# KUVEMPU UNIVERSITY

## Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori L.*

**A Thesis Submitted to the**
**Faculty of Science and Technology,**
**Kuvempu University for the Award of the Degree of**

**DOCTOR OF PHILOSOPHY**

**IN**

**BIOCHEMISTRY**


**Submitted by**

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**January-2018**

# DECLARATION

I hereby declare that the thesis entitled **"Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori L.*"** embodies the results of bonafide research work carried out by me under the guidance of **Dr. P. Niranjana,** Assistant Professor, Department of P.G. Studies and Research in Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shivamogga District, Karnataka and co-guidance of **Dr. Anitha Peter,** Professor, Department of Plant Biotechnology, UAS, GKVK, Bengaluru, Karnataka . I further certify that the results of this thesis have not been previously formed the basis of the award of any Degree, Diploma, or other similar title of in this University or other University/Institute of higher learning.

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*Place: Jnana Sahyadri* *(Manjunatha Gowda K.S)*

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| | | |
|---|---|---|
| A | - | Absorbance |
| Hr. | - | Hours |
| GFC | - | Gel Filtration Chromatography |
| Min | - | Minutes |
| Sec | - | Seconds |
| MALDI | - | Matrix Assisted Laser Desorption Ionization |
| TOF | - | Time of Flight |
| MS | - | Mass Spectrometry |
| DEPC | - | Di Ethyl Pyro Carbonate |
| UV | - | Ultra Violet |
| RFP | - | Red Fluorescent Protein |
| PM | - | Pure Mysore(multivoltine silkworm race) |
| CSR2 | - | CSR2 (bivoltine silkworm race) |
| SDS | - | Sodium Dodecyl Sulfate |
| PAGE | - | Poly Acrylamide Gel Electrophoresis |
| Pmol | - | Picomole/Picomolar |
| kDa | - | Kilo Dalton |
| Bm | - | Bombyx mori L. |
| µM | - | Micromolar/Micromole |
| µg | - | Microgram |
| Ng | - | Nanogram |
| mL | - | Milli-liter |
| nm | - | Nanometer |
| mM | - | Millimoles/Millimolar |
| CHBP | - | Chlorophyllide Binding Protein |
| Chlide | - | Chlorophyllide |
| BmKV | - | Bombyx Mori Kenchu Virus |

| | | |
|---|---|---|
| BmDNV | - | Bombyx mori Denso Nucleo Virus |
| BmIFV | - | Bombyx mori Infectious Flacherie Virus |
| BmCPV | - | Bombyx mori L. Cytoplasmic Polyhedro Virus |
| BmNPV | - | Bombyx mori Nuclear Polyhedrosis Virus |
| DNA | - | Deoxyribose Nucleic Acid |
| RNA | - | Ribose Nucleic Acid |
| PCR | - | Polymerase Chain Reaction |
| Pi | - | Post inoculation |
| PNPP | - | Para Nitro Phenyl Phosphate |
| PVDF | - | Polyvinylidene fluoride |
| NaCl | - | Sodium Chloride |
| Tris HCL | - | Tris Hydrochloric Acid |
| TE Buffer | - | Tris EDTA Buffer |
| CD | - | Circular Diachroism |
| EDTA | - | Ethylene Diamine Tetra Acetic Acid |
| Rpm | - | Revolution Per Minute |
| Kb/kbp | - | Kilo base Pair |
| bp | - | Base Pairs |
| O.D | - | Optical Density |
| °C | - | Degree Centigrade |
| cDNA | - | Complementary DNA |
| DEPC | - | Di Ethyl Pyro Carbonate |
| DMSO | - | Di Methyl Sulfoxide |
| dNTP's | - | Deoxy nucleotide Triphosphates (dATP,dTTP,dCTP,dGTP) |
| et al. | - | More than three authors |
| Fig. | - | Figure |
| G.K.V.K | - | Gandhi Krishi Vignana Kendra |
| IIHR | - | Indian Institute of Horticulture Research Institute |
| H2O2 | - | Hydrogen Peroxide |
| MMuLV-RT | - | Moloney Murine Leukemia Virus-Reverse Transcriptase |

| | | |
|---|---|---|
| LC50 | - | Lethal Dose Concentration 50% |
| PBS | - | Phosphate Buffer Saline |
| RNase | - | Ribonuclease |
| RT-PCR | - | Reverse Transcription PCR |
| qPCR/RT | - | quantitative PCR/Real Time PCR |
| TBE | - | Tris Borate EDTA |
| TE | - | Tris EDTA |
| TEMED | - | N,N,N,N-Tetra methyl ethylene diamine |
| V | - | Volts |
| mA | - | Milli Amps |
| V/V | - | Volume/Volume |
| W/V | - | Weight/Volume |
| HPLC | - | High Performance Liquid Chromatography |
| Cm | - | Centi Meter |
| KV | - | Kilo Vats |
| Hz | - | Hertz |
| BSA | - | Bovine Serum Albumin |
| TCA | - | Tri Chloro Acetic Acid |
| T | - | Treatments |
| C | - | Control |
| YL | - | Yellow mulberry leaf |
| GL | - | Green mulberry leaf |

# "Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori* L."

## ABSTRACT

Silkworm (*Bombyx mori.* L) is an economically important insect being a primary producer of silk. Sericulture is an important agro-based industry in India. Silkworm is susceptible to fungal, bacterial, viral and protozoan diseases. The *Bombyx mori* Nucleo Polyhedrosis Virus (*BmNPV)* is the most harmful virus to silkworm.

The digestive juice was collected by inducing fifth instar silkworm Kolar Gold breed to regurgitate by brief exposure of the larvae to chloroform vapours. The RFP was isolated and purified by Gel Filtration Chromatography on Sephadex G-100 column. The fractions collected have shown red fluorescence under U.V light was lyophilized and used for further analysis. The Purified RFP confirmed size of 40 kDa through SDS-PAGE and MALDI-TOF. The ten amino acids sequenced at the N-terminal and predicted a signal peptide and showed homology to Chlorophyllide Binding Protein (*ChBP*) gene. *In-silico* analysis revealed that the *ChBP* belongs to lipocalin group of protein family. Phylogenetic analysis showed that genes similar to *ChBP* exists in other group of insects as well and shows the antiviral, antimicrobial activity. ChBP gene expression found higher in green leaves fed silk worms and decreased expression in only yellow leaves fed worms.

## 1.1 Introduction

Silk, the queen of all fabrics is historically from one of India's most important industries. Sericulture spread across 52,360 villages in India providing employment to 7.9 million people and the Indian silk industry is one of the largest generators of employment. India is the second largest producer of silk and holds a unique global position in terms of production of all commercially useful varieties of silk.

India produces a variety of silks viz. eri, muga, tasar, and mulberry silkworm. Major silk production is in Karnataka, Andhra Pradesh, and Tamil Nadu and some extent towards Assam and West Bengal. Comparing to other states Karnataka alone produce 70% of the silk production (Silk industry in India, 2017).

The silkworm is the larvae of the domesticated silk moth, *Bombyx mori L.* It is an insect silk producer economically important. A silkworm prefers mulberry leaves (Morus Alba) and also they may eat other mulberry species and even Osage orange.

During the 1960s, many improved polyvoltine breeds like Kolar Gold, Kollegal Jawan, Mysore Princes and Hosa Mysore were developed which are having shorter larval duration, high silk content, better disease tolerance, better quality and higher yield. Among polyvoltine breeds, Kolar Gold developed by crossing CSR2

with Pure Mysore (CSR2 X PM) gives higher yield in all seasons due to its robustness, higher reliability and oblong shaped cocoons (Dandin et al., 2005).

Mulberry (*Morus* spp.) leaf is the sole food of the silkworm *Bombyx mori L.* The total area of mulberry cultivation in the country is around 2,82,244 ha. The growth and development of silkworm, cocoon crop and production of quality silk are mainly influenced by yield and nutritional quality of mulberry leaves. There are about 68 species of mulberry belonging to the genus *Morus*. In India, there are many species of *Morus*, of which *M.alba, M. indica. M. serrata* and *M. laevigata* are most common. Most of the Indian varieties of mulberry belong to *M. indica* (Dutta, 2012).

Mulberry leaf constitutes over 70% of the material required for the biosynthesis of silk proteins. Nutritive value of mulberry leaves is very important where it is utilized by the silkworm larvae for the nitrogenous matter for their growth, development and bio synthesis of silk protein (Khan et al., 2012). The biochemical constituents in carbohydrates, proteins and lipids play important role in biochemical changes in haemolymph and the silkworms growth and development (Ito and Horse, 1959: Wyatt, 1961 and 1967). Young leaves which have attained full size are best suited for the feeding of silkworm larvae (Koul et al., 1994). Chawki silkworms requires leaves with high moisture content to aid digestion rate whereas, aged silkworms

require mature leaves with less moisture content. On the other hand, matured and yellow leaves do not contain sufficient biochemical contents hence are not suitable to feed silkworms (Murthy et al., 2013).

Silkworm diseases are mainly caused by pathogens like virus (flacherie, grasserie), bacteria, fungus (muscardine), protozoa (pebrine) and parasites. The diseases caused by microbial pathogens are big threats to the sericulture industry which cause financial loss (www.government of Karnataka). The 70% of the silkworm crop loss is due to diseases caused by virus that accounts one of the major problems in silk industry. The two major viral diseases include grasserie caused by *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV) and flacherie caused by *Bombyx mori* Infectious Flacherie Virus (BmIFV) and *Bombyx mori* Denso Nucleosis Virus (BmDNV), *Bombyx mori* Bi Denso Virus (BmBDV), and *Bombyx mori* Kenchu Virus (BmKV).

The sever economic losses due to *Bombyx mori* Nucleo Polyhedrosis Virus (BmNPV) which is the most harmful virus in the sericulture industry (Ponnuvel et al., 2003). BmNPV infection is one major hurdle in trying to establish bivoltine sericulture in India and it is estimated that 40-50% crop losses occur in sericulture due to this disease. High temperature with high humidity is conducive for proliferation of this disease.

Thirty years ago, Japanese scientists studied the biochemistry and biological properties of a Red Fluorescent Protein (RFP) produced by the midgut of silkworm. They assigned antiviral properties to this protein. They revealed that the red fluorescence is due to the binding of a silkworm midgut protein with chlorophyllide-a, the prosthetic group of chlorophyll. This chlorophyllide results in the activity of Chlorophyllase, released from mulberry leaves during silkworm feeding. The Red Fluorescent Proteins (RFPs) present in silkworm are pigment protein complexes (Mauchamp et al., 2006: Suresh et al., 2007: Pandian et al., 2008: Matti et al., 2009). Owing to their antiviral, namely antiNPV properties, the RFPs have an important role in the immune system of the silkworm. The BmNPV is inactivated by Red Fluorescent Proteins (RFPs) have reported (Hayashiya et al., 1969: Hayashiya et al., 1976: Funakoshi and Aizawa 1989). The RFPs have been presented only in the mid gut juice of the silkworm and not in the haemolymph (Hayashiya et al., 1968: Nishida et al., 1973). In addition, a few laboratories have separated molecules containing chlorophyll like substance in silkworm having antiviral activity against selected human viruses (Hiraki et al., 1997). The antiviral protein isolated from healthy silkworm excreta was found to exhibit anti Nucleo Polyhedro Virus (antiNPV) activity (Hiraki et al., 2000: Neelgund et al., 2007: Matti et al., 2009). Supplementation of the artificial diet with the green pigment i.e. the alcohol extract of the mulberry leaf did not promote

the RFP synthesis suggesting that the mulberry leaf proteins are involved in RFP synthesis (Hayashiya, 1978). The RFP synthesis did not occur in silkworm reared in total darkness in spite of inclusion of fresh mulberry leaves in the artificial diet of silkworms (Hayashiya et al., 1968). Hence, light may be necessary for the formation of the green pigment, and/or for causing photodynamic inactivation of the viruses (Schnipper et al., 1980: Spikes, 1984: Lee and Lee, 1990). Uchida et al. (1984) have observed that the virus inactivating protein contained in the digestive juice of the silkworm was not a glycoprotein.

The native Indian multivoltine races of *B. mori* (Pure Mysore and Nistari) have been reported to be more resistant to NPV compared to the exotic, high- yielding bivoltine races ($NB_4D_2$) (Acharya et al., 2002). This resistance seems to be controlled by many genes that produce proteases and lipases in gut juice (Ponnuvel, 2003: Nakazawa et al., 2004). This polygenic control by the dominant and multiple micro-effector genes makes the understanding of the inheritance of resistance to NPV complicated (Goldsmith et al., 2005).

In the synthesis of RFP protein, Chlorophyll-a from mulberry leaf, in the presence of the enzyme Chlorophyllase is converted to chlorophyllide-a, which combines with a protein present in the larval midgut and is converted to RFP in the presence of light.

Chlorophyllase enzyme catalyses the hydrolytic cleavage of the phytol moiety from chlorophyllide setting free phytol and chlorophyllide.

For this reaction light and oxygen are most necessary. Both chlorophyll-a and Chlorophyllase are essential for RFP synthesis. Chlorophyllide-a pigment can be conjugated non specifically with more than one midgut protein, with the ingested chlorophyll merely providing a pigment moiety for RFP biosynthesis (fig. 1).

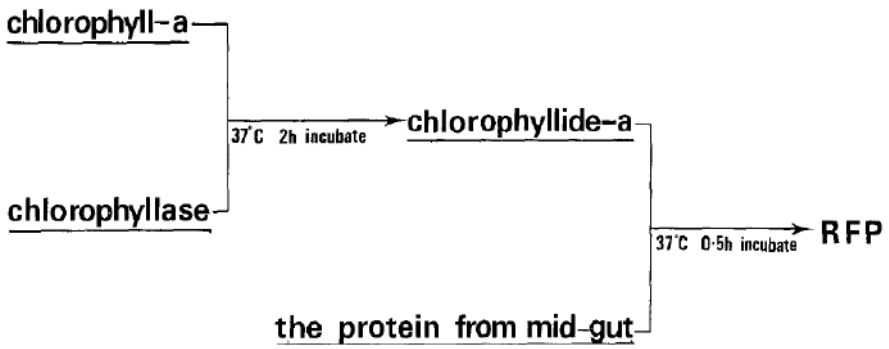


**Fig. 1:** Diagrammatic representation of biosynthesis of RFP.

The present investigation was thus undertaken for molecular characterization of RFP from the midgut of the silkworm *Bombyx mori L.* and an in-silico analysis and bioassay will be performed with the hypothesis that the antiviral RFP could also have antibacterial and antifungal properties which will perhaps lead to the bioprospecting of a moiety which can be of use in the silk industry and additionally explored for its importance and application as a

candidate antipathogenic molecule against other plant, animal and human pathogens of potential pharmacological significance that is in bioprospecting. The present study also aims at assessing the effect of feeding mulberry leaves of different maturity (green and yellow) on the growth and development of silkworms owing to the importance of its nutritional value.

