Asian Journal of Biological and Life Sciences | Original Research

Electrophoretic haemolymph protein profile in *Beauveria bassiana* inoculated and subsequent treatment of ethanolic plant extracts, dusting of plant powder in silkworm *Bombyx mori* L.

Chavan J. A., Gaikwad Y. B and Bhawane G. P.*

Department of Zoology, Shivaji University, Kolhapur (MS), India- 416004.

E-mail : drgpbhawane@rediffmail.com Contact No : +91 9421202739

Submitted : 14.06.2013 Accepted : 19.09.2013 Published : 31.12.2013

Abstract

The effect of *Beauveria bassiana* and subsequent treatment of ethanolic plant extracts and dusting of plant powder on concentration of the haemolymph proteins were examined in the larvae of *Bombyx mori* PM race on the 3rd and 5th day of 5th instar by SDS-PAGE. The electrophoretic analysis of haemolymph proteins revealed separation of maximum 18 protein bands among which some of them represents the storage proteins. The *B. bassiana* inoculation causes the decrease in number of protein bands and staining intensity was also low as compared to the control. The treatment of plant extracts orally and dusting of plant powder also shows the decreased protein bands and staining intensity loe but in *Curcuma longa* and *Argemone mexicana* trated group shows the similar protein bands when compared with the control group sample.

INTRODUCTION

The silkworm *Bombyx mori* considered as "Queen of textile" because it is one of the popular beneficial insect for the production of silk fibre. The silk fibre is produced from two kinds of silk proteins fibroin and sericin. The synthesis of fibroin and sericin is one of the major physiological phenomenons, which is involved sequential interlinked mechanism of silk production from mulberry as food to protein and protein into silk fibre. Depending on the stage of insect development and on its ecophysiological condition the number and quantity of different proteins vary [1].

The haemolymph is store house of various kinds of proteins and enzymes [2] play an important role during development and metamorphosis of insects. Several reports are available on haemolymph proteins in silkworm [3-5]. The immune system is effective against non-pathogenic organisms and suggests the insect immunity has more to do with regulating population dynamics of the non pathogenic bacteria that naturally occur in insect body.

During metamorphosis in insects various biochemical studies revealed perodic changes in the total concentration of haemolymph amino acids and proteins [6-9]. The rapid uptake of at least some amino acids from haemolymph to the silk gland cells in silkworm *B. mori* was reported [10]. In silkworm *B. mori* larval haemolymph proteins differ from the silkproteins that represents the storage proteins predominantly studied [4].

In larvae of holometabolus insects, storage proteins are the major haemolymph proteins and playing important role as reservoirs for amino acids that are utilized in adult development during metamorphosis [4]. There are two kinds of storage protein in *B. mori* SP-1 and SP-2 [3]. SP-1 is the female specific at the 5th instar and during subsequent metamorphosis, is present equally in both sexes from the 1st to 4th larval instar [11]. SP-2 an arylphorin, is most abundant protein in the haemolymph of 4th instar larvae. Storage proteins in the haemolymph have cyclic changes in quantity during larval molts [5].

The effective removal of the bacteria is due to the combined response of cell-free immunity and haemocytic immunity. The former is also regarded as the humoral immunity. The humoral facet of the insect host response is interesting in that it appear rapidly in the cell free haemolymph as potent antibacterial protein [12-13]. Antimicrobial proteins are small, basic, single gene-encoded peptides that are generally synthesized as preproteins and are activated as part of the host defense system in plants [14-15], Insect [16-18]. These small proteins primarily give immune protection to prevent, repair wound and defense against microbial pathogenesis [19-21]. The microbial membrane acting like disinfectants and killing rapidly. Most have broad spectrum against bacteria, fungi and viruses while they have low toxicity on most animal cells [22].

The characterization of the first antibacterial peptides, cecropin from lymph of the cecropia moth [23], the field of insect immunity has received increasing attention, which results the better understanding of innate immunity for both invertebrates and vertebrates. Most of the studies done on the biochemistry of isolated compounds [24] by injection of the non-pathogenic bacteria and pathogenic bacteria two different silkworm races was done.

