Int.J.Curr.Microbiol.App.Sci (2017) *6*(3): 2055-2059

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 3 (2017) pp. 2055-2059**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.603.234

In vitro Efficacy of Antibacterial Proteins from Haemolymph of Silkworm Breeds against Bacterial Pathogens of Mulberry Silkworm, *Bombyx mori* L.

S. Manimegalai[1], B. Bebitha[2] and P.Mohanraj[3*]

[1]Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore-3, India
[3]Department of Sericulture, Forest College and Research Institute, Mettupalayam, India
**Corresponding author*

Keywords

Bombyx mori, Antibacterial, Proteins, Mulberry, *Bacillus thuringiensis*.

Article Info

Accepted:
20 February 2017
Available Online:
10 March 2017

A B S T R A C T

Antibacterial activity of proteins eluted from immunized haemolymph collected from breeds of mulberry silkworm, *Bombyx mori* L., *viz.*, Rong daizo, CSR2 and double hybrid were studied against bacterial pathogens. The breeds were immunized with bacterial pathogens, *Escherichia coli* (K^R strain), *Bacillus thuringiensis* and *Staphylococcus aureus* . *In vitro* efficacy of purified antibacterial proteins from breeds of *B.mori* against *E.coli*, *S. aureus* and *B. thuringiensis* revealed that among the ten fractions of antibacterial proteins eluted from *B.mori* breeds, ninth fraction@ 200µl showed higher antibacterial activity in Rong daizo (3.01cm, 3.10 cm and 1.82cm) (2.71 cm, 3.01cm and 1.60 cm), CSR2 (2.98 cm, 3.05 cm and 1.81 cm) and double hybrid (2.89 cm, 2.98 cm and 1.79 cm) against *E.coli*, *S. aureus* and *B. thuringiensis* (Table 1,2&3).Lesser inhibitory activity was recorded in seventh fraction @ 200 µl concentration of Rong daizo (2.71 cm, 3.01 and 1.60 cm) followed by CSR2 (2.68 cm, 2.98cm and 1.59 cm) and DH (2.69 cm, 2.96 cm and 1.54 cm) respectively. Standard antibiotic streptomycin sulphate @ 200 µl concentration produced the inhibition zone of 7.12 cm, 8.12 cm and 6.32 cm. Purified antibacterial protein fractions showed higher antibacterial activity against gram negative *E.coli* and gram positive *S.aureus* and low activity against *B.thuringiensis*.

Introduction

Several self-defense proteins have been isolated from the silkworm, *Bombyx mori* and their amino acid sequences determined. These proteins include novel antibacterial proteins designated lebocin and moricin, Among the various defense mechanisms in *B.mori*, humoral responses involved phenoloxidase and immune proteins such as antimicrobial proteins (AMPs), lysozyme and lectins. In response to microbial infection, AMPs and lysozyme are rapidly produced primarily in the fat body and haemocytes and subsequently secreted into the haemolymph to eliminate invading pathogens (Tanaka and Yamakawa, 2011). The induced antibacterial protein of silkworm have shown antibacterial effect against a wide variety of gram positive bacteria such as *Agrobacterium tumefaciens, Enterobacter cloacae, Escherichia coli*, *Serratia marcescens* and gram negative bacteria such as *Micrococcus ureae, Cornybacterium equi* and *Staphylococcus aureus* (Morishima *et al.*,1988). Upon bacterial infection, antibacterial proteins,

lectins and lysozymes rapidly appear in the haemolymph (Boman and Hultmark,1987). Cecropins are family of highly potent bactericidal peptides with 35-37 amino acid residues (Boman *et al.,* 1991). *B.mori* was found to contain eleven types of cecropin (Tu *et al.,* 1989; Morishima *et al.,*1990; Hara *et al.,*1994).

Present study investigated the antibacterial activity of haemolymph of *B.mori* by inhibition zone assay. The haemolymph proteins obtained from Sephadex G-100 column (55x1.5cm) were used for SDS-PAGE analysis. Among the ten fractions isolated, fractions seven and nine were found to have antibacterial activity and were compared with streptomycin sulphate. The fractions isolated from infected breeds of *B.mori* were more effective at higher dose of 200 µl compared to 100 µl against *E.coli*, *S.aureus* and *Bacillus thuringiensis* in Rong Daizo (3.01±0.042, 3.10±0.04 and 1.82± 0.051), Double hybrid (2.89± 0.732, 2.98± 0.750 and1.79± 0.445) and CSR2 (2.98±0.748, 3.05±0.764 and 1.81±0.448) respectively.