## 1.2 Need and Significance of the Study

The silkworm *B. mori* was domesticated from *Bombyx mandarina,* a wild ancestor silk moth. From the past 5000 years the domestic *B. mori* has been used to produce many new hybrids to improve the disease resistance and quality of the cocoons. The Kolar Gold polyvoltine hybrid, developed from crossing with CSR2 x PM, with its high fecundity and tolerance to diseases comparatively produces 20 % more silk. It has been very much popularized and rearing in southern India particularly in Karnataka, Telangana, Andhra Pradesh and Tamil Nadu.

Demanding research has been going on in *B. mori* to understand Biochemistry, Physiology, Molecular biology and its genomics. Red Fluorescent Protein (RFP) with varied molecular weight were found in the midgut of silkworm and emits red fluorescence, show activity against BmNPV and also reported to have antimicrobial properties. The silkworm *B. mori* has a good innate defence system against invading pathogens namely viruses, bacteria and fungi. Several

antiviral factors have been reported that act against such pathogens, RFP is one of them.

Though, India rank second largest producer of silk but suffers 30 to 40 % loss in its produce mainly due to viral, bacterial, and fungal diseases of silkworm. Identification of biochemical factors and understanding molecular mechanism associated with such disease resistance directly increase the silk productivity and significantly contribute to the economy of the country and that would also show the way to develop further improved varieties. The main endeavour of the present study is to explore the RFP present in the midgut of the silkworm larvae of Kolar Gold breed, and assess its biochemical characterization.

Chlorophyllide-a, a prosthetic group of chlorophyll released in the silkworm midgut by the action of Chlorophyllase enzyme, binds to secretory RFP protein expressed in epithelial midgut of the silkworm. It combats against BmNPV, so its identification and biochemical characterization is utmost essential in Kolar Gold silkworm breed.

Identification of N-terminal amino acid sequences present in purified RFP may further identify its biochemical functions and various signalling pathways and also predicts its gene. To understand the mode of RFP action, its biochemical characterisation, amplification of its gene and its molecular characterisation are most important. Many midgut RFPs were identified and amplified the

*ChBP* gene and also proved it belongs to lipocalin family. To comeback with findings, the current study accepted to amplify the *ChBP* gene and set the target for its molecular characterisation.

Different rearing environmental conditions were adopted for the silkworm rearing. While rearing, silkworm larvae mainly fed with mulberry green leaves only. But sometime the larva may feed partially turned yellow leaves in addition to green leaves which may effect on the expression of *ChBP* gene. Mulberry leaf constitutes over 70 % of the material required for the biosynthesis of silk proteins. Nutritive value of mulberry leaves is very important where it is utilized by the silkworm larvae for the nitrogenous matter for their growth and development and also for the bio synthesis of silk protein. To realise this, in the present research work amidst to quantify the level of *ChBP* gene expression in the silkworm fed with yellow mulberry leaves records the growth of the silkworm, its survival rate, and also witness the disease prevalence.

Therefore, current investigation is undertaken and the study for research is stated as below.

## 1.3 Statement of the Problem

The statement of the problem of the present study is **"Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori L*".**

## 1.4 Scope and Limitations of the Study

The present investigation is confined to trace the presence of Red Fluorescent Protein (RFP) in the midgut juice of the silkworm *Bombyx mori* multivoltine hybrid Kolar Gold generated through CSR2 x PM breed. Purification and biochemical characterisation of RFP, sequencing of its N-terminal amino acids. Identification and amplification of Chlorophyll Binding Protein Gene (*ChBP*), molecular characterisation of *ChBP*. The study also confined to quantify the expression analysis of *ChBP* gene when silkworms fed with green and yellow mulberry leaves. The study further confined to check the antiviral characteristics of the RFP against *Bombyx mori* Nucleo Polyhedrosis Virus (BmNPV), and antibacterial activity against *E. coli* and *B. subtilis,* and antifungal activity against *Phytophthora meadii*.

## 1.5 Objectives of the Study

1) Isolation and purification of antiviral Red Fluorescent Protein (RFP) from the silkworm *Bombyx mori* L.
2) Biochemical characterization of RFP purified from silkworm *Bombyx mori* L.
3) Effect of yellow mulberry leaves feeds to silkworm larvae on *ChBP* Gene expression.
4) Evaluation of feeding green and yellow mulberry leaves on growth, development, Survival and disease prevalence.

## 1.6 Hypotheses

1) In silkworm, the major disease development and mortality is due to Baculovirus infection, develop resistance due to inhibitory and antiviral properties of midgut digestive juice.

2) In silkworm larvae, the protein expressed in midgut epithelial cells secretes into midgut and displays fluorescence when it binds to chlorophyllide-a and may acquire antimicrobial properties.

3) Lipocalin, a group of proteins involved in various physiological processes includes transportation, larval color, alfaction, immune response, etc.

4) Chlorophyllide-a, prosthetic group of chlorophyll formed by the action of Chlorophyllase bound to *ChBP* and may acquire antimicrobial properties and emits its fluorescence.

5) Expression of RFP in the midgut of silkworm larvae may depend upon availability of green pigment of the mulberry leaves. Identification, Biochemical characterisation of RFP and to judge its bioactive principle is important in silkworm Kolar Gold breed.

## 1.7 Methodology

The details of methodology for this study are described in detail in chapter 3. Material and Methods.

## 1.8 Chapterisation

The present study on the isolation, purification and biochemical characterization of RFP in silkworm (*Bombyx mori* L.) has been undertaken systematically.

For the systematic presentation, the present thesis has been organized into six chapters.

### Chapter-1: Introduction

This chapter includes introduction to the present study, need and significance of the study, statement of the problem, objectives of the study, scope and limitations of the study, methodology and chapterisation.

### Chapter-2: Review of Literature

The literature pertaining to Red Fluorescent Protein (RFP) in the mid gut juice of silkworm *Bombyx mori,* its purification, Antiviral and antimicrobial property of RFP, *ChBP* gene expression analysis on yellow fed worms and the importance of green mulberry leaf for silkworm growth and development are presented in this chapter.

### Chapter-3: Material and Methods

This section explains in detail the material, chemicals and equipments used in the present study. Protocols for the Isolation and Purification of Red Fluorescent Protein (RFP) from the Silkworm

Kolar Gold breed and its Biochemical characterization are presented. The determination of molecular weight and the purity analysis of RFP were done by using techniques like PAGE, MALDI-TOF, HPLC, etc. Antimicrobial property of purified RFP against BmNPV, Gram (-) bacteria *Escherichia coli,* Gram (+) bacteria *Bacillus subtilis* and fungus *Phytophthorameadii*. Effect of yellow fed leaves on the expression of *ChBP* gene and on growth, development and survival rate of the Silkworm *B. mori* L are described in detail.

## Chapter-4: Results

In this chapter, the results obtained from the experiments carried out on "Purification and characterization of an antiviral protein (Red Fluorescent Protein) from the silkworm *B. mori* L. are presented.

## Chapter-5: Discussion

The results obtained in each of the experiments conducted to meet the objectives of the present study are discussed and the salient findings of the study have been highlighted in this chapter.

## Chapter-6: Findings and Conclusion

In this chapter, the important findings are noted, future perspective of the work has been suggested and conclusions are drawn.

## Bibliography

# REVIEW OF LITERATURE

The literature pertaining to Red Fluorescent Protein (RFP) in the midgut juice of silkworm *Bombyx mori L,* its purification, biochemical characterization and ChBP gene expression fed with yellow and green mulberry leaves and its importance of growth and development are and Biochemical Constituents in haemolymph presented in this chapter.

## 2.1 Sericulture

The most common type of silk used by humans mainly is from the silkworm *Bombyx mori*. The *B. mori* is a cultivated silkworm and its silk fibres have very good mechanical, biocompatible, low biodegradable properties and low inflammatory reaction, it has been used as sutures, base for ointment and other biomedical applications (Wang et al., 2012).

Sericulture spread across 52,360 villages in India providing employment to 7.9 million people and the Indian silk industry is one of the largest generators of employment. India is the second largest producer of silk and holds a unique global position in terms of production of all commercially useful varieties of silk (Indian silk industry at a glance 2016 page 1-4).

## 2.2 Silkworm Breeds

Prior to the introduction of the breeds it was either the indigenous (Pure Mysore, Nistari) or the exotic (C.Nichi, Sarupat) pure races, which were exclusively used for rearing. Crossing these races with those from Italy, Russia, Japan and China led to the evolution of hybrids of several new polyvoltine races, which however could not make much impact. During the 60s many improved polyvoltine breeds like Kolar Gold, Kollegal Jawan, Mysore Princes, and Hossa Mysore were developed. During 1981, CSRTI, Mysore evolved polyvoltine breeds with shorter larval duration, high silk content, better disease tolerance, better silk quality and higher yield (ex. BL23 & BL24). Central Sericulture Research and Training Institute (CSRTI) with beneficial mutants evolved through x-ray and chemical mutagenesis: used these in further crossbreeding programs. Better silk quality and productivity were achieved by using the hybrids BL23 x NB4D2 and BL23 x NB4D2 (Mohandas, 2009).

## 2.3 Silkworm Life Cycle

Silkworm is an insect that completes its life cycle by four stages viz. egg-larvae-pupa-moth represented in Fig.2. A silkworm that under goes through its life-cycle one time per year is called univoltine, two times per year is called bivoltine and any number of times per year is called polyvoltine (http://www.zanzibartrading.com).

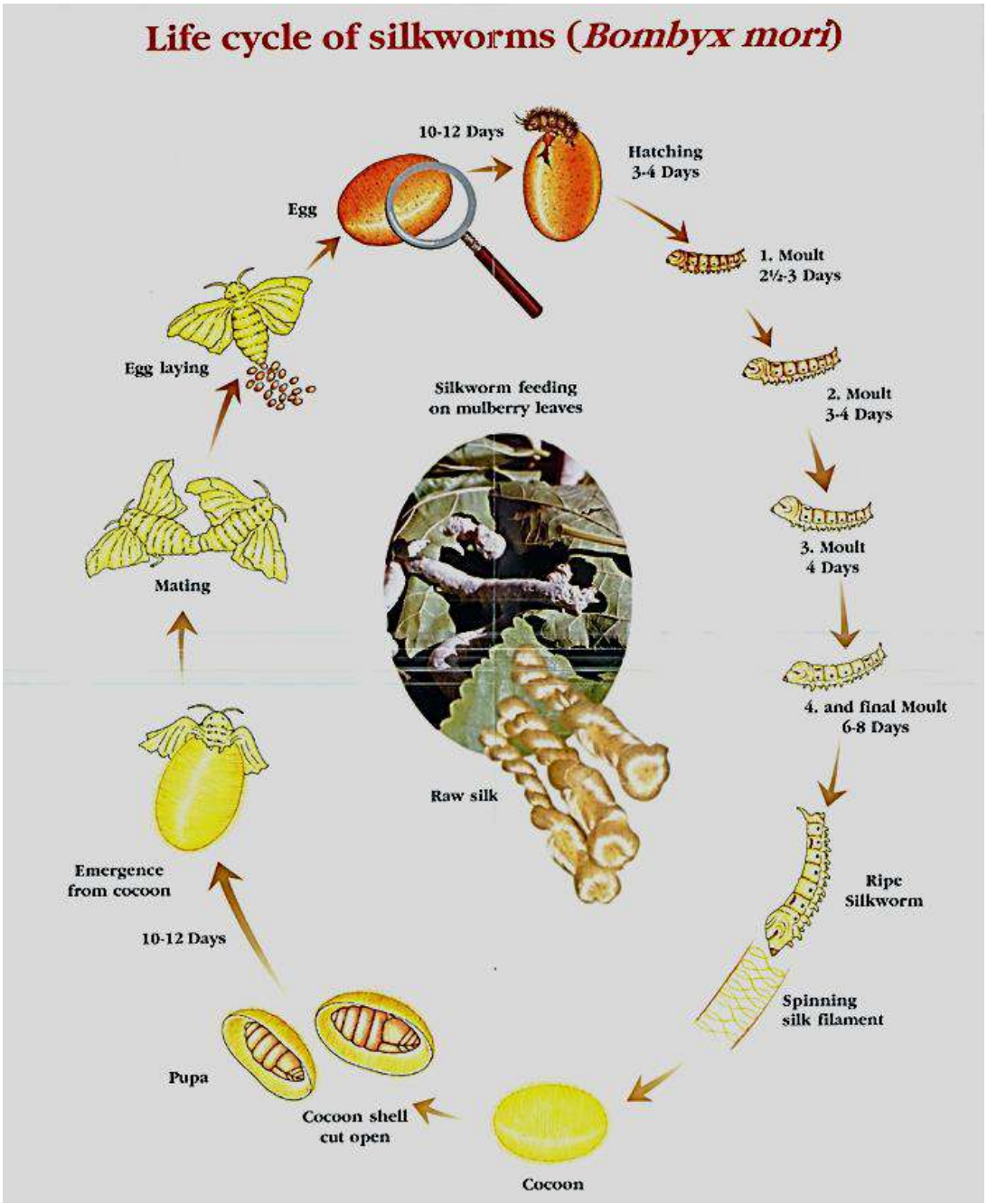


**Fig.2**. Life cycle of a silkworm *B.Mori* L.
The hatched silkworm goes through 5 different stages so called instar before it spin its cocoon and transform into a pupa which in turn metamorphoses into a moth.(http://www.zanzibartrading.com).

**Scientific Classification of Silkworm *Bombyx mori* L.**

Kingdom : Animalia

Phylum : Arthropoda

Class : Insecta

Order : Lepidoptera

Family : Bombycidae

Genus : *Bombyx*

Species : *B. mori*

**Binomial name:** *Bombyx mori* (Linnaeus, 1758)

## 2.4 Silkworm Diseases

Silkworm diseases are mainly caused by pathogens like virus (Flacherie, Grasserie), bacteria, fungus (Muscardine), protozoa (Pebrine) and parasites. Thus diseases are a big threat to the sericulture industry leading to financial loss caused by microbial pathogens. Cocoons production loss is mainly due to the occurrence of diseases, rather than by unfavourable weather conditions that lead to poor harvests of mulberry leaves. There is a significant decrease in the pathogens of biochemical substances among the infected silkworm due to different pathogen attack (Sharma et al., 2013).