Hence in the present investigation, studies were conducted on the effect of antifungal plant extracts and plant powder in *B. bassiana* inoculated larvae of silkworm *B. mori* L. in PM race.

MATERIALS AND METHODS

***Experimental animals*:** The disease free laying (dfls) of popular pure multivoltine race, Pure Mysore (PM) used in present study and was obtained from the Directorate, Government Sericulture Grainage Center, Shahupuri, Kolhapur, for laboratory rearing. The larvae were reared as per the method of Krishnaswamy [25-26].

Microorganisms: The fungus culture of *B. bassiana* was made available from Microbial Type Culture Collection (M.T.C.C), institute of Microbial Technology, Chandigarh, India. The fungus culture was maintained as per the procedure of

Govindan [27].

Antifungal plants: For the present work, three plants Viz., *C. longa, A. mexicana and C. multiflorum* were selected on the basis of earlier reports and by arranging preliminary screening experiments for plant extractives.

Preparation of plant extract: Fresh leaves of *C. multiflorum,* seeds of *A. mexicana* and rhizome of *C. longa* were collected from fields of Kolhapur District, Maharashtra, India. The taxonomic identification of the plants was made with available literature [28].

The collected plant material washed with distilled water and shade dried at room temperature. The materials were grinded to fine powder with the help of mixer grinder. Then these powdered materials were used for preparation of ethanolic extracts by using 50g powder mashed in 100 ml absolute ethanol for 72 hours. The mixture was stirred every 24 hour using a sterile glass rod and it was filtered by using Whatmans filter paper No.1. At the end of extraction, each extract was concentrated in oven at 30^0C and paste extract was stored at 4^0C until further use.

Treatment of pathogen and plant extracts to silkworm: The *B. bassiana* was cultured in petriplates using Sabouraud's dextrose agar. Conidia were harvested by scrapping the surface of 3 week old culture into a 250 ml glass beaker containing 50 ml sterile distilled water. A drop of tween -20 was then added to the beaker containing distilled water and conidia. The conidial suspension was prepared by mixing the solution using a magnetic stirrer for 5 min and was then diluted in sterilized distilled water to get the desired concentration based on counts made with an improved Neubauer's Haemocytometer. Nine spore concentration of the fungus Viz., 10^1 to 10^9 spores/ml suspension with three replication each. The experiment was repeated for four rearings. The LC_{50} was observed at $1x10^6$ spores/ml for PM race.

Newly ecdysed fifth instar larvae of PM were topically inoculated by dipping them in the *B. bassiana* inoculums suspension of $1x10^6$ spores/ml concentration respectively. Five groups were arranged control, inoculated and three groups inoculated and separately of plant extracts treated groups. After 6 hours of the treatment of pathogen, larvae were fed on the ethanolic plant extract 6000 ppm concentration of *C. longa*, *A. mexicana* and *C. multiflorum* 100µl/larva on $4cm^2$ piece of mulberry leaf separately and repeatedly for three days in the morning and also dusting of plant powder 5% with lime was done for three days. Normal and inoculated control groups were fed on normal leaves without any application or dusting.

Preparation of electrophoresis sample:

The haemolymph samples was collected from larvae of each group by pricking the prolegs in vial precoated with phenylthiurea on 3rd and 5th day of 5th instar larvae of PM race. The sample was prepared by using the 1:2 ratio of haemolymph and sample buffer. On 12% SDS PAGE, all lanes were loaded with uniform volume of haemolymph sample. The gel was stained by the silver blue stain for three hours, then destained and observed the obtained results by comparing with standard protein sample.

RESULTS

The haemolymph protein profile of control, *B. bassiana* inoculated and subsequent ethanolic plant extract treated groups of 3rd day and 5th day showed in Plate-I, Fig. 1. In control group haemolymph of 3rd day, proteins were separated distinctly into 15 bands. The haemolymph proteins observed thick band with molecular weight 105.30 KDa, 94 KDa, 63.78 KDa, 57.69 KDa, 44.45 KDa and 29.28 KDa. The *B. bassiana* inoculated and plant extract treated group observed similar bands except the one band, which absent in all groups with molecular weight 94 KDa as compared to control group. The staining intensity was observed similar in all groups while the low staining intensity was observed in *C. multiflorum* treated group. In haemolymph proteins, 12-15 bands observed among which the some represent the storage proteins and some sex specific proteins. The storage proteins can be divided into two groups as SP-I and SP-II. The storage protein I showed single band with molecular weight 63 KDa. and thick bands was observed while in SP-II showed thin bands with molecular weight 45 KDa to 38KDa.

