomicroscopic observation.

In vitro antiviral activity
Vero cells (CCl-81) between passages 75 and 86 were propagated in Eagle’s minimum essential medium (EMEM) supplemented with 10% FBS. Maintenance medium containing 2% FBS was used for maintenance of cells. The camelpox virus (CMLV) (Genus: Orthopoxvirus, family: Poxviridae) (CMLV/96, F60) and goatpox virus (Uttarkashi, GTPV/78) (Genus: Capripoxvirus, Family: Poxviridae) adapted to Vero cells were used as source of the viruses. The Vero cell monolayer was infected with CMLV at 0.01 multiplicity of infection and allowed to adsorb the virus on to cells at 37°C for 1 h. Then the monolayer was fed with maintenance medium and incubated for 2–3 days until >80%. Cytopathic effect (CPE) was observed for every medium on every alternate day. The virus was harvested when infected Vero cells showing virus specific CPE (syncytia, ballooning, and rounding of cells). After harvesting, the cells were freeze-thawed thrice and the virus was titrated in Vero cell monolayer. The titer was calculated as per Reed and Muench (1938) and expressed as log tissue culture infective dose (TCID50).

Cytotoxicity assay of the protein
Determination of the maximum nontoxic concentration/Dose
The cytotoxicity of partially purified 35 kDa protein to Vero cells was determined (Bhanuprakesh et al., 2007, 2008). Various concentrations of partially purified protein (25, 12.5, 6.25, 3.125, 1.56, 0.39 mg/mL with two-fold dilutions) were prepared in maintenance medium and added to the 24 h confluent Vero cell monolayer in 96-well tissue culture plates and incubated in an atmosphere of 5% CO2 at 37°C for 4 days. Each concentration was tested in triplicate along with controls. Cells were examined daily for morphological changes (rounding, degeneration), if any. After 96h, cell morphology was compared between treated and untreated cultures (control). The highest concentration of the protein that showed no cellular morphological changes was considered as the maximum nontoxic concentration/dose (MNTC/MNTD). Further, the medium was decanted from the wells and the cells were trypsinized and counted by the Trypan blue dye exclusion staining method to assess the viability of the cells. The concentration of the protein at which 50% cytotoxicity was observed (i.e., 50% viability of cells) was recorded as the CyC50 value. The concentration of protein at which cell viability was above 80% was further used in the study for assessment of antiviral activity of the protein against camelpox/goatpox viruses in a dose-dependent manner.

Antiviral effect (cytopathic effect inhibitory assay)
Assessment of in vitro antiviral activity of the partially purified protein was initially performed in 96-well microtiter plates in triplicate against camelpox and goatpox virus. Vero cells were seeded at 5 x 104 cells/well. After 24 h the growth medium was removed and replaced with serial dilutions of partially purified protein in maintenance medium. The pretitrated camelpox vaccine virus (CMLV) (10-3 TCID50/mL) and goatpox virus (10-3 TCID50/mL) at different dilutions (10-3 to 10-5) in 100 μL was used along with various non-toxic concentrations (1.56, 0.78, 0.39, 0.1953 mg/mL) of protein. To ensure the cytotoxic effect of the protein, a set of controls such as virus, protein (at different dilutions) and appropriate cell controls were included in the test. The plates were incubated at 37°C in a humidified CO2 incubator. The media was changed at every 48 h interval. The cells were observed for cytopathic effect (CPE) regularly under an inverted microscope.

Statistical analysis
The data of antioxidant activities were expressed as mean ± standard error mean (SEM). The difference among the was analyzed by one-way ANOVA test. Statistical significance was calculated at P <0.05.

Results
Hepatoprotective activity
The result of hepatoprotective activity of protein on CCl4-intoxicated rats is shown in Table 1. In the CCl4 intoxicated group (II), serum AST, ALT, ALP and total bilirubin were increased to 478.6, 660.8, 111.2 and 456.8% respectively, when compared to the control group. The elevated levels of serum AST, ALT, ALP and total bilirubin were significantly reduced to 110.2, 97, 63.4, 53.1% and 312.4, 349.2, 78.1, and 106.3% respectively in the animal groups treated with 50 and 100 mg dose of partially purified protein. Rats treated with partially purified protein 100 mg/kg bw showed highly significant hepatoprotective activity (P <0.001).
Table 1. Effect of protein on CCl₄-induced hepatotoxicity in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>Total bilirubin (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>242.02 ± 18.39</td>
<td>139.38 ± 14.6</td>
<td>456.08 ± 25.12</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>Group II</td>
<td>CCl₄</td>
<td>1400.03 ± 78.82***</td>
<td>1060.47 ± 85.67***</td>
<td>963.48 ± 55.22***</td>
<td>2.45 ± 0.16***</td>
</tr>
<tr>
<td>Group III</td>
<td>Protein (50 mg) + CCl₄</td>
<td>666.9 ± 43.04**</td>
<td>538.27 ± 67.88**</td>
<td>589.4 ± 34.85**</td>
<td>1.39 ± 0.09**</td>
</tr>
<tr>
<td>Group IV</td>
<td>Protein (100 mg) + CCl₄</td>
<td>339.5 ± 24.3**</td>
<td>236.6 ± 31.7**</td>
<td>541.4 ± 32.68**</td>
<td>1.19 ± 0.05**</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM of six rats; ***P < 0.001 significant compared to control; **P < 0.01 significant compared to CCl₄-intoxicated control.

