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ORIGINAL ARTICLE

Analysis of the total proteins in the bivoltine silkworm cocoon layers for their antibacterial properties

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ABSTRACT
The silkworm produces silk protein as a natural fibre to make a cocoon that will protect it from many threats when it is in the pupal stage. The components of silkworm cocoons contain antibacterial properties that protect pupae inside from microbial infection. Since the silkworm naturally uses antimicrobial proteins to prevent infection when creating its cocoon, they have been used employed as a biomaterial for a few decades. The current study attempts to understand the antimicrobial proteins found in the Bombyx mori silkworm's bivoltine cocoon. The various cocoon layers were collected from the cocoon, and then each layer's total proteins were extracted. Then the protein extracts were tested for antibacterial activity against two important silkworm pathogens, Serratia marcescens and Staphylococcus aureus. The disc diffusion method was employed to understand the microbial inhibition of cocoon proteins. The development of a zone of inhibition against the microorganisms demonstrated the presence of antibacterial activity in the cocoon shell extract. For further analysis, 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on the cocoon shell extract. According to the protein profile of the cocoon extract, bands stained with silver nitrate and resolved between 14 and 150 kDa were found. Most of the protein bands are localized around 10 to 90 kDa in size.
Keywords: *Cocoons, Antibacterial, Protein.*

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INTRODUCTION

The silkworm cocoon, a model lepidopteron insect that has been extensively studied, has economic importance. In India, the sericulture industry is largely concerned with boosting the production of high-quality silk. According to Akai (1997) and Bharathi ***et al***. (2020), India is the only country to produce all five types of commercial silk: Mulberry, Tropical Tasar, Oak Tasar, Eri, and Muga [1, 2]. Commercial silk cultivation is one of humanity's oldest agricultural business enterprises. [3].

The silkworm produces silk protein as a natural fibre to make a cocoon that will protect it from many threats when it is in the pupal stage. The proportions of sericin and fibroin, the two types of proteins that make up silk, differ depending on the races and breeds of silkworms, with fibroin typically comprising 70–80% and sericin 20–30%. Sericin is unique in that it contains 18 amino acids, including essential amino acids, and 32% serine. A total of 45.89% hydroxyl amino acids are included in sericin. However, it has been found to have important biological features. 18 amino acids make up the majority of its side chains, which are polar and made up of amino, hydroxyl, and carboxyl groups. These functional groups make it possible for sericin protein to easily co-polymerize, mix, and cross-link with other synthetic and natural polymers to create biomaterials with improved characteristics. Serine, aspartic acid, and glutamic acid are three polar amino acids that are abundant in the silk fibre [4]. Silk is a resource that is commercially utilized in India and is created by the silkworm by spinning cocoons utilizing silk fiber. Breed-to-breed differences exist in cocoon and raw silk quality and quantity characteristics [5]. While the

quantity characters are stated in terms of cocoon weight, shell weight, shell percentage, filament length, filament denier, and reliability, the quality of cocoons is defined in terms of cocoon colour, cocoon shape, grains, size, compactness, and dependability [6]. Quantitative characteristics such as cocoon weight, shell percentage, and filament length are given more attention in the current study because sericin content mostly depends on these characteristics [7].

MATERIAL AND METHODS

Bivoltine silkworm cocoon collection:

The bivoltine *Bombyx mori* silkworm cocoons were obtained from the Central Sericulture Research and Training Institute, Central Silk Board, Srirampura, Mysuru, India.

Total protein extraction from the cocoon layers:

The bivoltine cocoon layers were removed using sterile scissors, and the cut cocoon was weighed before being immersed in the Tris-HCl buffer (pH 7.5) for 6–12 hours in a rotary shaker at 120 rpm and 28°C. Following the incubation, the crude extract was centrifuged at 10,000 RPM for 10 to 15 minutes after being filtered through a sterile muslin cloth. A minimal amount of Tris-HCl buffer (pH 7.5) was used to suspend the resulting pellet.

Five layers of the cocoon were separated before being broken into smaller pieces and placed to the prescribed 25 mL flask along with 10 mL of the pH 7.5, 100 mM Tris-HCl buffer for overnight incubation in a rotary shaker at 220 RPM for 24 hours. To avoid protein denaturation, the proteins were collected after incubation and kept in the buffer at 4 °C.

Protein estimation:

Isolated protein samples were carried out for quantification by advanced nanodrop spectrophotometer analysis.