The storage proteins observed sharply in control group with SP-I and SP-II, the staining intensity was also high as compared to other groups. The *C. multiflorum* treated plant extract showed the disappearance of storage protein band from haemolymph sample on 3rd day of experiment. The calculated molecular weights of the proteins of respective band are given in Table No. 1.

The haemolymph proteins separate distinctly into 16-18 bands on 5th day, 5th instar larvae in control group. In *B. bassiana* inoculated and other plant extract treated group observed similar bands. But the treatment of *C. multiflorum* showed the fewer bands compared with control and the staining intensity was also less. The band with molecular weight 15.66 KDa observed in control group. However, it was disappear or absent in inoculated and plant extract groups. The storage protein bands with molecular weight 63 KDa. to 38 KD appear thick in control group but in other group it was less clear. In *C. multiflorum* treated groups the bands of storage proteins disappears. The thick bands with molecular weight observed in all groups i.e. 105.30 KDa, 94 KDa, 63.78 KDa, 57.69 KDa, 44.45 KDa and 29.28 KDa.

In haemolymph protein, band separation observed in both the days was similar but the more bands showed on the 5th day haemolymph sample. The thickness of bands observed on 5th day haemolymph was maximum as compared to 3rd day haemolymph sample. The storage proteins observed high thickness in 5th day haemolymph sample as compared to the 3rd day. On both days of the treatment of *C. multiflorum* plant extract showed the disappearance of bands as compared with their respective control group. The calculated molecular weights of protein bands which appear predominantly on 5th day of 5th instar haemolymph was given in Table No. 2.

The haemolymph protein profile showed the changes in appearance of band intensity in control, inoculated and plant powder treated group. The separation of haemolymph protein by electrophoretically shown in Plate-I, Fig. 2. The haemolymph sample of 3rd day 5th instar larvae of PM showed approximately 14-16 bands distinctly in all the groups. The protein bands of 140 KDa, 116.6 KDa, 87.58 KDa, 63.78 KDa and 57.69 KDa appeared as thick bands in all groups. The storage protein bands observed with a range of 63 KDa to 38 KDa. In all group similar separation of haemolymph proteins were observed on 3rd day sample.

In case of 5th day haemolymph sample variation among the bands observed. The control group noticed approximately 18-19 bands as per their staining intensity. The thick bands observed had molecular weight of 140.07 KDa, 113.69 KDa, 80.99 KDa, 76.87 KDa, 63.78 KDa, 46.83 KDa and 44.43 KDa. The thick bands