DPPH-scavenging activity

Figure 1 illustrates the dose-dependent decrease in the concentration of DPPH radical due to scavenging activity of the protein. The results indicate that the protein has better scavenging activity that was enhanced with increased concentration. The calculated inhibition concentration (IC₅₀) value for the protein was found to be 52 µg/mL.

Superoxide anion scavenging activity

The superoxide anion derived from dissolved oxygen by phenazine methosulfate/nicotinamide adenine dinucleotide coupling reaction reduces to nitroblue tetrazolium. The decreased absorbance at 560 nm indicates the consumption of superoxide anion in the reaction mixture. The partially purified protein showed the scavenging activity with an IC₅₀ value of 111 µg/mL (Figure 2).

Histopathological observations

Histologically, the liver from control animals (Group I) had normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus and visible central veins, whereas the liver collected from CCl₄-intoxicated rats showed massive fatty degenerations, necrosis, ballooning and infiltration of lymphocytes with the loss of cellular boundaries. However, the histological architecture of liver sections of the rats treated with different doses of protein showed more or less normal lobular pattern with mild degree of fatty tissue changes and necrosis, which were similar to the control group (Figure 3).

Cytotoxicity and antiviral activity

Cytotoxicity of partially purified 35 kDa protein on host cells (Vero cells from an African green monkey kidney cell line) was studied. The maximum non-toxic concentration/dose (MNTC/MNTD) of protein was found at a concentration of 1.56 mg/mL at which there was no visible toxicity to the cells. The cells tolerated MNTCs of protein and hence below MNTCs were utilized for antiviral efficiency testing. When the protein was tested for its antiviral activity below its MNTC against camel pox virus and goatpox virus it failed to inhibit the replication of these viruses in vitro. The titers of both the viruses remained 10⁶ and 10⁵ pfu/mL, respectively, both in control and treated wells. This indicates that the protein is non-inhibitory/avirucidal (data not shown) against these viruses; rather, infected and treated cells showed virus-specific cytopathic effects.

Discussion

In the present study, a partially purified 35 kDa protein from silk worm Bombyx mori fecal matter was investigated for its antioxidant and hepatoprotective activities in CCl₄-treated rats. This study proved the usefulness of partially purified 35 kDa protein from silk worm fecal matter in controlling the hepatic disorders induced by CCl₄. Further, the protein was found to have a major defense mechanism involving free radical scavenging potential as measured by DPPH and superoxide anion scavenging activities (Figures 1 and 2). It is clear from the experimental observation that the dose-dependent decrease in the concentration of DPPH radical was due to scavenging ability of the protein. The protein showed good scavenging activity that was enhanced by increasing concentration. The calculated IC₅₀ value for the protein was found to be 52 µg/mL for DPPH and 111 µg/mL for superoxide anion scavenging activity.
The antioxidant reacts with DPPH, which is a stable free radical, and converts DPPH into 1,1-diphenyl-2-picrylhydrazine (Chidambaram Murthy et al., 2002). DPPH is known to abstract labile hydrogen (Constantin et al., 1990; Matsubara et al., 1991; Jain et al., 2008) simultaneously. The hepatoprotective effects of partially purified protein were assessed by biochemical studies in terms of measuring the levels of AST, ALT, ALP and total bilirubin in the serum under experimental conditions. The protein also protected the liver against CCl₄-induced histopathological changes such as necrosis, fatty changes, ballooning and degeneration. In addition, CCl₄ damages the liver, and the changes induced resemble histologically that of the viral hepatitis (James & Pickering, 1976). The aforementioned enzymes normally remain in the cytosol of hepatocytes. But, when the hepatocyte membrane is damaged, these enzymes are released into the blood stream. Estimation of these enzymes in the serum is 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 CCl₄, radical is produced, which further reacts with oxygen to give trichloromethyl peroxyl radical. Cytochrome P450 2E1 is the enzyme responsible for this conversion. This radical binds covalently to the macromolecule and causes peroxidative degradation of the lipid membrane of the adipose tissue. In this view, the reduction in levels of AST and ALT by the protein is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl₄.

These findings are in agreement with the fact that the serum levels of AST and ALT return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew et al., 1987). A number of recent 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). However, these reports have not described the mechanism of action of these proteins. In this investigation, the purified protein did not inhibit the two pox viruses (DNA viruses), camelpox virus and goatpox virus, in vitro.

In conclusion, it is to be inferred that the partially purified 35 kDa protein obtained 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. Moreover, the protein was non-inhibitory to two pox viruses studied. Further, complete purification and characterization of the hepatoprotective protein is in progress and the intention is to screen different groups of viruses for antiviral activity in the future.

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Declaration of interest

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