SDS-PAGE analysis:

The isolated protein samples were denatured by heating at 100 °C for 5 minutes while being mixed with sample buffer, which contains 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris HCl, pH 6.8. The polyacrylamide gel was utilized up to 15%. The electrophoresis took place for 90 minutes at 150 V and 20 mA.

Protein Staining:

The SDS-PAGE gel was stained with silver nitrate. The methodology was followed by the standard method [8]. Finally, the gel was stained with 7% acetic acid.

Antibacterial assay:

The experiment had three independent replicates (n = 3). In brief, sterile nutrient agar was put onto a labeled sterile petri-plate (layers 1, 2, 3, 4, and 5) and allowed to settle. Using an L-shaped rod, bacterial inoculum (*Serratia marcescens* and *Staphylococcus aureus*) was dispersed over the agar plate (0.1 ml of each strain). Using sterile forceps, a sterile disc was dipped into the protein sample and placed on the infected plates of each sample. The plates were incubated for 24 hours at 37 °C. The inhibition zone was detected around the disc after incubation.

RESULTS

Sample collection:

Different cocoon layers were collected from the bivoltine cocoons (Fig.1) and incubated in Tris-HCl (pH 7.5) overnight for protein extraction.

Protein concentration:

The maximum total protein concentrations were found in layers 2 (0.92 mg/ ml) and 5 (0.73 mg/ml) (Fig. 2), whereas the minimum protein concentrations were associated with layers 1 (0.33 mg/ml) and 4 (0.48 mg/ml).

Protein profiling by SDS-PAGE analysis:

The total protein was extracted from the cocoon's various layers according to the technique. Protein bands were obtained after loading the sample into an SDS-PAGE gel (supplementary figure S1). Different bands at different molecular weights were found, indicating protein separation.

Layer 1 included 6 protein bands, Layer 2 contained 8 protein bands, Layer 3 contained 9 protein bands, Layer 4 contained 8 bands, and Layer 5 contained 4 protein bands. The total number of protein bands discovered in different levels of bivoltine cocoon samples was 35.

Anti-bacterial assay:

The anti-bacterial activity of cocoon proteins was tested against bacterial pathogens such as *Serratia marcescens* (gramme negative) and *Staphylococcus aureus* (gramme positive) in the current study. The findings demonstrated that proteins isolated from different cocoon layers effectively limit pathogen

growth to varying degrees (Table 1 and Figure 3). The highest antibacterial activity (0.4 0.33 cm) was discovered against *Serratia marcescens*, whereas the lowest activity (0.2 0.33 cm) was identified against *Staphylococcus aureus* (Fig. 3 and 4). Total protein antibacterial activity in distinct layers against both infections concluded that total proteins in the bivoltine cocoon have good antibacterial characteristics.

Table 1 contains the diameter of the inhibition zone of cocoon proteins against *Serratia marcescens.* Different layers of the cocoon had distinct inhibitory activity on bacterial pathogens. Among the 5 coon layers, layer 1 had shown maximum antibacterial activity (0.4± 0.33 cm), compared to layers 2, 4, and 5, which show minimum antibacterial activity.

Staphylococcus aureus. Table 2, shows the diameter of the inhibition zone against *Staphylococcus aureus,* shows that all the different layers of the cocoon have the minimum inhibitory concentration of protein.

The values are mean of three independent experiments with standard error. The values are significantly different when subjected to Duncan's Multiple Range Test, DMRT (P>0.05).

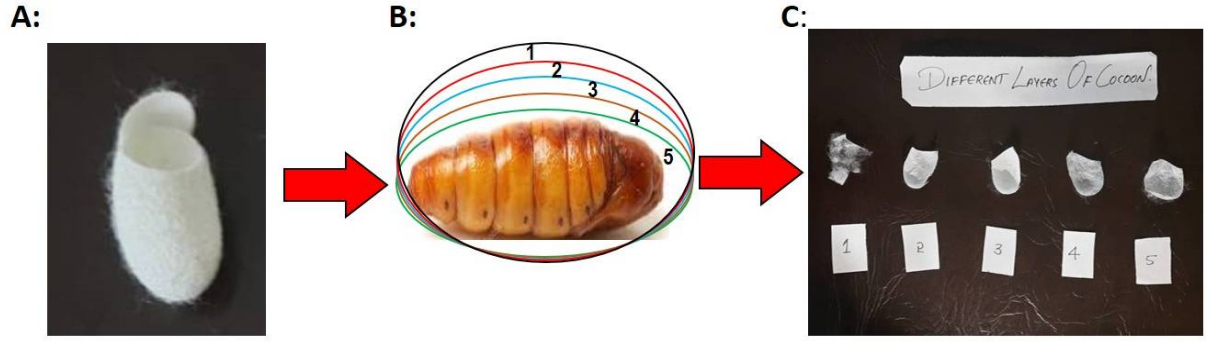

Fig 1: Representation of bivoltine *B. mori* silkworm cocoon (A), schematic cocoon layers (B) and separated cocoon layers (C)