This indirectly also affect food industry as pupae of both mulberry and non-mulberry silkworms were traditionally known as animal

feed and edible insects in some countries, such as Japan, Korea and Thailand (Zhou et al., 2006).

### 2.4.1 Viral Disease

Viral diseases of silkworm pose a major problem to sericulture as they account for almost 70% of the total loss. The two major viral diseases include grasserie and flacherie. *Bombyx mori* Denso Nucleolic Virus (BmDNV), *Bombyx mori* Bi Denso Virus (BmBDV), and *Bombyx mori* Kenchu Virus (BmKV) which is a BmBDV and *Bombyx mori* Cytoplasmic Polyhedrosis Virus (CPV). Virus infecting silkworm comprise of inclusions and non-inclusion types. The *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV) and Cytoplasmic Polyhedrosis Virus (CPV) can be easily identified through an ordinary microscopy (Mukha et al., 2006). The non-inclusion *Bombyx mori* type consists of Infectious Flacherie Virus (BmIFV) and Kenchu virus which can be detected only through electron/fluorescent microscopy and serological tests.

### 2.4.2 Management of Diseases

Among the hundreds of species of silkworms available worldwide, the most commonly commercially used silkworm is *B. mori* (mulberry silkworm). The mulberry silkworm (*B. mori L.)* is very delicate and highly sensitive to environmental fluctuations and are unable to survive in extreme natural fluctuations in the temperature, humidity

and infectious pathogens *viz*. protozoa, bacteria, virus and fungus (Peter et al., 2000).

Micro sporidiosis or Pebrine is trans-ovarially transmitted and thus the most serious disease in silkworm is almost fully controlled by the various mother moth examination methods. Immune and molecular diagnosis developed for precise identification of the virulent of the strains protozoa (Mohan Das, 2009). Chemicals like bleaching powder, sodium hypochlorite, mixture of formalin and bleaching powder are found to be more effective to NPV and are used as disinfectants for silkworm rearing houses (Shimizu et al., 1975).

Proper disinfection using slaked lime, formalin and bleaching powder and chlorine dioxide (500 ppm) are found to be most effective. Rearing rooms, appliances and the premises are to be disinfected thoroughly before the start of the crop. Many rearing bed disinfectants (under the commercial names like Resham Keed Oushad, Resham Jyothi and Vetcare Vijetha) are developed which are very useful in the management of various diseases (Mohandas et al., 2011).

## 2.5 Red Fluorescent Protein (RFP) in Silkworm

A Red Fluorescent Protein (RFP) is present in the digestive juice of silkworm larvae when they are raised on fresh mulberry leaves. It is an antiviral substance and is absent in the insects reared on artificial diet. The biosynthesis of red fluorescent protein has been

investigated (*in vitro*) which showed the requirement of chlorophyll-a for its formation. A larval midgut protein reacts with the chlorophyll-a to give the final molecule (Hayashiya, 1978).

Midgut proteins from the silkworm, *Bombyx mori,* fed on an artificial diet containing dried powder of the spiral blue – green alga, *Spirulina platensis,* had very low fluorescence intensity. This indicates that chlorophyll in this alga is not utilizable for synthesizing the Red Fluorescent Protein (RFP), which has been considered as an effective antiviral protein in the midgut of silkworm. Many electrophoretic bands of the midgut proteins from the mulberry fed silkworm emitted red fluorescence under UV light. It is thus concluded that the RFP as a whole is more than one midgut protein. Enzymatic analysis showed that silkworms fed on mulberry leaves on artificial diets had similar protease activities, but they had different phospholipase activities (Hou & Chiu, 1986)**.**

## 2.6 Purification of RFP

In the course of the purification of a nuclease from the digestive juice of silkworm larvae *Bombyx mori* L. a red surface coloured, fluorescent protein with interesting chemical and physiological properties was encountered (Mukai et al., 1969).

A protein that can precipitate Nuclear Polyhedrosis Virus (NPV) *in vitro* was isolated from the digestive juice of silkworm larvae (*Bombyx mori*) by the procedures of gel filtration and ion exchange and

hydroxyl apatite column chromatography (Uchida et al., 1984). The SDS polyacrylamide gel electrophoresis and the ultra-centrifugal analysis showed that the purified substance was a homogenous simple protein. The molecular weight of the purified protein was 27,000 – 28,000 and the sedimentation coefficient was 2.61S. This protein had an additional activity to inactivate NPV of *B. mori in vitro,* which was somewhat analogous to serological neutralization by serum proteins (Uchida et al., 1984).

Sethuraman et al. (1993) purified the RFP in silkworm gut juice by Sephadex G-100 column chromatography. The elution profile showed three peaks at 280 nm. However, only one peak showed high absorbance both at 280 and 605 nm, which is characteristic of RFP. Electrophoretic separation of purified RFP showed only a single protein of 65 kDa, which is different from the 28 kDa RFP purified earlier by Uchida et al. (1984) indicating that the RFP includes more than one protein. The antiviral nature of the isolated protein was confirmed against BmNPV.

A lutein binding protein was purified from fifth instar larval midgut of *Bombyx mori* by a combination of ammonium sulphate fractionation and three chromatographic procedures, gel filtration, chromate focusing and anion exchange chromatography. The protein had a PI of 5.4 and an apparent molecular mass of 35,000 kDa as determined by a linear gradient SDS–polyacrylamide gel

electrophoresis, lutein is specifically and stoichiometrically bound to the protein, with a ratio of 3 mol of lutein per mol of protein. Binding of lutein to the Apo protein results in a marked red spectral shift of about 38 nm, giving rise to absorption maxima at 432, 462 and 492 m in 20 mM Tris H Cl pH 7.0. This protein is distributed in equal amounts throughout the midgut and in all developmental stages of the larvae of B. *mori* (Jouni and Wells, 1996).

Yamamoto et al. (1999) studied the isolation and characterization of prophenol oxidase isoforms from the silkworm, *Bombyx mori.* Three isoforms of prophenol oxidase a proenzyme of phenol oxidase were isolated and characterized from the haemolymph of the a80 strain of silkworm *B. mori*. They also studied the pH activation of prophenol oxidases. Each oxidase prophenol was activated most efficiently at pH 6.5.Upon activation these enzymes catalyze the oxidation of dopamine–L and N-acetyl dopamine but not hydroquinone. They also noticed a significant difference in catalytic efficiency among the three isoforms.

Datta et al. (2000) studied the purification and characterization of antiviral protein in the gut juice of silkworm, *Bombyx mori* L. In order to understand the mechanism of differential tolerance in silkworms. They extracted antiviral protein from the gut juice of silkworms in a step wise (10-60%) ammonium sulphate precipitation. The partially

purified proteins were filtered through Sephadex G-100 and the fractions were tested for antiviral activity.

Kaji et al. (2004) purified a serine protease with a molecular mass of 37KDa in the midgut of silkworm *B. mori*. The sequence analysis of the purified protein revealed it to the trypsin like serine protease.

Mauchamp et al. (2006) reported a protein from the midgut of silkworm characterized by its ability to bind to the prosthetic group of chlorophyll that confers fluorescence properties to the protein. The protein was identified as a lipocalin with a molecular size of 25kDa. Suresh et al. (2007) reported a 66 kDa RFP from the midgut of the silkworm. A 26.5 kDa protein that is overexpressed in the gut juice of silkworm *B. mori* was identified by Selot et al. (2007). Sunagar et al. (2008) found the presence of multiple forms of (RFP) in the gut juice of the silkworm, *Bombyx mori* L. as 3 electrophoretically separable bands A, B, C. These bands were found to be heterogeneous with respect to their components namely the protein and the tetrapyrole pigment moiety. Among the three RFPs, band C was found to be a glycoprotein.

Partially purified (RFP) secured from the gut juice of 5th-instar multivoltine and bivoltine silkworm races were observed as several bands in electrophoretograms and chromatographic elutes (Sunagar et al., 2011). Matti et al. (2009) purified a RFP from the excreta. The apparent mass of the protein was about 1100 kDa. The bioactive RFP

has two absorption peaks at 280 and 603 nm. Neelagund and Hinchigeri (2011) purified and characterized a RFP from the silkworm faecal matter of *Bombyx mori*. The purified protein was found to be associated with tetrapyrole pigments.

A novel bioactive RFP was purified by using Sephadex G-75 gel filtration column followed by reverse-phase high-performance liquid chromatography in a C18 column. The molecular weight of the protein was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Raghavendra & Neelagund, 2009).

## 2.7 Antiviral Proteins

### 2.7.1 In Haemolymph

Two different haemolymph proteins (P-I and P-II) of the Japanese silkworm *A.* yamamai were purified from the haemolymph of the 5$^{th}$ instar larvae. P-I was shown to have an apparent molecular weight of 31000 and 35000 Daltons, as determined by gel-filtration and SDS-PAGE respectively. P-II showed a molecular weight of 22000 and 25000 Daltons by gel-filtration and SDS-PAGE respectively (Saito et al., 1998).

### 2.7.2 In Midgut

Suzuki (1936) investigated the inhibitory and antiviral property of digestive juices of *Bombyx mori* larvae and reported that an unknown

substance with high molecular weight in the gut juice could inactivate the virus *in-vitro*.

Aizawa (1962) reported that unknown substance with high and low molecular weight in the digestive juice of silkworm had anti-viral activities. The RFP is able to inactivate BmNPV extracted from the polyhedral bodies' *in-vitro* and a white precipitate occurred at the bottom of the wells of the agglutination plate when RFP and NPV were mixed in the wells indicating a similarity with the antigen antibody reaction. This precipitation was also observed BmIFV but not with Tobacco Mosaic Virus (TMV). The RFP activity is reduced when silkworms were reared on artificial diets even when they contain mulberry leaf powder and when reared in complete darkness (Hayashiya et al., 1967, 1968, 1969, 1971, 1978).

Sasaki et al. (1993) found that the catalytic triad Asp-His-Ser is responsible for the activity of the alkaline protease P-IIc obtained from the digestive juice of the silkworm *B. mori*, which is also invariably found in the serine proteases as well.

A protein showing strong antiviral activity against BmNPV was purified from the digestive juice of *B. mori* larvae. As lipase activity of the protein was confirmed *in vitro,* the protein was designated as Bmlipase-1, Northern blot analysis showed that Bmlipase-1 gene was expressed in the midgut but not in the other tissues. The digestive

enzyme has potential as a physiological barrier against BmNPV at the initial site of viral infection (Ponnuvel et al., 2003).

Nakazawa et al. (2004) showed the presence of a serine protease in the digestive juice of silkworm that has strong antiviral activity against BmNPV. The molecular mass and partial N- terminal sequence was also determined. Since the deduced amino acid sequence of cDNA showed 94% homology with *B. mori* serine protease, it was designated as BmSP-2 and was reported to have a molecular mass of 24271 Daltons.

Lee et al. (2005) purified a thioredoxin peroxidase from the silkworm *B. mori* (BmTPx) with a molecular mass of 25kDa which was shown to play a protective role in oxidative stress caused by temperature stimuli and viral infections.

A significant decrease in the incidence of the nucleopolyhedrosis virus was observed when the susceptible strains of the silkworms were orally fed with the RFP. The antiviral activity of the protein was also observed against a densonucleo virus and infectious flacherie virus (Lakkappan et al., 2007).

A novel gene was characterized and revealed to encode a putative BmS3a protein i.e named so because it has highly homologous to insect S3a protein, the S3a protein is known to play crucial role in protein synthesis and is related to apoptosis. It is differentially expressed in resistance breeds of silkworms and therefore, is

perhaps involved in silkworm resistance to BmNPV (Ping et al., 2008).

A new protein RNase III that confers antiviral property to the silkworm *B. mori* against the IFV was identified (Ponnuvel et al., 2008). Ponnuvel et al. (2009) also reported an alkaline protease activity against BmNPV from the digestive juice of silkworm.

A 26.5 kDa protein named BmNOx was identified in the gut fluid of Nistari strain of *B. mori* which possessed antiviral activity against BmNPV in vitro. The BmNOx expression was evaluated for Nistari and CSR2 strain of which Nistari was found to be highly resistant due to higher expression of BmNOx (Selot et al., 2010).

The SDS – PAGE pattern for the RFP from the gut fluid revealed the presence of four different bands one of which coincides with 14.4 kDa of the marker whereas the other three bands observed were smaller than 14.4 kDa (Ashish et al., 2015)

The formation of a Red Fluorescent Protein (RFP), an anti-viral substance present in the digestive juice, was studied *in-vitro*. An active protein was isolated from the midgut of the larvae and two active substances, chlorophyll-a and basic protein, were obtained from the chloroplast of the spinach. The RFP was produced by incubating the mixture of these three pure active components in the same alkaline state as mentioned above. It was highly probable that the protein from the midgut was the protein-part of the RFP and the

basic protein from the spinach was an enzyme which converts chlorophyll-a into the pigment-part of the RFP (Nishida, 1970).

A red fluorescent protein is present in the digestive juice of silkworm larvae when they are raised on fresh mulberry leaves. It is an antiviral substance and is absent in the insects reared on artificial diet. The biosynthesis of red fluorescent protein has been investigated (*in- vitro*) which showed the requirement of chlorophyll-a for its formation. A larval midgut protein reacts with the chlorophyll-a to give the final product (Hayashiya, 1978).

Horie and Watanabe (1980) reported that RFP synthesis requires chlorophyll and Chlorophyllase present in the mulberry leaves and light. Chlorophyll-a from mulberry leaf, in the presence of the enzyme Chlorophyllase is converted to chlorophyllide-a, which combines with a protein present in the larval midgut and is converted to RFP in the presence of light.

The presence of a promising broad spectrum antimicrobial red fluorescent protein was also confirmed by Matti et al. (2010). Kang et al. (2011) identified a gene encoding *B.mori* Arginine Kinase (BmAK) differentially expressed in the midgut of *Bombyx mori* strain NB which is resistant to Nucleo Polyhedron Virus (BmNPV).

Chen et al. (2012) identified 9 differentially expressed proteins in the resistant strains of the silkworm *B.mori* infected with denso nucleosis virus, which included the heat-shock 70-KDa protein

cognate, cytochrome P450, vascular ATP synthase subunit B, arginine kinase, vascular ATP synthase subunit D and glutathione S-transferase.

### 2.7.3 RFP in Silkworm Faeces

Hirayama et al. (1993) purified an antiviral protein from the silkworm faecal matter and reported it to be a glycoprotein. Hiraki et al. (1996) obtained an antiviral substance from the silkworm faecal matter extract. The active substance was found to be chlorophyll like molecule with a molecular mass of 350 (Hiraki et al., 1997).