PLATE-I

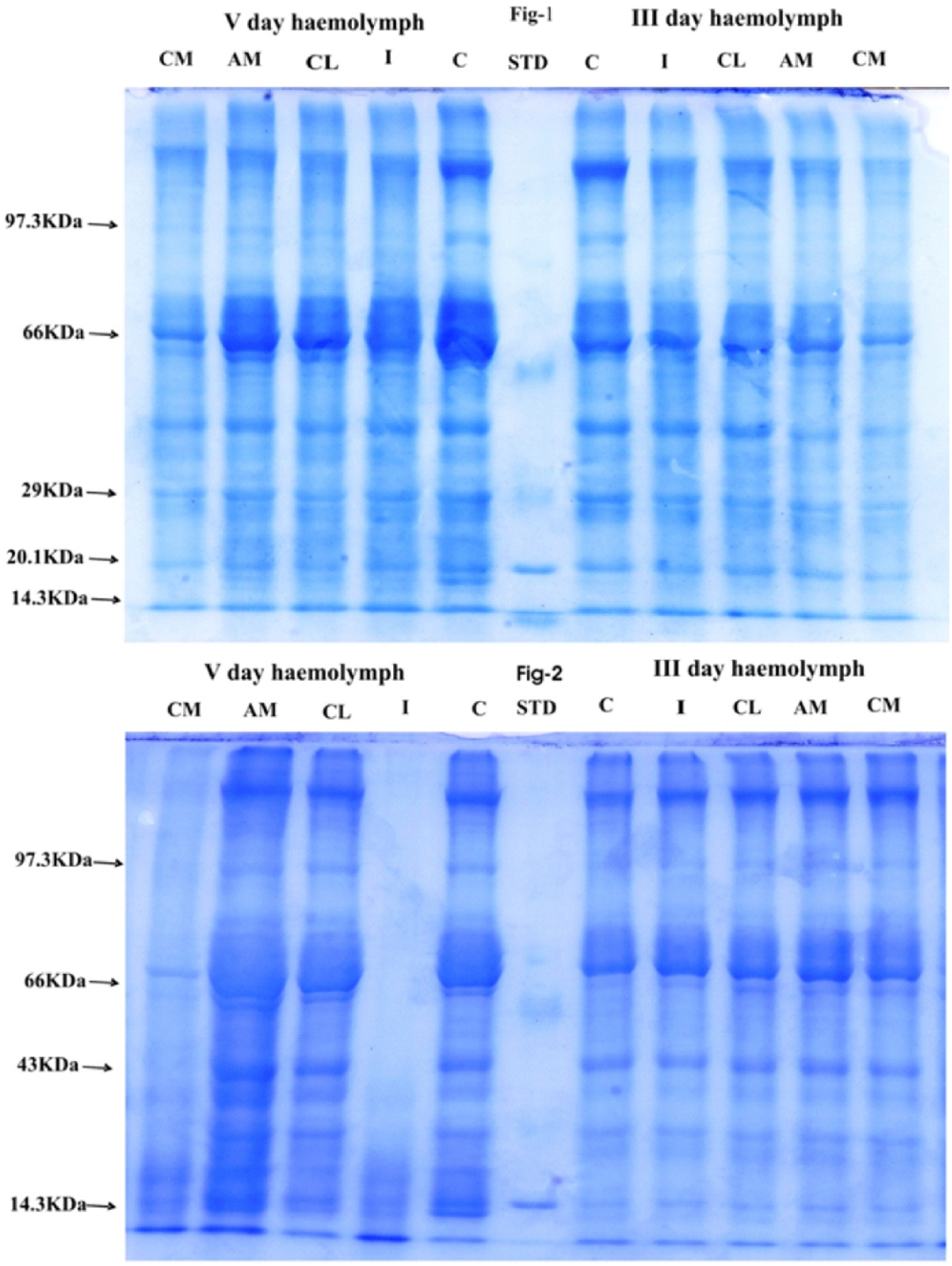

Fig. 1. Electrophoretic protein profile of haemolymph on 3rd and 5th day inoculated with B. bassiana and subsequent oral treatment of plant extracts in PM race of B. mori

Fig. 2. Electrophoretic protein profile of haemolymph on 3rd and 5th day inoculated with B. bassiana and subsequent dusting of plant extracts in PM race of B. mori

Std	-	Standard	**CL**	-	Curcuma longa
C	-	Control	**AM**	-	Argemone mexicana
I	-	Inoculated	**CM**	-	Clerodendrum multiflorum

Table 1. Effect of *B. bassiana* infection and subsequent oral treatment of ethanolic plant extracts on SDS-PAGE protein profile of midgut tissue on 3rd day in PM B. mori L.