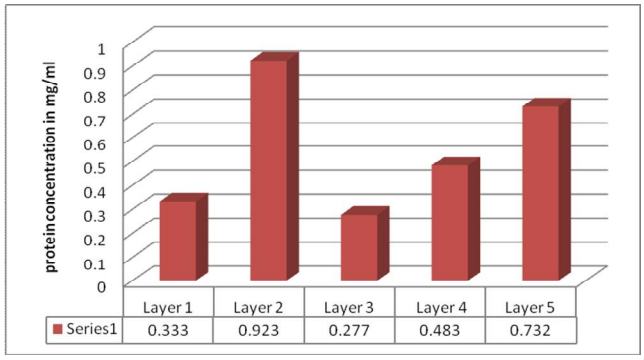

Fig 2: Protein concentration (mg/ml) in different layers of bivoltine **cocoon sample**

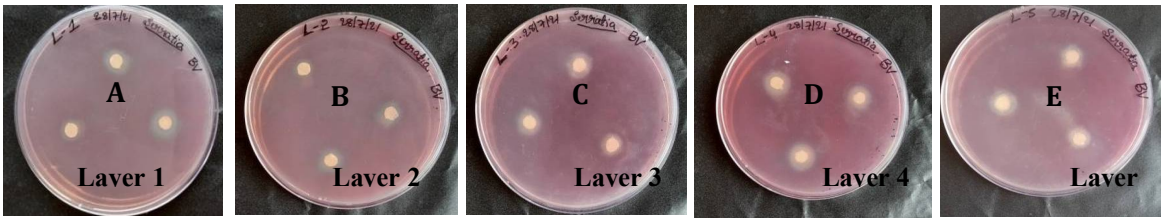

Fig 3: Antibacterial activity of bivoltine cocoon proteins against *Serratia marcescens*

Fig 4: Antibacterial activity of bivoltine cocoon proteins against ***Staphylococcus aureus.***

Table 1: Inhibition assay result against *Serratia marcescens*

No of layers	Zone of inhibition against *Serratia marcescens*		
	Disc 1(cm)	Disc 2 (cm)	Disc 3 (cm)
Layer-1	0.4 ± 0.33^{b}	0.3 ± 0.33^{b}	0.3 ± 0.32^{b}
Layer-2	0.3 ± 0.34^{b}	0.3 ± 0.32^{b}	0.3 ± 0.33^{b}
Layer-3	0.3 ± 0.31^{b}	0.4 ± 0.33^{b}	0.3 ± 0.33^{b}
Layer-4	0.3 ± 0.35^{b}	0.3 ± 0.34^{b}	0.3 ± 0.34^{b}
Layer-5	0.3 ± 0.31^{b}	0.3 ± 0.32^{b}	0.3 ± 0.31^{b}

Table 2: Inhibition assay result against *Staphylococcus aureus*

No of layers	Zone of inhibition against *Staphylococcus aurous*		
	Disc 1(cm)	Disc 2(cm)	Disc 3(cm)
Layer-1	0.2 ± 0.33^{b}	0.2 ± 0.32^{b}	0.2 ± 0.33^{b}
Layer-2	0.2 ± 0.32^{b}	0.2 ± 0.31^{b}	0.2 ± 0.31^{b}
Layer-3	0.2 ± 0.33^{b}	0.2 ± 0.33^{b}	0.2 ± 0.34^{b}
Layer-4	0.2 ± 0.32^{b}	0.2 ± 0.32^{b}	0.2 ± 0.33^{b}
Layer-5	0.2 ± 0.32^{b}	0.2 ± 0.32^{b}	0.2 ± 0.33^{b}