Neelagund et al. (2007) purified an antiviral protein from the faecal matter of the silkworm *B. mori*. In SDS-PAGE the purified antiviral protein revealed two protein bands of 23 kDa and 66 kDa.

Raghavendra et al. (2009) studied the protective effect of partially purified 35 kDa proteins from silkworm faecal matter against carbon tetrachloride induced hepatotoxicity and in vitro anti-viral properties. An RFP (65 kDa) having antiviral activity against BmNPV was purified (Sethuraman et al., 1993).

Ashish et al. (2015) purified the antiviral protein from faecal matter of the silkworm *Bombyx mori*. In SDS PAGE two bands of which one was found to be around 28 kDa and the other band was smaller than 14.4 kDa.

### 2.7.4 Antiviral Activity of Insect Proteins against other Important Viruses

Hiraki et al, (1996) have opined that rarely the silkworm antiviral protein found to be effective against Herpes Simplex Virus (HSV) type-1 and Human Immunodeficiency Virus (HIV) type-1. The antiviral activity was found to be light irradiation and temperature dependent (Hiraki et al., 1997)

A photodynamic agent which is a chlorophyll derivative from the silkworm excreta was studied and was shown to be involved in photo inactivation of Vesicular Stomatitis Virus (VSV) (Lim et al., 2002).

A 916 Daltons N-myristolyated peptide obtained from the haemolymph of tobacco cut worm Heliothis virescens was found to possess antiviral activity against seven DNA and RNA viruses including HIV (Ourth et al., 2004).

An antiviral protein from the haemolymph of *Lonomia oblique* was isolated and purified by gel filtration chromatography using a gel filtration column system (superdex-75). The protein was shown to possess potent antiviral activity against measles, influenza and polio virus. The molecular mass of the protein was approximated to be 20 kDa (Greco et al., 2009).

The defence reaction in the silkworm *B. mori* against Nuclear Polyhedrosis Virus (NPV) includes antiviral substances in gut juice and Viral Inhibitory Factors (VIF) produced in haemolymph. Red

Fluorescent Protein (RFP) and alkaline protease in the silkworm gut juice showed the antiviral activity.

Hiraki et al. (2000) studied the antiviral activity of a substance (L 4-1) purified from silkworm faeces in a hem agglutinating virus (HVJ), (Sendai virus) – LLC – MK2 cell system. The antiviral effect depended on the period of light irradiation and was inhibited by sodium sulphite and anaerobic conditions.

## 2.8 Expression of Chlorophyllide Binding Protein (*ChBP*)

Chlorophyllide Binding Protein *(ChBP)* was recently characterized by its ability to bind the prosthetic group of chlorophylls and little information is known regarding its expression, Chen et al. (2014) found that was expressed highly and exclusively in the midgut of silkworm *B. mori*. The expression level of it was very high in the newly moulted fifth instar larvae followed by gradual decline in the same instar. Results demonstrated that was a secretory protein and was located mainly in the apical region of the midgut epithelial cells. Real-Time Polymerase Chain Reaction (RT-PCR) analysis results showed that was highly expressed in the anterior midgut, threefold to six fold higher than that of the middle and posterior region of the midgut, and the expression declined in darkness and its expression was affected by high-dose of virus or bacterial infection.

## 2.9 Biochemical Constituents of Mulberry

Nutrient management in mulberry is utmost important in sericulture, since the growth and development of the silkworm and cocoon production depends on the quality of the mulberry leaf.

Mulberry leaf being the only food of silkworm, its nutrient management should aim at making available all the nutritional requirements of silkworms like carbohydrates, proteins, vitamins, minerals and adequate water in the mulberry leaves. It should also ensure quantity and quality of leaves. (Bose et al.,1991).

According to Chaluvachari et al. (1992) the soluble proteins, chlorophyll and moisture content were higher with 200 kg N compared to control without Nitrogen input, sugar content and root biomass were reduced with the consequent increase in N levels. Increase in N content to mulberry significantly increases the sugar and soluble protein content in mulberry leaf (Das et al., 1993).

Shankar and Shivashankar (1993) reported that CAN recorded higher N of 3.43 and 3.03 percent and phosphorus (P) content of 0.72 and 0.49 percent in leaf followed by urea. Application of urea and urea gypsum produced higher crude protein (19.7 to 21.3%) and calcium content of 2.70 to 2.28 percent respectively over control (9.6 to 21.3% of crude proteins and 1.4 to 1.6 percent Ca, respectively).

The crude protein and total sugar content in mulberry leaf increased with an increase in nitrogen level. However, leaf moisture

and total chlorophyll content were unaffected by an increase in nitrogen level (Potdar 1994: Siddappa Kore, 1992). Rangaswamy (1997) noticed higher nitrogen, crude protein, phosphorus, potassium, calcium, magnesium, sulphur and chlorophyll content of leaf due to the application of N and K at different levels.

Shankar (1990) studied the role of potassium in mulberry and found that increased levels of potassium resulted in an increase of crude protein, magnesium, potassium and total soluble sugars and decrease in crude fibre content of leaves. Yogananda murthy et al. (2013) studied that the nutritive requirement of silkworm larvae vary with the maturity of leaves fed. Chawki silk worms require leaves with high moisture content to digest, too much mature leaves and yellow leaves which do not contain sufficient biochemical contents are not suitable to feed silkworms.

## 2.10 Biochemical Studies of Silkworm

Shankar and Shivashankar (1994) opined that CAN and urea granules were the best sources of N to mulberry ($M_5$ variety) in harvesting the best quality leaf obtained for late-age silkworm ($NB_4D_2$ breed) and industrial properties of silk.

As the larval age increases there was a constituent increase in the level of organic constituents *viz*. total proteins, total carbohydrates and total lipids in both control and BmIFV treated batches (Mamatha et al., 2014).

Sugars and proteins are major components responsible for the silkworm growth, development and silk production. Nutritive values of proteins are very important as the silkworm larva utilizes the leaf nitrogenous matter for their growth, development and synthesis of silk protein (Khan et al., 2012).

Silkworms fed with mulberry raised at various levels of nitrogen had improved cocoon characters. When fed with mulberry fertilized with N @ 125 kg/ha/yr under rain fed condition with shoot feeding method of silkworm rearing, superior cocoon weight (2.23 g), shell weight (0.44g) and shell ratio (19.82%) were recorded. Nitrogen is the basic elementary constituent of silk proteins (fibroin and serein), its availability in higher amount in the leaf could have significantly increased the silk synthesis in the silkworm resulting in higher content of fibroin in the silk glands which ultimately improve the shell weight of silk cocoons (Sannappa et al., 2002).

## 2.11 Silkworm Growth and Development

Shankar (1990) opined that the silkworm fed with mulberry leaves fertilized with CAN and urea gypsum significantly increase higher cocoon and shell weight.

Among the different sources of nitrogenous fertilizers, CAN application significantly increased the full grown larval weight, cocoon weight, shell weight, shell ratio and single cocoon filament length when compared to other sources, namely, urea ammonium

sulphate and ammonium nitrate.(Manjula,1993: Subbarayappa et al., 1992) Shell and pupal weight increased with its application to mulberry (Das et al.,1993).

According to Potdar (1994), Sudharshana Reddy (1994) and Potdar et al. (1997) higher dose of N (400 kg /ha/yr) improved the quality of mulberry leaves and subsequently had a positive effect on the growth and development of silkworm and on the cocoon parameters.

The use of CAN was found to increase significantly the fecundity and hatching percentage and reduce the number of dead eggs of $NB_4D_2$ breed of silkworms in rabi and summer season (Shankar et al., 1999). Further, rearing results indicated that cocoon yield/1000 DFLs increased substantially when silkworms were fed with leaf from ammonium sulphate treated plot compared to urea. Thus, the effectiveness of these fertilizers in mulberry varied in the sequence of ammonium sulphate>urea>CAN under irrigated conditions (Subbaswamy et al., 1999b).

Sannappa et al. (2003) observed that silkworm fed with mulberry at 5 feeds/day raised with application of N @ 125 kg/ha/yr recorded higher fifth instar larval weight (43.83 g/10), larval survival (92.33%), ERR (Effective rate of rearing) (89.50%) and cocoon yield (38.19 kg/50 DFL's) with reduced total larval duration (25.32 days) together with 4 and 3 feeds/day at the same level of nitrogen.

Among major elements the deficiency of K badly affected the cocoon characters and silk quality due to an imbalance in the uptake of other elements in mulberry leaves (Shankar, 1990). It has been reported that larval weight differed in all instars when worms were fed with mulberry leaves fertilized with graded level of potassium. In addition, they also reported improvement in mature worm weight and cocoon weight (Leina Mary Joseph, 1991: Puttaswamy, 1993 and Shriharsha, 1996).

Nagaraju (1997) reported that under rain fed condition the mulberry plants fertilized with potassium sulphate significantly increased a single cocoon weight (1.778 g), shell weight (0.307 g), filament length (792.8 m) and denier (2.33). Rangaswami (1997) also reported that feeding of worms on such mulberry leaves recorded higher cocoon (1.649 g), pupal (1.370 g), shell weight (0.278 g) and filament length (880.0 m). Low intake of P and K was found to decrease body weight of silkworm (Singhal et al., 1999).

Shankar et al. (2002) revealed that application of sulphate of potash to mulberry led to an increase in single ripened worm weight, single cocoon weight, shell percentage, filament length and lesser denier as compared to murate of potash.

## 2.12 Biochemical Constituents of Silkworm Haemolymph

Nirwani and Kaliwal (1996) indicated that potassium sulphate stimulated the metabolic activity of bivoltine breed by induction of changes in carbohydrates and proteins, in the fat body and haemolymph, thereby increasing the economic parameters.

Banno et al. (1993) analysed the haemolymph proteins quantitatively throughout the larval development of *B. mori.* The haemolymph protein concentration decreased by the 4$^{th}$ larval instar with cyclic increases until moulting and decreases after the 4$^{th}$molt and rearing a maximum value at the end of the 5$^{th}$ instar, with female larvae showing higher concentration than the male.

Mahadev Kumar et al. (2002) studied the changes in protein and reducing sugar content of the haemolymph during 5$^{th}$ instar development of *Bombyx mori* L. The level of protein and reducing sugar in the haemolymph of different varieties of silkworm *Bombyx mori, viz.* Bivoltine (BV), Multivoltine (MV) and Cross breed (CB) were investigated during 5$^{th}$ instar development. The concentration of protein increased from the first day to reach its peak on seventh day in all the three varieties. Bivoltine showed a higher protein content in the haemolymph followed by cross breed and multivoltine. The concentration of decreasing sugar also showed a similar trend in the respective varieties being higher in BV followed by CB and MV.

Sinha and Sinha (1994) studied the changes in concentration of proteins and carbohydrates in developing embryos and larval haemolymph of temperate tasar silkworm, A*ntherae aproyleii.* They observed that concentration of both protein and carbohydrates in the haemolymph increased progressively during larval development and reaches maximum in the late fifth instar larvae. Increase in protein content may be attributed to the development of reproductive organs. Carbohydrates widely occur in plants in which their quality and quantity vary enormously. Larvae of 5$^{th}$ instar consume enormous quantity of leaf and perhaps this is the reason for the highest concentration of carbohydrates in the haemolymph of late fifth instar larvae.

# MATERIAL AND METHODS

The experiments were conducted to study the "**Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori* L."** was undertaken in the Department of Biochemistry, Jnana Sahyadri, Kuvempu University, Shankaraghatta, Shivamogga, Karnataka, India and the Department of Plant Biotechnology, University of Agricultural Sciences (UAS), Gandhi Krishi Vignana Kendra (GKVK), Bengaluru, Karnataka, India

## 3.1 Materials

### 3.1.1 Chemicals

All the chemicals used in this study were of molecular biology grade and analytical grade. The chemical TEMED was obtained from Koch – Light laboratories Ltd. England, and chemicals, Sephadex G-100, Ethidium bromide, PCR primers, isopropanol, Diethyl Pyro Carbonate (DEPC), Poly Vinyl Pyrrolidone (PVP), Dialysis tube, etc., were obtained from Sigma Chemicals Co., St. Louis, USA. The DNA ladder, chloroform, agarose, ammonium sulphate, sodium chloride, Ethylene Diamine Tetra Acetic Acid (EDTA), potassium phosphate, sucrose, *Taq polymerase*, *Taq* buffer, nuclease free water, $MgCl_2$, dNTPs, ribonuclease inhibitor, reverse transcriptase (MMuLv), Triton X-100, Tris-HCl, glycine, SDS, β-mercapto ethanol, glycerol,

bromophenol blue, ethanol, Protein molecular weight marker and all other chemicals used in this study were from Bangalore GeNei, Chromous Biotech and Himedia labs (India). The aqueous solutions were prepared by using deionized water from Milli-Q ultrapure water system.

### 3.1.2 Glasses and Plastic Wares

All the glass ware used in the study was from Borosil India Ltd. They were thoroughly washed and sterilized as per standard procedure. Plastic ware (micro-centrifuge tubes and micropipette tips) were from Tarsons (India).

### 3.1.3 Equipments

- Mini spin – Eppendorf.
- Cooling centrifuge-REMI.
- UV trans Illuminator-Genie.
- Pipettes capacity of 2-20µl, 50-100 µl, 100-200 µl, and 1000 µl from Tarsons.
- Horizontal Agarose Gel Electrophoresis apparatus with power supply- Genie.
- -80ºC Ultra Low freezer-Esco Lexicon ultra-low temperature freezer.
- Microwave Oven-Samsung.
- Electronic Shaking Incubator-Wise cube.

- Incubator-ILE (The industrial & Laboratory Equipment).
- Horizontal laminar air flow.
- PCR Machine-ABI Thermal Cycler 2700.
- SDS-PAGE unit- Genie.
- Spectrophotometer- Varian Cary 50 UV-visible spectrophotometer.
- Lyophilize- Vertis wizard 2.0.
- Gel Documentation system- Alpha InfoTech Corporation, USA.
- HPLC- ShimadzuLC-10AVP series, Japan.
- Real Time PCR- ABI Step-one Real Time PCR machine.
- Sequencing machine – ABI 3500 XL Genetic Analyzer.

### 3.1.4 Reagents and Buffers

Composition of Reagents, Buffers and Solutions used in this study are given in the appendix or at appropriate places.