SR. NO.	RF	MW (KDa)	DAY III				
			C	I	CL	AM	CM
1.	0.04	132.9	+	-	-	-	-
2.	0.11	113.6	+	-	-	-	-
3.	0.18	97.21	+	-	-	-	-
4.	0.25	83.12	+	-	-	-	+
5.	0.27	78.90	-	-	-	-	+
6.	0.47	49.33	+	-	-	-	-
7.	0.48	48.06	-	-	-	-	+
8.	0.52	44.44	+	-	-	-	-
9.	0.56	40.04	+	-	-	+	+
10.	0.61	36.07	-	-	-	-	+
11.	0.69	30.05	+	-	-	+	-
12.	0.70	29.27	+	+	+	+	+
13.	0.71	28.52	+	-	+	-	+
14.	0.72	27.79	+	-	+	+	+
15.	0.75	26.37	-	-	-	-	+
16.	0.78	24.39	-	-	-	-	+

'+' present, '_' indicates the present or absent of protein band, + - low, ++ - medium and +++ - high staining intensity

Table 2. Effect of *B. bassiana* infection and subsequent oral treatment of ethanolic plant extracts on SDS-PAGE protein profile of midgut tissue on 5th day in PM B. mori L.

SR. NO.	RF	MW(KDa)	DAY V				
			C	I	CL	AM	CM
1.	0.23	87.58	+	–	-	-	-
2.	0.24	85.33	-	-	-	-	+
3.	0.24	85.33	+	-	-	-	-
4.	0.26	80.99	+	–	-	-	-
5.	0.27	78.90	+	-	-	-	-
6.	0.28	76.87	-	-	+	+	+
7.	0.30	74.89	-	-	-	-	-
8.	0.39	60.78	-	-	-	-	+
9.	0.47	50.64	-	++	-	-	-
10.	0.52	44.45	-	++	+	+	-
11.	0.55	42.19	-	++	-	-	+
12.	0.56	41.10	+	++	-	-	-
13.	0.57	40.04	-	-	-	+	-
14.	0.58	39.01	-	-	+	-	-
15.	0.59	38.01	-	–	-	-	-
16.	0.63	35.15	+	++	-	+	-
17.	0.64	34.24	-	++	-	-	-
18.	0.70	29.28	++	++	+	+	+
19.	0.72	28.53	++	++	+	+	-
20.	0.73	27.79	++	++	+	+	-
21.	0.75	26.38	++	-	+	-	-
22.	0.76	25.70	-	-	-	-	+
23.	0.77	25.04	-	–	-	-	-
24.	0.80	23.76	+	-	+	-	-
25.	0.81	23.15	-	–	-	+	+

'+' present, '_' indicates the present or absent of protein band, + - low, ++ - medium and +++ - high staining intensity

observed in control were absent in *B. bassiana* inoculated group. In inoculated group only 3 bands observed of 18.79 KDa, 16.93 KDa and 15.66 KDa. The dusting of *C. longa* powder showed the similar separation of protein bands as compared to control. However the *A. mexicana* treated group showed more bands than its control. The storage protein bands observed thicker as in control group. The dustings of *C. multiflorum* dusting plant powder only one thick band observe of 63 KDa.

The haemolymph protein profile change was not observed in all groups on 3rd day of experiment. While in 5th day haemolymph samples, the inoculated group and *C. multiflorum* group showed the absence of bands as compared to control group. The bands

Table 3. Effect of *B. bassiana* infection and subsequent dusting plant powder on SDS-PAGE protein profile of midgut tissue on 3rd day in PM B. mori L.

SR. NO.	RF	MW(KDa)	DAY III				
			C	I	CL	AM	CM
1.	0.14	107.91	+–	+	+	+	++
2.	0.15	105.13	-	-	-	-	+
3.	0.24	85.33	+	-	-	-	-
4.	0.27	78.90	-	-	-	+	-
5.	0.28	76.87	+	+	+	-	-
6.	0.35	65.73	+	-	-	-	+
7.	0.41	57.69	+	-	+	-	-
8.	0.43	54.76	+	+	+	+	+
9.	0.49	48.07	+	+	+	+	+
10.	0.58	39.01	-	-	-	+	-
11.	0.60	37.03	+	-	-	-	++
12.	0.61	31.66	-	-	-	+	-
13.	0.67	30.85	+	+	+	-	-
14.	0.68	29.32	-	-	-	-	-
15.	0.76	26.38	-	+	+	+	-
16.	0.78	25.70	+	-	-	+	+
17.	0.80	24.39	+–	-	-	+	-
18.	0.81	23.76	+	+	+	+	+
19.	0.82	20.86	-	-	+	-	-
20.	0.85	20.32	-	-	-	+	+
21.	0.86	19.80	-	+	-	-	-
22.	0.88	18.31	+	-	-	-	-
23.	0.91	16.07	+	+	+	+	+

'+' present, '_' indicates the present or absent of protein band, + - low, ++ - medium and +++ - high staining intensity

Table 4. Effect of B. bassiana infection and subsequent dusting plant powder on SDS-PAGE protein profile of midgut tissue on 5th day in PM B. mori L.