DISCUSSION

Antimicrobial proteins such protease inhibitors and seroins have been discovered in silkworm cocoons [9]. These proteins' functioning and processes have been investigated [10]. Protease inhibitors include a wide range of sequences and domains, and the majority of them have numerous pairs of disulfide bonds. As a result, their structures and activities are stable. Seroins have two motifs: one that is proline-rich at the N-terminus and one that is sequence-conserved at the C-terminus. Seroins have broad-spectrum antimicrobial activity against bacteria, fungi, and viruses, whereas protease inhibitors are primarily antifungal [10]. These antimicrobial proteins are more prevalent in the sericin layers than in the fibroin layers and in the outer cocoon layer than in the inner cocoon layer. The protein in the cocoon may hinder the germination of Beauveria bassiana spores. In addition to silk proteins, the silkworm cocoon includes trace levels of antimicrobial non-protein components such as organic acids, alkaloids, flavonoids, and heterocyclic compounds [11]. Zang et al. (2015) discovered 169 proteins in the silk of the Bombyx mori cocoon using shotgun liquid chromatography-tandem mass spectrometry. The assay for protease inhibitor action included four proteases, including trypsin, chymotrypsin, elastase, and protease K. To protect the cocoon, anticipating the principal effects of protease inhibitors that result in protease K inhibitors may be more important than with other inhibitors [12].

An earlier study identified 129 proteins, 30 of which are classed as protease inhibitors. To maintain structural integrity, protease inhibitors feature multiple intramolecular disulfide connections [13, 14]. After being released from the cocoon, the seroin protein is divided into the Sn1, Sn2, and Sn3 subfamilies. Seroins 1 and 2 were further subdivided into Sn1 and Sn3 subfamilies, respectively, while Sn2 subfamilies Sn2a, Sn2b, and Sn2c were discovered. The researchers concluded that seroins are more successful than *E. coli* at preventing *S. aureus* growth, with seroin 1 having no bacteriostatic action against gram-negative E. coli but having an effect on gram-positive S. aureus [15]. Silkworm cocoons contain the protein serotonin. Seroins 1 and 2 have considerable antibacterial and antiviral action among those 1, 2, and 3 [16]. All three seroins showed superior antibacterial activity against gram-negative bacteria. Peptidoglycan rendered seroin 2's antibacterial activities ineffective, limiting seroin 2's ability to bind to bacteria. The three silkworm seroins' N-terminal domains limit bacterial growth, whereas the C-terminal domain binds to bacterial peptidoglycan, establishing a common antibacterial mechanism [17,18]. We isolated the protein from the cocoon using a Tris-HCl buffer (pH 7.5) and tested its impact on bacterial growth to determine the resistance functions of the cocoon protein. The microbiological susceptibility of cocoon shell extract to bacteria such as *E. coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* was examined using the disc diffusion method. The establishment of a zone of inhibition against the microorganisms further demonstrated the antibacterial and immunogenic effects of cocoon shell extract.

Pandiarajan et al., (2011) worked on the cocoon total proteins. The disc diffusion technique and SDS-PAGE protein profiling were used to test the antibacterial activity of cocoon extracts. To extract the peptide sequence and determine the functional characteristics of the peptide, protein samples were exposed to MALDI-TOF and recorded on the Ultraflex MALDI mass spectrometer. After using the disc diffusion method to test its antibacterial capabilities, discovered that the cocoon shell extract inhibits the

growth of bacteria such as *E. coli*, *B. cereus*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*. Invaders such as bacteria may be repelled by chemicals or enzymes in the outer layer [19]. Two protease inhibitors and two seroins were among the small-molecular-weight proteins discovered in the cocoon [20].
The majority of cocoon flavanol glycosides were related to plant phenolic compounds and had chemical structures similar to insect metabolites. The flavonoids contained in cocoons are critical for shielding silk from UV damage and microbial attack. An antibacterial protein has been discovered in the silkworm *B. mori's* silk gland, fat body, hemocytes, testes, and ovary. The cocoon of the non-motile silkworm *B. mori*, which produces high-quality silk, was investigated in order to identify and describe the immunological and antimicrobial proteins present [13]. The two proteins that makeup silk cocoons are fibroin and sericin, a gum-coated protein that is sticky in nature and aids in the growth of the fibre by glueing the fibroin strands into a single unit. Additionally, several proteins with modest molecular weight were discovered in the cocoon, along with some proteins with strong physical qualities to protect pupae.

CONCLUSION

Pathogen growth is effectively suppressed by proteins isolated from different cocoon layers, with varying degrees of efficacy. The antibacterial potential of silkworm proteins derived from different cocoon layers is promising, and it could lead to novel biomedical solutions, particularly in the fight against bacterial infections. Nonetheless, more study is required to fully exploit its antimicrobial properties and transfer them into practical uses. This is the initial research necessary to understand silkworm pupal survival strategies.

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