## 3.2 Experimental Methods

### 3.2.1 Isolation and Purification of Red Fluorescent Protein (RFP) from the Silkworm *Bombyx mori L*

**a. Establishment of Mulberry Garden for Rearing of Silkworms**

In the present study, the hybrid variety of mulberry Victory-1 (V-1) was undertaken (Fig.3) and garden was established and maintained as per regular package of practices in the Department of Plant Biotechnology, U.A.S., G.K.V.K, Bengaluru. The silkworm polyvoltine

**Fig. 3.** Victory-1 (V1) mulberry garden.

**Fig. 4.**Fifth instar Polyvoltine Silkworm (*Bombyx Mori* L)Kolar Gold hybrid (PM X $CSR_2$) breed.

hybrid Kolar Gold (PM X $CSR_2$) (Fig. 4) was selected for the study and reared as per the standard of practice adopted by Krishna Swami et al. (1978).

**b. Preparation and Rearing of Silkworm**

The rearing room was fumigated, bleached and treated with 5 % formaldehyde and the containers and instruments were washed with running water and wiped with 5 % formaldehyde and then air dried. During silkworm rearing the larvae fed on clean and fresh leaves, the maintained temperature and RH was 25±2°C and 65±10% , and the light and dark ratio of 12:12 photoperiod was maintained as per the procedure adopted (Krishna Swamy et al., 1978).

### 3.2.2 Extraction of Midgut Digestive Juice and Purification

Isolation and purification of RFP from the midgut digestive juice of silkworm was carried out by adopting the modified procedure (Sunagar et al., 2011). Fifth instar larvae of silkworm selected and exposed them to chloroform to regurgitate and collected the vomited gut juice in glass vials. Further, it was processed (immediately after collection without storing at 4°C) under nitrogenous condition with subdued light at 4°C. To remove the undigested mulberry leaves and other observable solid particles the gut juice was centrifuged at 1500 g, and the supernatant was neutralized to pH 7.0 with 2M acetic acid. The obtained supernatant was saturated to 40% ammonium sulphate for 3 hours (hrs) at 4°C for precipitation and it was

centrifuged at 14,000 g for 40 min at 4°C and suspended in a minimum amount of deionized water and volume was measured to which half volume of n-butanol was added and kept for 3 hrs. at 4°C, centrifuged again at 14,000 g at 4°C for 40 min. The lower aqueous layer was collected and equal volume of cold acetone was added and kept it for 4°C for 30 min, the obtained precipitate was collected by centrifugation at 14,000 g for 40 min and then dissolved in 40 mM sodium phosphate buffer (pH 7.0±0.1) and subsequently dialysed against the same buffer (2-3 times) using a dialysis membrane having 15,000 kDa cut-off, for a duration of 48 hours. Further, the dialyzed was centrifuged at $4^0$C for 14000 g for 10 min. The supernatant was taken in fresh clean vial and exposed to the UV-trans illuminator for the confirmation for the RFP followed by the lyophilisation before subjected to the column chromatography and protein concentration of lyophilized protein sample was estimated (Lowry et al., 1951).

**Gel Filtration Chromatography (GFC)**

In excess volume of distilled water, Sephadex G-100 was dissolved and allowed to swell for 72 hrs. The saturated Sephadex was poured into the column (70 x 1.5) to the required level and was equilibrated with 40 mM phosphate buffer (pH 7) for an overnight. On to the column, one ml (10 mg/ml) of the dialysed protein sample was loaded and elutes were collected (flow rate 10 ml/h) in fractions of

fixed volumes in eppendorf tubes were exposed to UV light to monitor the elution of RFP fractions. The glowing fractions were pooled and again screened under UV to reconfirm and absorbance was recorded at 280 nm by using spectrophotometer eluted RFP was concentrated and lyophilized protein content was estimated (Lowry et al., 1951).

## 3.3 Biochemical Characterization of the Purified RFP from Silkworm *B. mori* L.

### 3.3.1 Native Poly Acrylamide Gel Electrophoresis (Native-PAGE)

The purified RFP by GFC was subjected to Native-PAGE (Laemmle 1970). Using separating gel (12%), stacking gel (5%), and the RFP sample (40 µg) in glycerol (30%) were loaded and was ran by applying an 100 V for about 30 minutes to allow the tracking dye (Bromo phenol blue) to enter the separating gel. Subsequently, the current was increased to 150V and maintained until the tracking dye reaches the bottom of the gel. The separated RFP band was visualized under UV trans illuminator and the gel was stained using Coomassie Brilliant Blue R-250 (CBB R-250). Images were captured using Alpha Digi documentation system.

### 3.3.2 SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

The purified RFP by GFC was subjected to the SDS-PAGE (Laemmle 1970). The aliquots of 30 µg of purified RFP and partially purified RFP with 10 µl of 4x SDS sample buffer was mixed and boiled at 100°C for 10 min and cooled to room temperature before loading into

the gel. The vertical slab gel unit was assembled in the casting mode by keeping 1 mM Teflon spacers in between the electrophoresis plates. After sealing the plates with agarose, the resolving gel solutions were mixed in a beaker, leaving out acrylamide and TEMED which were added later with gentle swirling of the flask. The SDS discontinuous buffer system with five per cent stacking gel (pH 6.8) and 12 per cent resolving gel (pH 8.8) and Tris-glycine (pH 8.3) act as electrode buffer was used. The Teflon comb was inserted quickly, taking care not to trap any air bubbles and the gel was allowed to set for 30 min. The comb from the gel was slowly removed. The samples were loaded into the wells along with pre-stained protein standard molecular weight marker that was used to determine molecular weight. The two buffer chambers were connected to power supply and electrophoresis was carried out at voltage of 100 Volts (V) until bromo phenol blue dye reaches the bottom of the resolving gel.

### 3.3.3 Protein Staining

#### a. Coomassie Brilliant Blue R-250 (CBB-R 250) Staining

After completion of SDS-PAGE, the gel was carefully transferred from the gel plate assembly into a tray containing CBB-R 250 staining solution and kept on gel rack for an overnight. The orientation of the gel was marked by cutting the top left hand corner of the gel. The gel was removed from staining solution and transferred into destaining

solution. Further, the gel was destained and the blue coloured protein bands appeared against the clear background. Then the gel was transferred to storage solution and images were captured using Gel Doc System. The molecular weight of RFP protein was determined by comparing it with standard protein marker.

**b. Silver Staining**

For the silver staining of the RFP, the RFP was resolved by SDS-PAGE (as described in above section 3.2.2). After running the gel silver staining was performed (Shevchenko et al., 1996). Required solutions were freshly prepared and steps were performed at room temperature.

| | |
|---|---|
| Step 1 | The gel was soaked for one hour in Fixative solution (Methanol Acetic Acid water in ratio 25:15:60) to that 50 µl Formaldehyde was added. |
| Step 2 | The gel was washed with 50 % ethanol three times (20 minutes each). |
| Step 3 | The gel was treated precisely one minute with hypo solution (Sodium Thiosulfate solution 20 mg/100 ml) shaking by hand. |
| Step 4 | The gel was washed with water three times (20 seconds each) shaking by hand. |
| Step 5 | The gel was treated with Silver Nitrite solution (100 mg/100 ml) for 30 minutes. |
| Step 6 | The gel was washed with water three times (20 seconds each) shaking by hand. |
| Step 7 | The gel was developed in 100 ml developing solution (6 g Sodium Carbonate, 2 ml of the hypo solution, 50 µl Formaldehyde) for 10 min. |
| Step 8 | The gel was stored in 7 % acetic acid. Image was taken. |

### 3.3.4 High-Performance Liquid Chromatography (HPLC) Analysis

An analytical HPLC was used to confirm the purity of the RFP (Shimadzu LC-10AVP series, Japan). The RFP was dissolved in the required quantity of HPLC grade water and filtered through the 0.45 mM filters (Millipore) and injected into a C18 (Shimadzu) narrow bore column of 250 x 10mM with a 5µ particle size with a linear gradient of 30-70% acetonitrile (ACN)/0.1% tri-fluoroacetic acid (TFA) in water and at a flow rate of one ml/min. The column effluent was monitored under UV detection (280 nm). Each peak was manually collected and lyophilized for further analysis.

### 3.3.5 Matrix-Assisted Laser Desorption/Ionization (MALDI)-Time of Flight (TOF)-Mass Spectra (MS) (MALDI-TOF-MS)

Purified RFP by HPLC was subjected to MALDI-TOF in the reflector positive ion mode with an acceleration voltage of 20 KV, delay time of 150 ns and acquisition mass range of 8-70 kDa. One microliter portion was spotted onto the stainless steel target plate and air dried. The MALDI-TOF/MS analysis was performed using Ultraflextreme (Bruker Daltonics, Btrmen, Germany) which was operated in a linear mode of 25 KV accelerating voltage and 400 ns ion extraction delay with the nitrogen laser working at 337 nm and 3 Hz. Thousand shots/sec of laser of 35% strength were accumulated per spectrum.

### 3.3.6 Bioassay Studies of RFP

#### a. Antibacterial Activity

The RFP's antibacterial activity was determined by using bacterial strains like *E. coli* (ATCC number 25922) and *Bacillus subtilis* (ATCC number 6633) procured from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India using modified cup plate method (Collins et al., 1995). An inoculum of $10^8$ colony forming units per millilitre (cfu/ml) of each of the organism was prepared by suspending loopful of inoculum from the stock into different labelled test tubes, each containing 10 ml of the nutrient broth (Bauer et al., 1996). In three test tubes per each test organisms were incubated at $37^0$C for 24 hours. The cultures diluted with nutrient broth and 50 µl of each of these was evenly smeared on the petri plates, and these were air dried for 5 minutes and each plate was bored by using sterile cork borer (number 3) and was used as the control. A volume of 25 µg (R1) and 50 µg (R2) of the prepared dilutions of the RFP, 25 µg of Ampicillin (A) and Phosphate Buffer (P) as a positive control were poured into each of the aseptically bored holes separately and were incubated at $37^0$C for 24 hrs. Five replications per organism were done. The diameters of the zones of inhibition were measured using Himedia Zone scale.

**b. Antifungal Activity**

By adopting Using radial growth technique (Zambonelli et al., 1996 and Bajpai.2007) RFP's antifungal activity against plant pathogenic fungus *Phytophthora meadii* the causal organism of 'Koleroga' affecting arecanut was studied which was obtained from Indian Institute of Horticulture Research (IIHR), Bangalore). Metalaxyl water soluble fungicide is effective against all species of *Phytophthora* (Cohen et al., 1986) was used as the control. The protein and fungicide were added to the cooled carrot agar medium separately which was then poured into the petriplate. Discs of mycelia (0.5 cm diameter) of the plant pathogenic fungi taken from the 8-day old culture were transferred aseptically to the centre of the petriplate. The treatments were incubated at 27˚C in the dark and the diameter of the grown colony was measured on the eighth day. The percentage inhibition of mycelia growth was calculated using the formula (DC-DT/DC) *100 where DC-Diameter growth in the control and DT-Diameter growth in the treatment respectively.

**c. Antiviral Activity**

**Determination of the $LC_{50}$ Value for *Bm*NPV Virus**

In order to study the inhibitory effect of RFP on *Bm*NPV a $LC_{50}$ study was carried out to fix up the viral concentration which gives 50% mortality, the details of experiment are given below. The viral polyhedral suspension was purified as per the method described by

Palhan and Gopinathan (1996). The purified *Bm*NPV virus serially diluted to give $10^{-1}$,$10^{-3}$, $10^{-5}$ and $10^{-7}$ dilutions by using stock (undiluted) and distilled water which served as the control (Bancroft,1970).

**Mode of *Bm*NPV Infection**

The matured mulberry leaves were collected from the field and washed with tap water, shade dried and surfaces sterilized with 70 % ethyl alcohol and shade dried again. These leaves were smeared evenly with the virus suspension (0.5 ml/$cm^2$) using non-absorbent cotton. The infection was carried out by feeding orally the virus suspension smeared mulberry leaves to the $4^{th}$ instar $1^{st}$ day larvae and the mulberry leaf smeared with distilled water served as control. Three replications were maintained for each treatment with 30 worms per replication, and their mortality was recorded. The $LC_{50}$ value was determined by the Reed and Meunch method (Bancroft, 1970). The arithmetic estimate of the $LC_{50}$ value was obtained directly from the tabular arrangement of the data. The exact 50 per cent mortality was estimated using the following formula.

$$L = \frac{A - B}{C - B}$$

Where, L = $LC_{50}$ value

B = Per cent mortality just below 50

C = Per cent mortality at next higher dose above 50

A = 50

To make use of the assumption that the expression in the region of 50 per cent is linear, linear doses were used for the interpolation of the $LC_{50}$ with the following calculations.

Log $LC_{50}$ value = Log dose corresponding to b + 'L' log of number of folds increase at C compared with B. The $LC_{50}$ = antilog of Log $LC_{50}$.

**Per os (oral administration) Assay of RFP**

*In vivo* antiviral assays were carried out by following the method Nagraja et al. (1993). The following solutions were prepared and used

1. The NPV solution ($OD_{260}$ = 0.925) was mixed with an equal volume of the purified RFP solution ($OD_{280}$ = 2.5). (RFP+NPV)
2. The NPV solution ($OD_{260}$ = 0.925) was mixed with an equal volume of BSA (Bovine Serum Albumin) ($OD_{280}$ = 2.5) served as negative control. (NPV+BSA).
3. The NPV solution ($OD_{260}$ = 0.925) was mixed with an equal volume of 40 mM phosphate buffer at pH 7.5 which served as negative control (NPV+PB).
4. The BSA solution ($OD_{280}$ = 2.5) was mixed with an equal volume of 40 mM phosphate buffer at pH 7.5 to examine whether the protein affects the normal worms or not (BSA+PB).
5. Only 40 mM phosphate buffer (pH 7.5) served as positive control (PB).

6. Untreated larvae were also kept along with above to serve as untreated control.

All the solutions were incubated at 25$^{O}$C for 60 min. Each mixture was smeared on mulberry leaves that were fed to 4$^{th}$ instar larvae, which were maintained in a batch of 30 worms separately. After few days, (in 5$^{th}$ instar) the larvae were examined for nuclear polyhedral infection.

### 3.3.7 N-terminal Sequence Analysis

After reconfirmation of purity RFP by HPLC, the RFP was separated in denatured SDS-PAGE and the band was eluted and subjected to Edman degradation for its N-terminal amino acids sequencing. The protein band was transferred to polyvinylidene fluoride (PVDF) membrane in a transfer buffer containing 10 mM 3-Cyclogexyl Amino-1 Propane Sulfonic acid (CAPS) at a constant current of 350 mA for 90 minutes in a mini Trans-Blot® Cell (Bio-Rad Laboratories). The membrane was subsequently stained with CBB staining solution and de-stained subsequently. The bands excised from the membrane acted as a source for N-terminal sequence analysis. The sequencing was accomplished by using procise (pulsed liquid PVDF) 494 Procise protein sequencer/140C analyzer from Applied Bio systems, Inc, USA. To identify the protein from the sequencing results and confirm the RFP, Homology search was performed using Basic Local

Alignment Search Tool (BLAST) at National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/).