Materials and Methods

Purification of induced antibacterial protein from silkworm

Collection of haemolymph

Silkworm breeds, Rong Daizo, CSR 2 and double hybrid were reared on mulberry leaves under ambient conditions as per the standard rearing method (Krishnaswami,1978). Bacterial pathogens, *Escherichia coli* (K^R strain), *Bacillus thuringiensis* and *Staphylococcus aureus* were used for antibacterial assay**.** Three day old fifth instar larvae were fed with 10 µl suspension of log phase *E.coli* at $1x10^3$ cells/ml by spraying on mulberry leaves. Haemolymph samples were collected after 24 h of treatment into pre cooled tubes containing one per cent phenylthiourea, centrifuged at 10000 rpm at 4° C for 10 min and stored at -20° C. Haemolymph from untreated larvae served as control.

Purification of antibacterial protien

Step I: Haemolymph was collected from 30 immunized larvae at 24 h after treatment and diluted five times in 0.3M ammonium acetate maintaining a pH of 7.0 and applied to a Sephadex G-75 (20x1cm) column equilibrated in 0.3M ammonium acetate at a flow rate of 10ml/hr. The column was washed several times with 0.3M ammonium acetate. The bound proteins were eluted stepwise using the same buffer and the obtained fractions were observed at 280 nm (Abraham *et al*.,1995).

Step II: Antibacterial proteins obtained from the step I was applied onto a Sephadex G-100 column (55x1.5cm) in 0.1M phosphate buffer (pH 7.0) at a flow rate of 18ml/hr and eluted fractions were observed in UV-spectrophotometer at 280 nm. Haemolymph fractions were also collected from larvae treated with saline to compare the antibacterial protein induction with response to oral administration of bacteria. The fractions collected from step I and step II were tested for its antibacterial activity.

Antibacterial activity was assayed by measuring the zone of growth inhibition on thin agar plates with *E.coli, S.aureus* and *B.thuringiensis*. Serially diluted control and immunized protein samples were applied into wells on a thin agar plate seeded with bacteria. The zone of inhibition was measured and compared to that of control protein. Extracted fractions were compared with the standard antibiotic, Streptomycin sulphate.

Results and Discussion

Studies on i*n vitro* efficacy of purified antibacterial proteins from Rong daizo, CSR2 and double hybrid of *B.mori* against *E.coli, S. aureus* and *B. thuringiensis* revealed that among the ten fractions of antibacterial proteins eluted from *B.mori* breeds, ninth fraction @ 200µl showed higher antibacterial activity in Rong daizo (3.01cm, 3.10 cm and 1.82 cm) (2.71 cm, 3.01cm and 1.60 cm), CSR2 (2.98 cm, 3.05 cm and 1.81 cm) and double hybrid (2.89 cm, 2.98 cm and 1.79 cm) against *E.coli, S. aureus* and *B. thuringiensis* (Table 1,2&3)

Lesser inhibitory activity was recorded in seventh fraction compared to ninth fraction @ 200 µl concentration of Rong daizo (2.71 cm, 3.01 and 1.60 cm) followed by CSR2 (2.68 cm, 2.98cm and 1.59 cm) and DH (2.69 cm, 2.96 cm and 1.54 cm) respectively. Standard antibiotic streptomycin sulphate @ 200 µl concentration produced the inhibition zone of 7.12 cm, 8.12 cm and 6.32 cm. The present results are in conformity with the findings of (Hoffmann and Hetru,1992.) who reported that protein purified from different insect sources are reported to attack only gram-positive bacteria..

Antibacterial activity of obtained proteins also corroborated with the findings of Morishima *et al.* (1988) who reported that purified antibacterial protein fractions showed higher antibacterial activity against gram negative *E.coli* and gram positive *S.aureus* and low activity against *B.thuringiensis*. which is well supported by the present study.

