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

Ramappa Raghavendra • Shivayogeeswar Neelagund*

Department of PG Studies and Research in Biochemistry, Jnana Sahyadri, Kuvempu University, Shankaraghatta Shivamogga, Karnataka, 577 451 India

Corresponding author: * neelgund@gmail.com

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 clear supernatant was subjected to 50% ammonium sulphate precipitation, dialyzed and lyophilized. The above preparation was subjected to further purification by column chromatography. SDS-PAGE analysis showed a major band at 35 kDa along with other associated high molecular weight proteins. The UV-visible spectrum of the partially purified protein indicated the association of tetrapyrrole pigment. This protein showed excellent antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus haemolytius* and Gram-negative bacteria (*Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, 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; Amit *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), HVJ (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 (Akhiro *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 bed. 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.

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Original Research Paper

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 silk worm fecal matter were manually separated. The above cleaned silkworm fecal matter was stored at 4°C in a closed polythene container. The stored silk worm 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 refrigerated 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 × 40 cm and matrix was 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 distained 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 haemolytius*) 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 µg in 100 µL.) in phosphate buffer solution (Sharath *et al.* 2008).

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 (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 writhes 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:

$$(1-V_c/V_t) \times 100$$

where V_t = Mean number of writhing in test animals and V_c = 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.

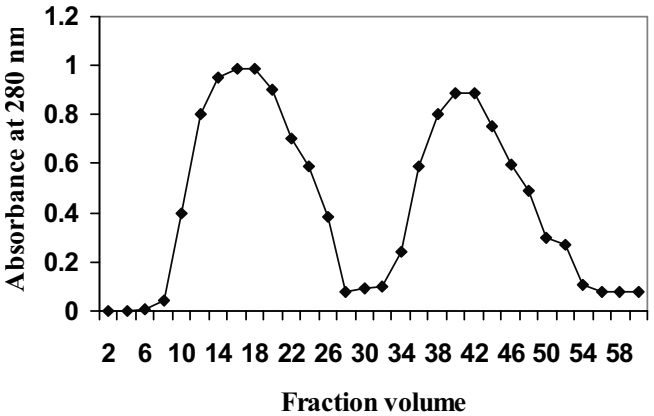

Fig. 1 Adsorption chromatography was performed using silica gel mesh 60/120. Novel Bioactive Protein was eluted using phosphate buffer, 0.02 N (pH 7.4). Two peaks, A and B, were obtained. Antimicrobial and analgesic activity was observed in the peak A fraction.

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**).

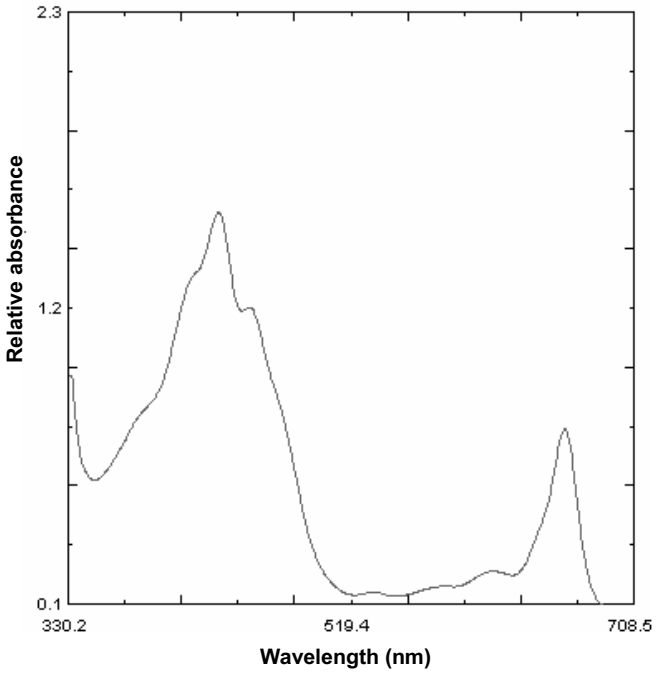

Fig. 2 Partially purified bioactive protein showing two peaks of UV-visible spectrum at 430 and 663 nm in phosphate buffer.