### 3.4 Identification and Molecular Characterization of *ChBP* Gene

#### 3.4.1 Silkworm Midgut Dissection

The fifth day old fifth instar larvae was selected and anaesthetized for mid gut dissection. The larvae were stretched and pinned at the head region and anal flap, fixed it on the dissection tray with upside dorsal. Skin was opened till posterior end of the head and pinned laterally. Then the specimen was immersed completely in water. The fat bodies, muscle, trachea, etc., were removed carefully and the midgut was dissected out, dipped in liquid nitrogen and used for RNA isolation.

#### 3.4.2 RNA Extraction Analysis

Total RNA was extracted from the dissected midgut using Chromous RNA isolation Kit-46. The required quantity of midgut tissue was taken (stored in liquid nitrogen) and transferred to a DEPC treated mortar placed on dry ice, grinded to a fine powder by pestle using liquid nitrogen and to this added 600 µl of total RNA extraction buffer then the same was again grinded to a fine paste, it was transferred into 1.5 ml vial and vortexed thoroughly, 200 µl of Chloroform: iso amyl alcohol mixture (24:1: v/v) was added and vortexed then centrifuged at 10,000 g for 10 minutes at room temperature. The clear upper aqueous layer was carefully transferred

into a fresh eppendorf tube and equal volume of iso propanol was added. The mini-spin column was placed into 2 ml collection tube and the above mixture was loaded on the spin column. It was centrifuged at 10,000 g for one minute at room temperature. The contents of the collection tube were discarded. The spin column was placed back in the collection tube. The bound RNA was washed with 500 µl of 1X RNA Wash Buffer and centrifuged at 10,000 g for one minute at RT. The contents of the collection tube were discarded. The empty column was spun at 10,000 g for three min at RT. Then the spin column was placed in a fresh 1.5ml vial. The elution buffer was warmed to 65°C for one minute, bound RNA was eluted with 30 µl warmed elution buffer and spin column was centrifuged at 10,000 g for one minute at RT and eluted total RNA was collected and stored at -80 $^{o}$C till further use.

### 3.4.3 Quality Analysis of RNA

Extracted RNA was subjected to its purity analysis by taking absorbance at 260/280 nm and running denatured formaldehyde agarose gel electrophoresis. One gm. agarose was weighed and dissolved in 100 ml of DEPC treated water and boiled for five minutes and then allowed to cool to 60°C. The 10 ml of 10X MOPS and 5.6 ml of formaldehyde was mixed and pre-warmed to 60°C and was added to the gel solution along with Ethidium bromide (10 mg/ml) and mixed gently and casted the gel immediately fitted with

comb and allowed the gel to set at RT and after the gel set perfectly, the comb was carefully removed. The tray with gel was then transferred to electrophoresis tank containing running buffer and submerged in 1x MOPS running buffer. 20 µg of the total RNA was added to the loading buffer (1X) and the resulting mixture was heated at 65 °C for 10 minutes and the same was cooled on ice immediately, loaded into wells alongside the molecular weight marker. Electrophoreses was carried out at 80 V in 1X MOPS buffer until the tracking dye has just passed out of the gel. The gel was visualized and documented under Alpha InfoTech gel documentation system.

### 3.4.4 Amplification of Chlorophyll Binding Protein Gene

The N-terminal sequence of RFP was obtained by Edman degradation method and sequence was subjected to the BLAST analysis using default parameter at NCBI and significantly predicted the *ChBP* genes of silkworm, based on this result, primers were designed and synthesized for ChBP gene.

### 3.4.5 cDNA Synthesis and Amplification of *ChBP* Gene

The purified total RNA was used for the synthesis of cDNA by using Chromous kit (RKN kit-65) as per the manufacturer's instructions. Two µg of RNA (10 µl) and 20 pmol of oligo dT (5 µl) was heat denatured at 65°C for 15 min and tubes were cooled on ice for 5 minutes then subsequently 5 µl of Reverse-Transcriptase buffer

(10X), 2 µl of dNTPs mix (2.5mM each), 0.25 µl RNasin (40U/µl), 5 µl of DTT (0.1M) was added making the final reaction 50 µl. The reaction mix was spin mixed and incubated at 37ºC for ten min. 2 µl MMuLV reverse transcriptase (20U/µl) was added to the above mix and cDNA synthesis allowed to proceed at 42ºC for one hour. The reaction was stopped by heat inactivating the enzyme at 94ºC for 5 min. The tubes were snap cooled on ice and subjected to polymerase chain reaction (PCR).

First strand of cDNA synthesized by reverse transcription was subjected to PCR amplification by using gene specific forward primer 5'-GCTGGAAGATGAGCAGAACGAACAC-3' and reverse primer 5'-GTTGTTATAATCGGTGCTCACGATCCAG-3'. As per the manufacture's instructions the PCR reactions were carried out (Chromous biotech kit RKN-65). The reaction mix consisted of 1.0 µl of cDNA as a template, 1.0 µl of each primer (100ng/µl), 1.0 µl of dNTPs (10mM), 2.5 µl of 10X Chromous Taq assay buffer, 0.25 µl Chromous Taq polymerase Enzyme (3U/mL) in a total volume of 25.0 µl adjusted with nuclease free water and mix was subjected to 35 cycles of amplification in thermal cycler after initial denaturation of the template at 95°C for 5 min. Each cycle consisted a denaturation phase at 94°C for 30 seconds followed by annealing temperature at 55°C for 30 seconds. Primer extension at 72°C for 1 min and the final extension phase was at 72°C for 10 min. The amplified product

was analysed by using 2.0 per cent (w/v) Agarose gel electrophoresis. Agarose 2% solution (100 ml) was prepared in 1X TAE by gently heating to boiling. After cooling it to 40°C, 5 µl EtBr (10 mg/ml) was added, mixed and poured into the cast. The comb of desired type was inserted to allow the formation of sample loading well. Once the gel was set properly (after 1/2 h), the comb was removed carefully and transferred to the gel electrophoresis unit. 5 µl of DNA sample was mixed with 2.5 µl of Bromo phenol dye in micro centrifuge tube and spun at low speed for a few seconds and loaded to the wells along with Step-up 500 bp DNA ladder. The gel electrophoresis tray was filled with 0.5X TAE buffer to cover the gel. The gel was run at 75 volts current till the tracking dye has just crossed out of the gel. The gel was carefully removed from the tank, visualized and images were captured (Alpha InfoTech Corporation, USA).

### 3.4.6 Elution of DNA from Agarose Gel

The amplified DNA fragment was eluted from the agarose gel using gel extraction kit (Chromous gel extraction kit-35) instructions. The separated DNA fragment from agarose gel was carefully cut out under UV-trans illuminator by using clean sharp cutter and the gel slice was taken to 2ml micro centrifuge tube and weighed. To the one volume of gel (100 mg-100 µl) 3 volumes of gel extraction buffer was added. The tube was incubated at 55°C for 10 min by inverting at every 2-3 min during the incubation to solubilize agarose completely.

After the gel piece dissolved completely, one gel volume of isopropanol (100 mg-100 µl) was added to the tube and transferred to spin column fixed with 2 ml collection tube (600 µl each time) and was spun at 10,000 g for 10 min at RT and discarded the contents of the collection tube. Further, 500 µl of wash buffer was added to the column and was spun at 10,000 g for 1 min at RT and the contents of the collection tube was discarded. Then placed the spin column back in the same collection tube and spun the empty column with the collection tube at 10,000 g for 3 min at RT. The spin column was placed into a fresh 1.5 ml micro centrifuge tube to which 20µl of elution buffer was added and kept at RT for 2 min and then again spun at 10,000 g for 1 min. Again 20µl of elution buffer was added to the spin column and kept for 2 min at RT with the column and spun at 10,000 g for 1 min to obtain the DNA. The gel purified product was taken for sequencing.

## 3.5. ChBP Gene Sequence and Bioinformatics Analysis

### 3.5.1 *ChBP* Gene Sequence

The amplified PCR product was sequenced (ABI 3500 XL Genetic Analyzer sequencing machine, Big Dye Terminator version 3.1 cycle sequencing kit, POP_7 polymers 50 cm Capillary Array). The reaction mix for sequence consisted of Big Dye terminator ready reaction mix 4µl, Template (100ng/µl) 1µl, Primer (10pmol/λ) 2µl and Milli Q water 3µl making total volume to 10µl. The mix was subjected to 25

cycles Initial denaturation-96°C for 1 min, denaturation-96°C for 10 Sec. Annealing-50°C for 5 seconds and extension-60°C for 4 min and was used for analysis protocol and the software Seq Scape-v 5.2 was used for data analysis and Applied Biosystems Micro Amp optical 96 well reaction plates was used. The obtained sequence has been deposited to the entrez gene bank under the accession number KX186723 at NCBI (www.ncbi.nlm.nih.gov).

### 3.5.2 *ChBP* Gene Analysis

The DNA and the deduced protein sequence have been analysed using *in-silico* approaches. Blast search was performed using default parameters against NCBI- database (http: //www.ncbi. nlm.nih.gov/) to identify the homologous sequence to obtain the *ChBP* homologous sequences and understand its diversity in other genomes. The deduced amino acid sequence of the novel *ChBP* protein of *B. mori* Kolar Gold breed with other representative groups were extracted from the Gene Bank Repository at NCBI web-site (http://www.ncbi.nlm.nih.gov/pubmed).

### 3.5.3 Domain Analysis

The obtained *ChBP* protein sequences were subjected to RPS-BLAST search versus the position-specific scoring matrices in CDD (v3. 16-50369 PSSMs) in the NCBI Conserved Domains Database (CDD) (Marchler-Bauer et al., 2005). Based on the identification of domains that is specific for the lipocalin superfamily (cl21528).

### 3.5.4 Phylogenetic Analysis

The protein sequence of *ChBP* gene is used to determine the evolutionary trend in insects. Using ClustalW multiple sequence alignment was performed and unrooted phylogenetic tree was constructed applying the maximum likelihood method with bootstrap value 500 in MEGA 5.05 tool (Thompson et al., 1994).

### 3.5.5 Homology Modelling of a ChBP Gene

*ChBP* protein sequence was used for the homology modelling using SWISS-MODEL (https://swissmodel.expasy.org/interactive) online web server which predicts the structure and function based on sequence homology A theoretical model was predicted for novel *ChBP* protein from *B. mori* based on template Z224 at X-ray diffraction (2.60 A resolution) a protein which is an insecticyanin A from tobacco hornworm, Manducasexta L (Arnold et al., 2006), with 26.44 % sequence identity with *ChBP* was used the template for comparison.

## 3.6 Effect of Yellow Mulberry Leaves Feeds to Silkworm Larvae on *ChBP* Gene Expression.

For the study, green and yellow leaves of mulberry were selected to see the level of expression of *ChBP* gene. The 5$^{th}$ instar 1$^{st}$ day larvae were selected and divided them into three groups. Each group consists of 30 larvae as follows: T1 green leaves (control), T2 - only

yellow leaves, T3- yellow leaves for 2 days and green leaves for 3 days.

### 3.6.1 RNA extraction, Real Time-PCR and Expression Analysis of *ChBP*

Total RNA was isolated from the 5$^{th}$ day old 5$^{th}$ instar larval midgut of the silkworm by using Chromous RNA isolation Kit-46 and the cDNA synthesized as explained in section 3.4. To determine the effect of yellow leaves feeds to the larvae on *ChBP* gene expression levels with the *ChBP*-specific primers. Forward Primer 5'-CCGTCTTAGCGACCACTGACGGAAGC-3' and Reverse Primer 5'-CCACTGCTTGGTCCTGCTAAGCTTCCAGC-3'. RT-PCR was carried out on an ABI step- one real time RT-PCR thermo cycler in a total volume of 50 µl consisting of 1 µl 1:5 diluted *ChBP* cDNA template, 2 µl of each of the primers (100ng/µl), 25 µl of 2 X PCR SYBR green ready mixes and 20 µl of water. The thermal cycling profile consisted of initial denaturation at 94ºC for 5 min and 40 cycles at 94ºC for 5 seconds, 55ºC for 10 seconds and at 72ºC for 10 seconds. The PCR reactions were performed in Applied Biosystems Micro Amp optical 96-well reaction plate using SYBR green to detect ds DNA synthesis. PCR amplifications were performed in triplicates. All samples were normalized against the silkworm cytoplasmic tubulin. Tub-FP-5'-TCGTCGAGCCCTACAACTCT-3',Tub-RP-5'-ACTCGGTGAGGTCCACATTC-3'. Relative gene expression levels were calculated using the comparative CT method (ΔΔCT).

## 3.7 Evaluation of Feeding Green and Yellow Mulberry Leaves on Growth, Development, Survival and Disease Prevalence

The larvae were fed daily 3 times and divided into 3 batches for treatments (T) with yellow leaves and green leaves fed silkworms viz. 1st batch (3rd to 5th instar), 2nd batch (4th to 5th instar), 3rd batch (5th instar only) and control (only green leaves fed worms) were maintained for each batch. Triplicate of 30 worms for replicate was maintained, in all the batches growth, survival and mortality rate was recorded.

**Batches and Treatment (T)**

**Batch-1 & $T_1$**: silkworm larvae fed with yellow mulberry leaves from 1st day of 3rd instar to till the end of 5th instar.

**Batch-2 & $T_2$**: silkworm larvae fed with yellow mulberry leaves from 1st day of 4th instar to till the end of 5th instar.

**Batch-3 & $T_3$**: silkworm larvae fed with yellow mulberry leaves from 1st day of 5th instar to till the end of 5th instar.

**Control:** silkworm larvae fed with only green mulberry leaves in all instars.

### 3.7.1 Evaluation of Survival Rate

The survival rate was observed daily with a regular interval of 24 hours from all the three batches mentioned above with control in all the replications and the results expressed in percentage (%).

### 3.7.2 Evaluation of Growth Rate

The silkworm growth performance in all the three batches with control was recorded by measuring the weight in grams daily with a regular interval of 24 hours.

**Statistical Analysis**: The data obtained from the biochemical experiments were statistically analysed by t-test and one-way ANOVA using through SPSS to determine the significant difference among the treatments and control batches.