SR. NO.	RF	MW(KDa)	DAY V				
			C	I	CL	AM	CM
1.	0.15	105.13	-	-	-	+	-
2.	0.25	83.13	+	-	-	-	-
3.	0.27	78.90	-	+	+	+	-
4.	0.36	76.87	+	-	-	-	-
5.	0.44	64.04	-	-	-	+	-
6.	0.47	53.35	+	-	-	-	-
7.	0.52	50.64	-	-	-	+	-
8.	0.53	44.45	-	-	-	+	-
9.	0.55	43.30	-	+	+	-	-
10.	0.58	42.19	+	-	-	-	-
11.	0.65	39.01	-	-	-	+	-
12.	0.68	33.36	+	-	-	-	-
13.	0.69	30.85	+	-	+	-	-
14.	0.77	30.05	+	+	++	++	-
15.	0.82	25.04	-	-	+	++	-
16.	0.85	20.86	-	+	+	++	-
17.	0.88	19.80	-	-	-	-	+
18.	0.95	16.49	-	+	+	-	-
19.	0.97	16.07	-	-	-	+	-

'+' present, '_' indicates the present or absent of protein band, + - low, ++ - medium and +++ - high staining intensity

appear on 5th day haemolymph samples were very thick and dark stained as compared to 3rd day haemolymph sample. The bands observed predominantly in samples of all the groups of their molecular weight were given in Table No. 3 and 4.

DISCUSSION

In the present study, the effect of *B. bassiana* infection on 3rd and 5th day old 5th instar larvae of PM race haemolymph protein profile in control, inoculated, and plant extracts treated groups

showed the variation in protein bands and also the variation in staining intensity on 3rd and 5th day of 5th instars larvae of silkworm. In all groups, the bands observe more in control group than inoculated and plant treated group. The banding pattern was different in all groups of haemolymph sample. During the present study, in haemolymph samples from each group analysed on 3rd and 5th day showed storage protein bands ranges from 63KDa to 38KDa. In control group the dark stained storage protein band observe as compared to other groups. On 3rd day the thickness of storage protein bands was less as compared to 5th day haemolymph sample. These Changes observed because of alteration in physiological condition. After the inoculation of PIBs of CPV, the infection causes changes in concentration of the haemolymph protein profile in the larvae *Antheraea mylitta*. After the inoculation of PIBs of CPV appearances of ovarian protein bands become reduced in intensity and their number. Their number reduced up to 5, 7 and 9 on 12th, 16th and 19th day after post inoculation respectively reported [29]. These results are similar with the present findings. The storage proteins are the major haemolymph proteins in the silkworm larvae playing important role as reservoirs for amino acids that used for development of adult organs [4]. There are two kinds of storage Proteins SP-I female specific protein observed in 5th instar.

During the metamorphosis the SP I is present equally in both sexes from 1st to 4th larval instar [30]. The most abundant protein in haemolymph of 4th instar larvae showed the SP II an arylphorin. The cyclic changes in quantity haemolymph storage protein during larval moults. The storage proteins are highly sensitive to the dietary protein content reported [5]. The middle and posterior silkgland protein concentration rising gradually during the secretary phase of the silk gland. Barsagade and Tembhare (2000) [31] reported that the haemolymph amino acids concentration is inversely correlated with the silkgland protein and suggesting the consumption of amino acids in the synthesis of protein during initial period of first six days and thereafter their absorption into the silkgland and lastly the gradual accumulation of amino acids into the haemolymph forming a amino acid pool reported [6, 9]. The presence of storage protein I and II in *B. mori* during larval stage similar with the *A. mylitta* [3, 30] and SP I can be considered as a vitellogenin in *B. mori*. The larval haemolymph proteins different from the silk protein not only in molecular weight but also in their mRNAs and tRNAs as well as in their genes [4]. An injection of bacteria into an insect increases the haemolymph lysozyme activity [32-33]. It has been reported that factors such as pressure, temperature, pH medium composition and cell growth stage can sensitize an organism for lysosomal synthesis [34-35].