Table.1 *In vitro* efficacy of purified antibacterial proteins (ABP) from Rong Daizo against bacterial pathogens

Treatments	**Zone of inhibition (cm±S.D.)* by ABP from Rong Daizo**		
	E.coli	***S.aureus***	***B.thuringiensis***
ABP eluted fractions (µl)			
A1. Fraction -7			
i. 100	2.30±0.032	2.90±0.013	1.41± 0.033
ii. 200	2.71±0.008	3.01±0.014	1.60± 0.012
A2. Fraction -9			
i. 100	2.81±0.014	2.92±0.008	1.55± 0.008
ii. 200	3.01±0.042	3.10±0.04	1.82± 0.051
B. Streptomycin Sulphate			
i. 100	3.51±0.005	3.61±0.008	3.21±0.005
ii. 200	7.12±0.015	8.12±0.017	6.32±0.008

*Values are the diameter of zone of inhibition (mean of three replications) in centimeter ± standard deviation

Table.2 *In vitro* efficacy of purified antibacterial proteins (ABP) from CSR 2 silkworm breed against bacterial pathogens

Treatments	**Zone of inhibition (cm±S.D.)* by ABP from CSR 2**		
	E.coli	***S.aureus***	***B.thuringiensis***
ABP eluted fractions (µl)			
Fraction -7			
i. 100	2.28± 0.570	2.87± 0.720	1.39± 0.348
ii. 200	2.68 ±0.674	2.98±0.748	1.59±0.398
Fraction -9			
i. 100	2.76 ±0.693	2.89±0.720	1.53±0.383
ii. 200	2.98±0.748	3.05±0.764	1.81±0.448
Streptomycin Sulphate			
i. 100	3.51±0.005	3.61±0.008	3.21±0.005
ii. 200	7.12±0.015	8.12±0.017	6.32±0.008

*Values are the diameter of zone of inhibition (mean of three replications) in centimetre ± standard deviation

Table.3 *In vitro* efficacy of purified antibacterial proteins (ABP) from Double hybrid of *Bombyx mori* against bacterial pathogens

Treatments	**Zone of inhibition (cm±S.D.)* by ABP from Double hybrid**		
ABP eluted fractions (µl)	Bacterial pathogens		
A1.Fraction 7	***E.coli***	***S.aureus***	***B.thuringiensis***
i. 100	2.27± 0.565	2.86± 0.712	1.41± 0.348
ii. 200	2.69± 0.667	2.96± 0.737	1.54± 0.386
A2.Fraction 9	***E.coli***	***S.aureus***	***B.thuringiensis***
i. 100	2.74± 0.680	2.86± 0.715	1.51± 0.373
ii. 200	2.89± 0.732	2.98± 0.750	1.79± 0.445
B. Streptomycin Sulphate			
i. 100	3.51±0.005	3.61±0.008	3.21±0.005
ii. 200	7.12±0.015	8.12±0.017	6.32±0.008

* Values are the diameter of zone of inhibition (mean of three replications) in centimeter ± standard deviation

The present results on antibacterial activity of antibacterial protein isolated from *B.mori* is in line with the findings of Pandia rajan *et al*.(2011) who reported that *cocoon* shell extract had antimicrobial activity against *E. coli, Bacillus cereus, S. aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae*.

Abraham *et al*. (1995) reported induction of antibacterial activity in the haemolymph of silkworm by injection with live cells of *E.coli* which was purified as antibacterial protein by Sephadex G-100 column chromatography, exhibited antibacterial activity against both *E.coli* and *M. luteus.*

The present result on antibacterial activity of ABP against bacterial pathogens were supported with the findings of Sharma *et al*. (2000) who reported the effectiveness of

proteins in inhibiting the growth of *P. aeruginosa* (AC-3) and also attributed that low molecular weight proteins were found to be effective against them; the diameter of the inhibition zone was found to be 3.5 cm.

In conclusion, proteins extracted from haemolymph of mulberry silkworm breeds, Rong daizo, CSR2 and double hybrid immunized with bacterial pathogens, *Escherichia coli* (K^R strain), *Bacillus thuringiensis* and *Staphylococcus aureus* showed antibacterial activity through inhibition zone assays. Antibacterial activity was found to be higher against *E.coli* and *S.aureus* compared to *B.thuringiensis* for all the breeds studied. The lower efficacy might be due to higher virulence of *B.thuringiensis.* Thuss exploring the mechanism of induction of antibacterial proteins upon immunization of silkworm breeds with *E.coli* and *S.aureus* may pave way for development of disease tolerant breeds.

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How to cite this article:

Manimegalai, S., B. Bebitha and Mohanraj, P. 2017. *In vitro* Efficacy of Antibacterial Proteins from Haemolymph of Silkworm Breeds against Bacterial Pathogens of Mulberry Silkworm, *Bombyx mori L. Int.J.Curr.Microbiol.App.Sci.* 6(3): 2055-2059.
doi: https://doi.org/10.20546/ijcmas.2017.603.234