# EXPERIMENTAL RESULTS

In this chapter, the results obtained from the experiments carried out on "**Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori* L."** using polyvoltine hybrid Kolar Gold (CSR2 x PM). Different analysis have been performed, presented the results on biochemical characterization of RFP and its bioassay studies, Molecular characterisation and its expression of *ChBP* on yellow and green leaves feed larvae and effects on growth are presented.

## 4.1 Isolation and Purification of Red Fluorescent Protein (RFP) from the Silkworm *Bombyx mori L*

The midgut digestive juice of the silkworm is one of the rich and promising sources for utilizing proteins having antiviral and antimicrobial activities. For the present study silkworm polyvoltine Kolar Gold (CSR2 x PM) hybrid breed was selected. The midgut digestive juice was collected by inducing regurgitation in fifth instar larvae by brief exposure to chloroform vapors (Fig. 5A). Initially, the protein was purified by ammonium sulphate precipitation and GFC (Fig. 5B). The absorbance of the collected fraction was measured at 280 nm and a graph of the OD values for the different fractions was plotted. The results showed that three peaks were observed at 280

nm, of these only one peak showed high absorbance (Fig. 6). The fractions 30-60 showed red fluorescence under UV light (Fig. 5C). These fractions were pooled (Fig. 5D) and lyophilized for further analysis.

### 4.2 Poly Acrylamide Gel Electrophoresis (PAGE)

#### 4.2.1 Native Poly Acrylamide Gel Electrophoresis (Native-PAGE)

The Purified RFP from the midgut juice of silkworm larvae through GFC was subjected to native-PAGE. The gel was exposed to UV light and one fluorescent band was observed. The same gel was stained with Coomassie Brilliant Blue R-250 (CBB-R-250), the result showed that only one single RFP band was observed and presented in Fig. 7.

#### 4.2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

**a. CBB-R 250 Staining Gel**

The purified RFP by GFC was subjected to SDS-PAGE stained with CBB R-250 presented in Fig.8. Only one single RFP band was observed which coincides with 40 kDa of the protein marker. 40% ammonium sulphate precipitation and dialysis (partially purified) showed additional protein bands.

**b. Silver Staining Gel**

The purified RFP by GFC was subjected to SDS-PAGE stained with silver staining solution presented in Fig.9. The only one single RFP band was observed in purified RFP protein and in 40% ammonium

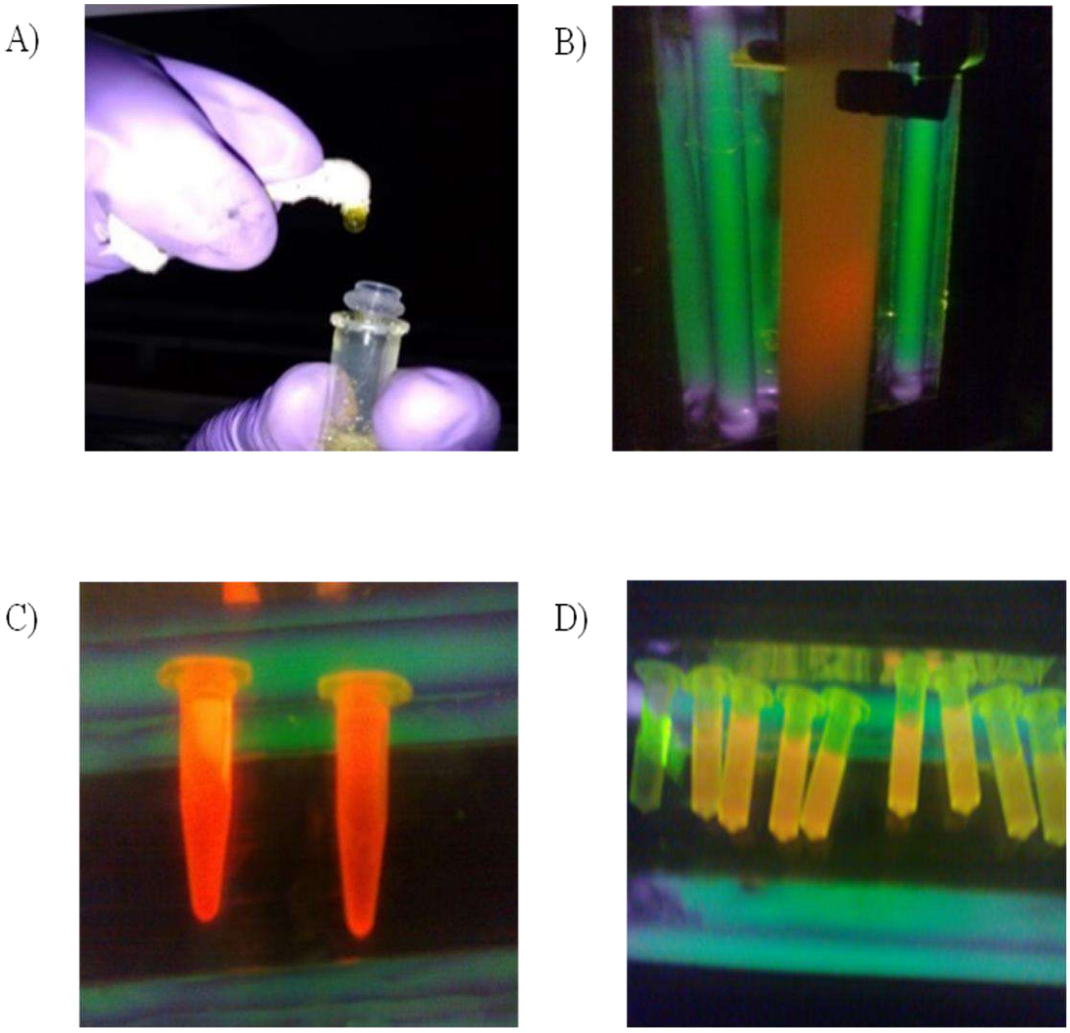


**Fig. 5A.** Collection of the midgut juice from silkworm *B. mori* L.

**Fig. 5B.** Purification of RFP by GFC under UV light.

**Fig.5C.** RFP eluted fractions by GFC were pooled for lyophilisation showed red fluorescence under UV light.

**Fig.5D.** RFP fractions eluted by GFC showed fluorescence under UV light.

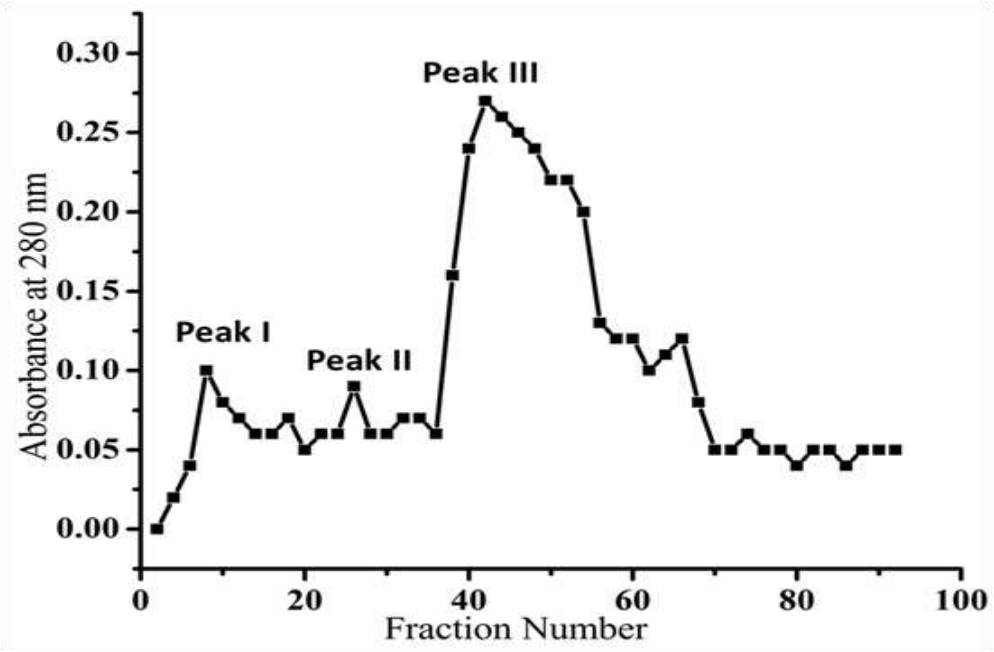


**Fig.6.** Elution RFP fractions profile of by gel filtration chromatography

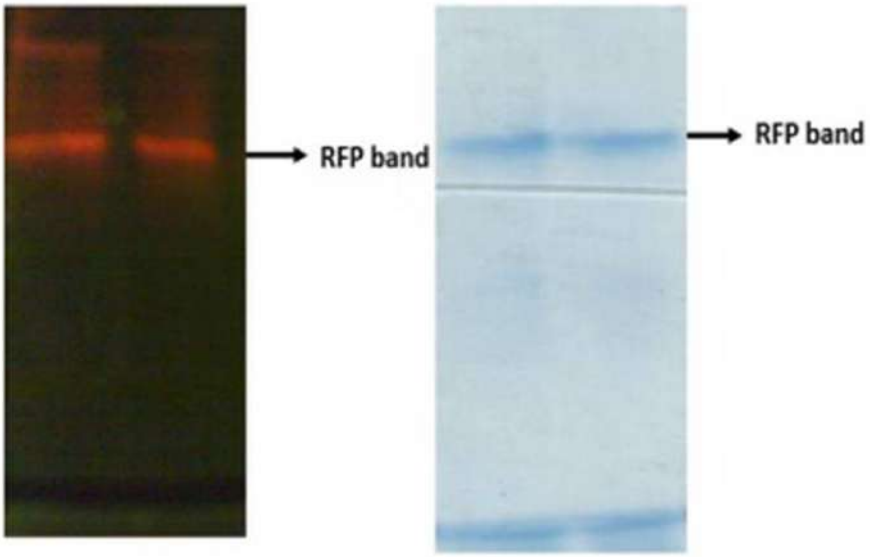


**Fig. 7.** Native PAGE separation of purified RFP .
(a) Fluorescent image of protein band under UV transilluminator.
(b) Electrophoretrogram after staining with CBB-R 250.

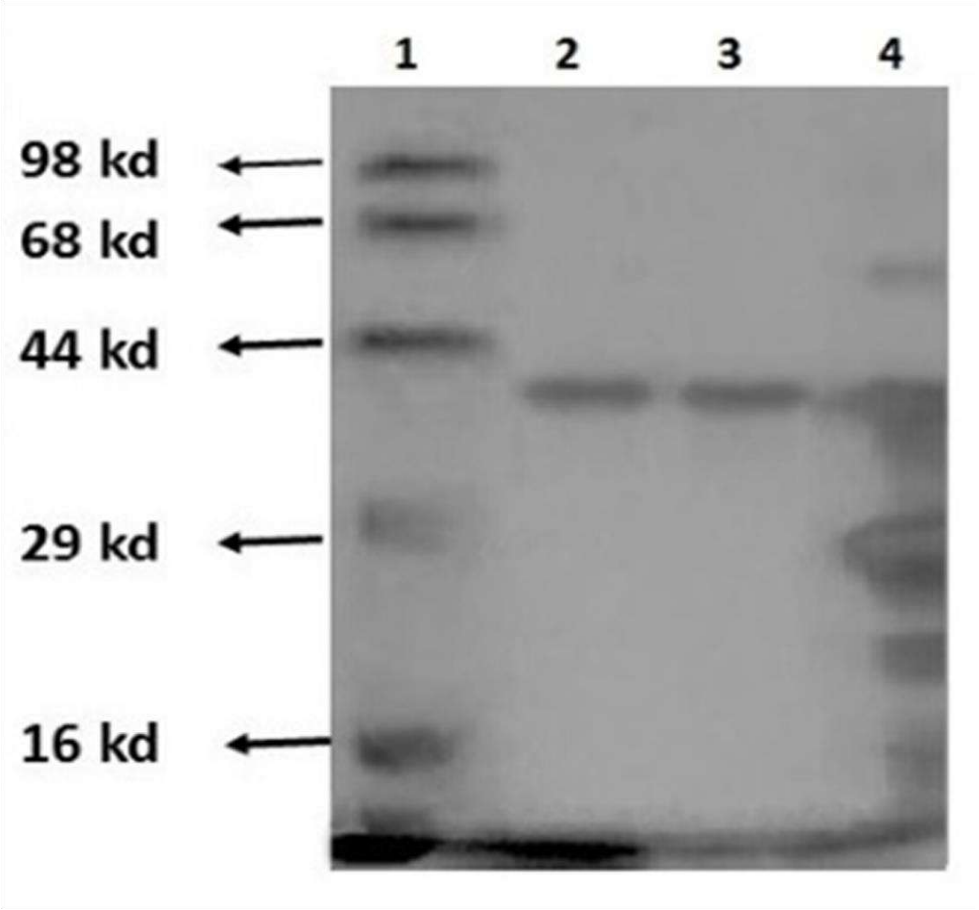


**Fig. 8.** SDS-PAGE was carried out using 12% polyacrylamide gel and stained with CBB-R 250.

Lane 1: Protein molecular marker .

Lane 2: and 3: Purified RFP from silkworm midgut.

Lane 4: Partially purified RFP from silkworm midgut.

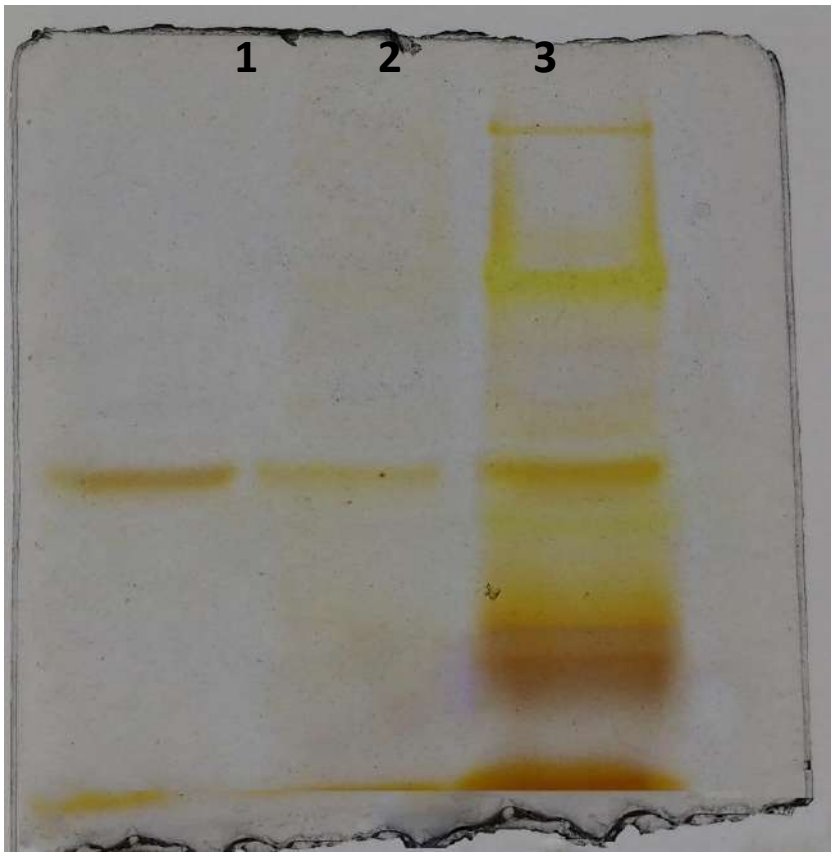


**Fig. 9.** Silver staining gel of RFP protein
Lane 1 and 2 : Purified RFP from silkworm midgut.
Lane 3 : Partially purified RFP from silkworm midgut

sulphate precipitation and dialysis (partially purified) shows additional protein bands.