Difference in susceptibility of test bacteria can be attributed of test bacteria can be attributed to the molecular characteristics of antibacterial factors in the immune haemolymph. The insect antibacterial factors are nonspecific in nature [12]. Immune haemolymph from *Hyalophora cecropia* [36], *Glossina morsitans* [37] showed a broad spectrum antimicrobial action. At molecular level this action is attributed to two main classes of antibacterial factor namely cecropins and attacins [12].

CONCLUSION

Therefore, from the above study, it is concluded that after the inoculation of pathogens like *B. bassiana* causes the alteration in protein profiles in haemolymph. After using the plant extract for curing the pathogen infection also causes the changes in the larval body and therefore, changes occurs in protein, which is separated by electrophoresis. Therefore, the application of plant extracts showed the more or less similar protein separation in all groups of haemolymph sample when compared to their control groups.

ACKNOWLEDGMENTS

Authors are thankful to UGC, New Delhi for financial assistance and Head Department of Zoology, Shivaji University, Kolhapur for providing laboratory and other infrastructural facilities.

REFERENCES

1. Wyatt GR, Pan ML. Insect plasma proteins; A. Rev. Biochem. 1978; 47: 779-817.

2. Wigglesworth VB. *The principles of Insect physiology,* 7th ed. Chapman and Hall, London. 1972.

3. Tojo S, Nagata M, Kobayashi M. Storage proteins in the Silkworm. *Bombyx mori* L. Insect Biochem. 1980; 10: 289-303.

4. Levenbook L. In comprehensive Insect Physiology, Biochemistry and pharmacology, Kerkut, G. A. and Gilbert, L. I. (eds). Pergamon Press: Oxford. 1985; 307-346.

5. Nagata M, Kobayashi M. Quantitative changes in storage proteins during larval development of the silkworm, *B. mori.* The J. of Seric. Sci. of Jap. 1990; 59: 461- 468.

6. Gilmour D. The biochemistry of Insects, Academic press: New York. 1965.

7. Chen PS. *Biochemical aspects of Insect Development* (Basel: S Karger). 1971; 230.

8. Rockstein M. Biochemistry of insects, Academic Press New York. 1978.

9. Kilby BA. Intermediary metabolism and the insect fatbody. Biochem. Soc. Sym. 1965; 25: 39-48.

10. Prudhomme JC, Couble P. The adaptation of the silk gland cell to the production of fibroin in *Bombyx mori* L. Biochimie.1979; 61: 215-227.

11. Kawaguchi Y, Fuji H, Doira, H. J. of Seric. Sci. Japan. 1983; 52: 517-523.

12. Boman HG, Hultmark D. Cell free immunity in insects. Annu. Rev. Microbiol. 1987; 41: 103-126.

13. Gotz P, Boman HG. *Insect immunity.* In: sinology, Comprehensive insect physiology biochemistry and pharmacology. G. A. Kertut, L. I. Gillbert, Eds., Pergamon Press, Oxford, 1985; 454-485.

14. Cammue BP, De Bolle MF, Terras MF. Isolation and characterization of a novel of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J. Biol. Chem. 1992; 267(4): 2228-2233.

15. Tailer RH, Acland DP, Attenborough S. A novel family of small cysteine-rich antimicrobial peptides from seed of *Impatiens balsamia* is derived from a single precursor protein. J. Biol. Chem. 1997; 272: 24480-24487.