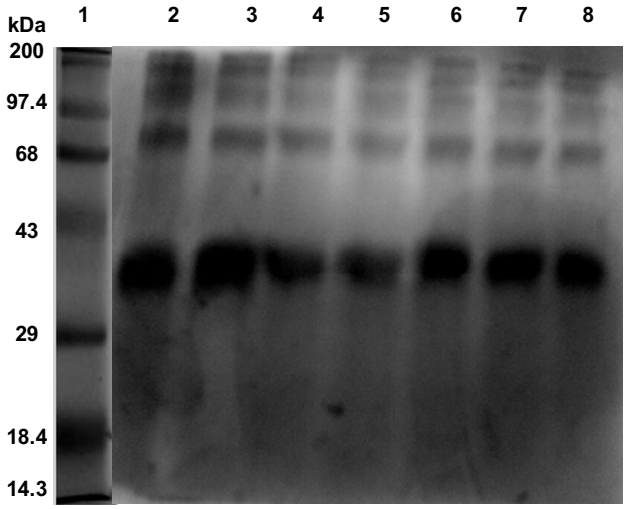

Fig. 3 SDS-PAGE was carried out using 6% polyacrylamide gel and stained with Coomassie brilliant blue. Lane 1: standard molecular weight protein markers. Lanes 2-8: partially purified novel bioactive protein. A major band showing molecular mass around 35 kDa protein, along with some high molecular weight minor protein bands.

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 ciprofloxcin (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 tetrapyrrole 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-lactoalbumins 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
Standard	22.58 ± 0.23	22.67 ± 0.16	22.81 ± 0.09	21.67 ± 0.03	21.68 ± 0.08	22.61 ± 0.05	22.68 ± 0.08
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 constituents 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

Table 2 MIC of partially purified Novel Bioactive Protein.

Protein concentration (µg)	*Streptococcus-haemolyticus*	*Staphylococcus aureus*	*Salmonella typhi*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Bacillus subtilis*	*Klebsiella pneumonia*
10	16.28 ± 0.04	16.41 ± 0.07	16.32 ± 0.08	16.44 ± 0.08	16.45 ± 0.04	16.43 ± 0.05	16.34 ± 0.10
20	20.21 ± 0.02	20.47 ± 0.09	20.37 ± 0.04	20.74 ± 0.02	20.38 ± 0.02	20.42 ± 0.05	20.38 ± 0.06
30	24.26 ± 0.06	23.19 ± 0.02	24.50 ± 0.10	24.21 ± 0.05	24.36 ± 0.02	23.31 ± 0.06	24.56 ± 0.06
40	24.63 ± 0.01	24.40 ± 0.02	25.37 ± 0.05	24.68 ± 0.03	25.15 ± 0.02	24.49 ± 0.16	24.53 ± 0.09
50	24.78 ± 0.10	24.68 ± 0.04	26.39 ± 0.01	24.60 ± 0.10	24.57 ± 0.07	26.77 ± 0.05	24.65 ± 0.04
Standard	22.33 ± 0.09	21.74 ± 0.03	22.77 ± 0.05	20.83 ± 0.09	21.48 ± 0.24	21.57 ± 0.13	22.55 ± 0.13
Control (PBS)	0.27 ± 0.02	0.31 ± 0.02	0.19 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.28 ± 0.02

The value of each constituents 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

Table 3 Analgesic activity of partially purified Novel Bioactive Protein.

Compounds	Dose (mg/kg)	Mean no. of writhes		% Protection
		Before administration of drug (mean ± SE)	After administration of drug [PBS as control. Aspirin as standard. Protein as test sample]	
Control ((PBS)	100	33.66 ± 0.816	33.33 ± 1.0	1
Standard (aspirin)	100	34.66 ± 0.93	16.66 ± 0.44	52.00
Test sample (protein)	100	34.16 ± 0.70	15.5 ± 0.447	54.63

Values are mean ± SEM (Standard Error of the Mean)
Index for analgesic activity
Method: Acetic acid induced writhing (acetic acid-0.6%)
Animal: Swiss Albino male mice.
No. of animals per group: 6 (25-30 g each)
Route of administration: IP (Interperitoneally)
Standard drug used: acetyl salicylic acid (Aspirin)

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 algesia 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 involved association of increased level of prostaglandins (PGE_2 and $PGE_{2\alpha}$) in peritoneal fluids and lipoxygenease metabolites (Dhara *et al*. 2000). Hence, the purified bioactive protein suppressed the synthesis or release of the above stated endogenous substances. From the above facts and 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 highly 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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