16. Hoffmann JA, Reichart JM, Hetru C. Innate immunity in higher Insects. Curr Opin Immunol. 1996; 8: 8-13.

17. Mak P, Chmiel D, Gacek GJ. Antibacterial peptides of the moth *Galleria mellonella.* Acta Biochimica Polonica. 2001; 48 (4): 1191-1195

18. Laszlootvos JR. Antibacterial peptides isolated from insects. J. peptide Sci. 2000; 6: 497-511.

19. Ganz T, Selsted ME. Lehrer RI. Antimicrobial activity of Phagocyte granule proteins. Semin Respire Infect. 1986; 1: 107-117.

20. Ganz T, Lehrer R. Antimicrobial peptides of leukocytes. Curropin hematol. 1997; 4: 53-58.

21. Gallo RL, Huttner KM. Antimicrobial peptides: an emerging concept in cutanious biology. J. Invest. Dermatol. 1998; 111: 739-743.

22. Hetru C, Hoffmann D, Bulet P. Antimicrobial peptides from insects. in *Molecular Mechanisms of Immune Responses in Insects* (Ed by Paul T. Brey and Dan Hultmark). Chapmann and Hall, London. 1998; 40-66.

23. Steiner H, Hultmark D, Engstrom A, Bennich H, Boman, HG. Sequence and specificity of two antibacterial proteins involved on Insect immunity. Nature (London). 1981; 292: 246-248.

24. Boman HG, Faye I, Gudmundson GH, Lee JY, Lidholm, DA .Eur. J. Biochem. 1991; 201: 23-31.

25. Krishnaswami S. New Technology of silkworm rearing. CSR and TI Bulletin. 1978; 2: 1-23.

26. Krishnaswami S. Improved method of rearing youngage silkworms, CSR and TI Bulletin. 1979; 3: 1-24.

27. Govindon R, Narayanaswamy TK Devaiah MC. *Principles of Silkworm pathology,* SERI Scientific Publishers, Bangalore. 1998.

28. Yadav SR, Sardesai MM. Flora of Kolhapur District, Shivaji Univerity Publication, pp. 2002; 32, 39, 191, 193.

29. Kadwey M. Studies on the effect of pathogens on some vital organs of tropical Tasar silkworm *Antherea mylitta* (D) (Lepidoptera: Saturniidae) Ph. D. Thesis Rashtrasanth Tukdoji Maharaj, Nagpur University, Nagpur. 2009.

30. Mine E, Izumi Si, Katsuki M, Tomio S. Develop. Biol. 1983; 97: 329-337.

31. Barsagade DD, Tembhare DB. Development, structure and secretory activity of silkgland complex in the tropical tasar silkworm, *Antheraea mylitta* (Drury). (Lepidoptera: Saturnidae). Seric. 2000; 40: 231-245.

32. Powing RF, Davidn WJ. Studies on insect bacterilytic enzymes. Lysozymes in haemolymph of *Galleria mellonella* and *B.mori.* L. Comp. Biochem. Physiol. 1973; 45: 669-686.

33. Croizer G, Groizer L. Etude du phenomena de I'induction de facteurs bacteirlytiques chez les lepidopteres : inhibition de la puction du lysozyme chez les lerves de *Galleia mellonella* (Lep: Pyralidae) par I' actinomycine D et la cycloheximide. Entomophaga. 1980; 25: 219-228.

34. Ibrahim H, Higashiguchi S, Koketsu M, Juneja F, Kim M, Yamamoto T, Sugimoto Y, Aoki T. Partially unfolded lysozymes at neutral pH agglutinartes and kills gram-negative and gram-positive bacteria through membrane damage mechanism. J. Agric. Food Chem. 1996; 44: 3799-3806.

35. Garcia-Graells C, Masschalck B, Michiels, C. Inactivation of *Escherichia coli* in milk by high hydrostatic pressure treatment in combination with antimicrobial peptides. J. food prot. 1999; 62: 1248-1254.

36. Hultmark D, Engstrom A, Anderson K, Steiner H, Bennich H, Boman HG.. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia.* EMBO J. 1983; 2: 571-576.

37. Kaaya GP. Inducibal humoral antibacterial immunity in insects. *In insects Immunity* (J. P. N. Pathak, ed). Oxford and IBH publishing Co. Pvt. Ltd. 1993; 69-89.