### 4.3 Purity Analysis

#### 4.3.1 High-Performance Liquid Chromatography (HPLC) Analysis

To confirm the purity of RFP protein purified by GFC was further reconfirmed by HPLC. A single peak with a retention time of 8.755 min was obtained and it was shown in Fig. 10.

#### 4.3.2 Matrix-Assisted Laser Desorption/Ionization (MALDI) Time of Flight (TOF)/ Mass Spectra

MALDI-TOF/MS protein mass information acts as a means of analysis of the intact protein and represents the second dimension of protein separation within the vacuum phase of the mass spectrometer. The column purified RFP protein from the midgut digestive juice of silkworm produced a peak at a molecular mass of 40 kDa shown in Fig. 11.

#### 4.3.3 Bioassay Studies of RFP

**a. Antibacterial Activity**

The purified RFP protein was studied for its activity against Gram negative bacteria, *Escherichia coli,* and Gram positive bacteria, *Bacillus subtilis* (Fig.12). The purified RFP protein from the silkworm midgut showed antibacterial activity against the selected bacterial strains. The significant value of antibacterial activity of the RFP

protein has also been compared with Ampicillin as a standard antibiotic. Zone of inhibition is measured and results are expressed in centimetre (cm) and the results are presented in Table 1.

**b. Antifungal Activity**

The results of antifungal assays of the purified RFP protein are presented in Fig.13. The RFP showed that antifungal activity against fungus *Phytophthora meadii.* The mean radial growth of the fungus had reduced by 32.66, 41.66 and 54.55 per cent on third day and 20.33, 38.33 and 49.44 per cent on fifth day after inoculation at 10, 20 and 30 µg/ml of RFP respectively (Table 2). Thus RFP inhibited the growth of the fungus and no growth in control Metalaxyl.

**c. Antiviral Activity**

The antiviral assay of purified RFP against silkworm virus *Bm*NPV. Results pertaining to the per cent survival of the silkworms, orally administered with the NPV are shown in Table 3. In negative control (BSA + BmNPV and phosphate buffer + BmNPV) cent percent mortality was observed. Whereas in positive control (BSA+ phosphate buffer and phosphate buffer only) and untreated control cent percent of survivability was observed, where the larvae were fed with RFP + BmNPV there was eighty per cent survivability, which clearly demonstrate that the observed antinuclear polyhedrosis activity was only due to purified RFP.

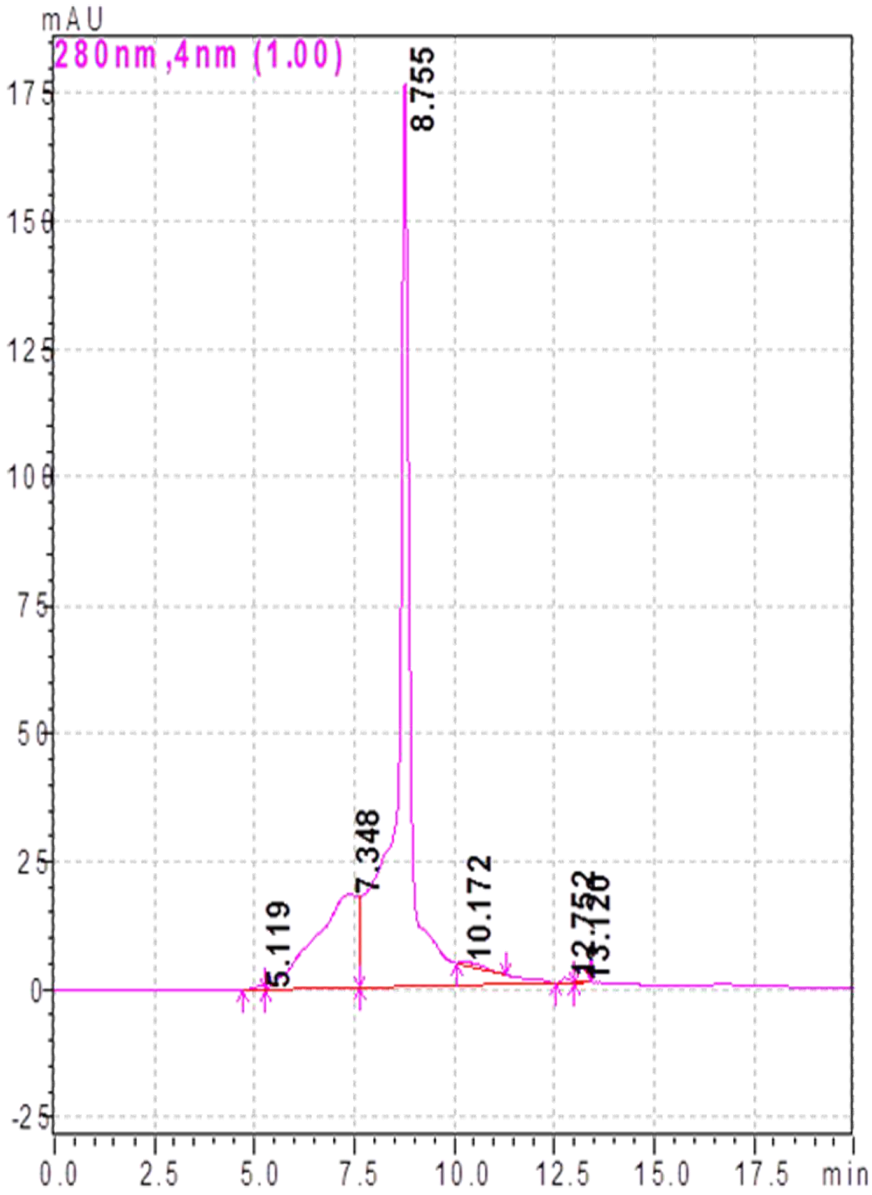


**Fig.10.** Analytical HPLC profile of Purified RFP.

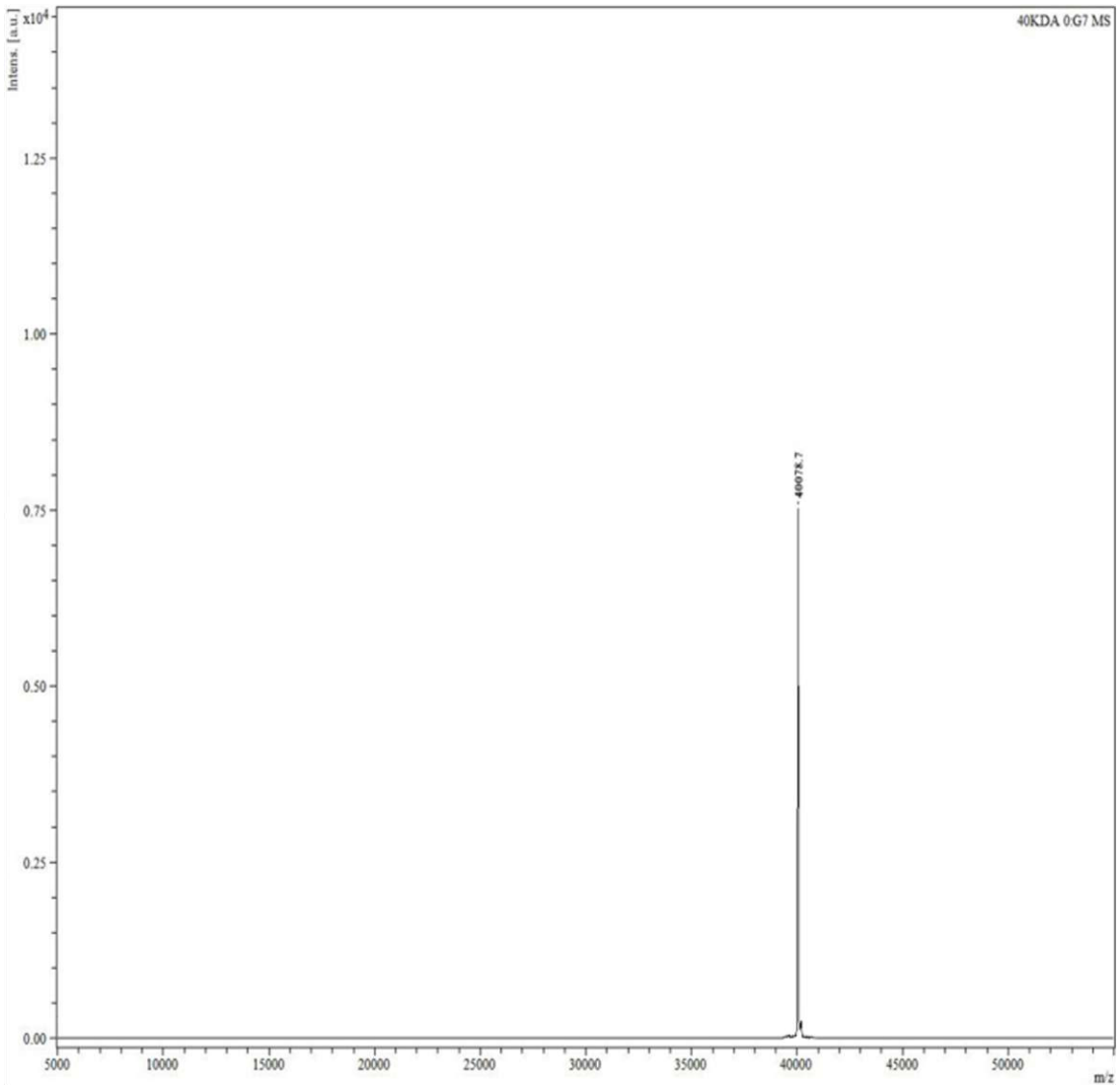


**Fig.11.**MALDI-TOF analysis of purified RFP protein was produced a peak at a molecular mass of 40 kDa.

**Table 1.** Inhibition of growth of different bacteria with purified RFP.

| | **Zone of Inhibition in cm** | | |
|---|---|---|---|
| **Compounds** | **Concentration used µg** | ***Bacillus subtilis* (ATCC Number 6633)** | ***E.coli* (ATCC Number 25922)** |
| Ampicillin | 25 µg | 3.0±0.00 | 3.2±0.00 |
| RFP | 25 µg | 2.1±0.00 | 2.4±0.00 |
| | 50 µg | 2.8±0.00 | 2.6±0.00 |

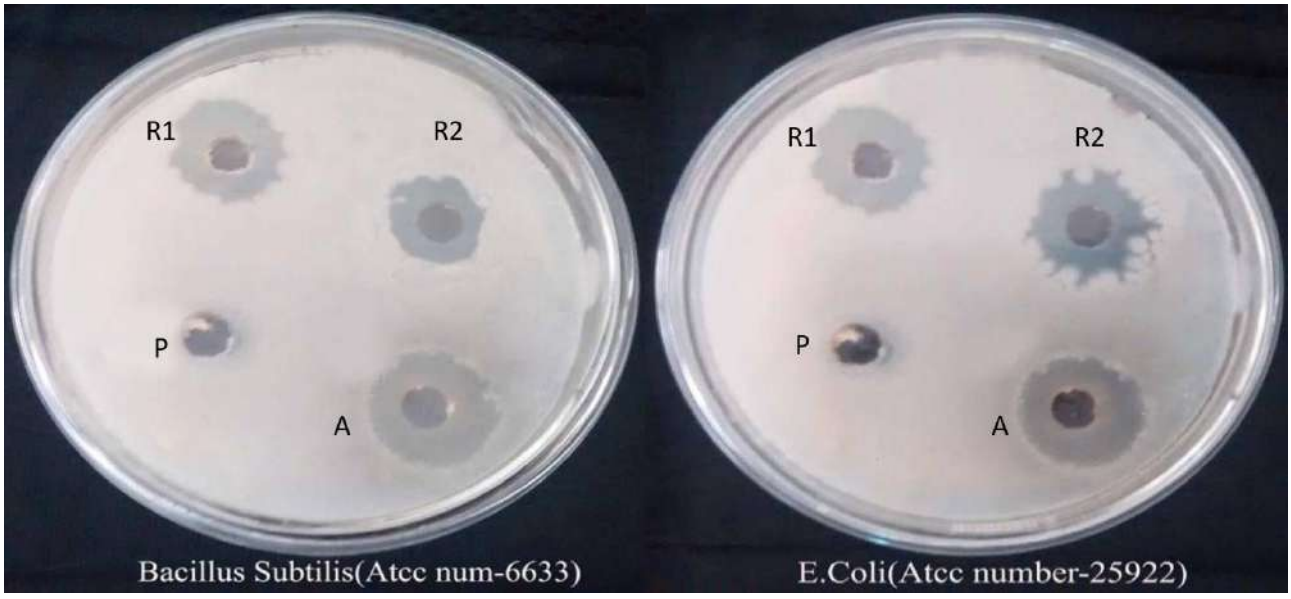


**Fig. 12.** Inhibition of growth of different bacteria with RFP.

**R1-**RFP concentration 25 µg. **R2-** RFP concentration 50 µg.

**A -** Ampicillin 25 µg. and **P-** Phosphate Buffer

**Table.2.** Inhibition of radial mycelia growth of *Phytophthorameadii* by purified RFP.

| Sl.No. | Fungus | RFP concentration (µg/ml) | Radial growth inhibition | |
|---|---|---|---|---|
| | | | **3rd Day (Mean ±SEM) in %** | **5th Day (Mean ±SEM) in %** |
| 1 | *Phytophthorameadii* | 10 | 32.66 per cent | 20.33 per cent |
| 2 | | 20 | 41.66 per cent | 38.33 per cent |
| 3 | | 30 | 54.55 per cent | 49.44 per cent |

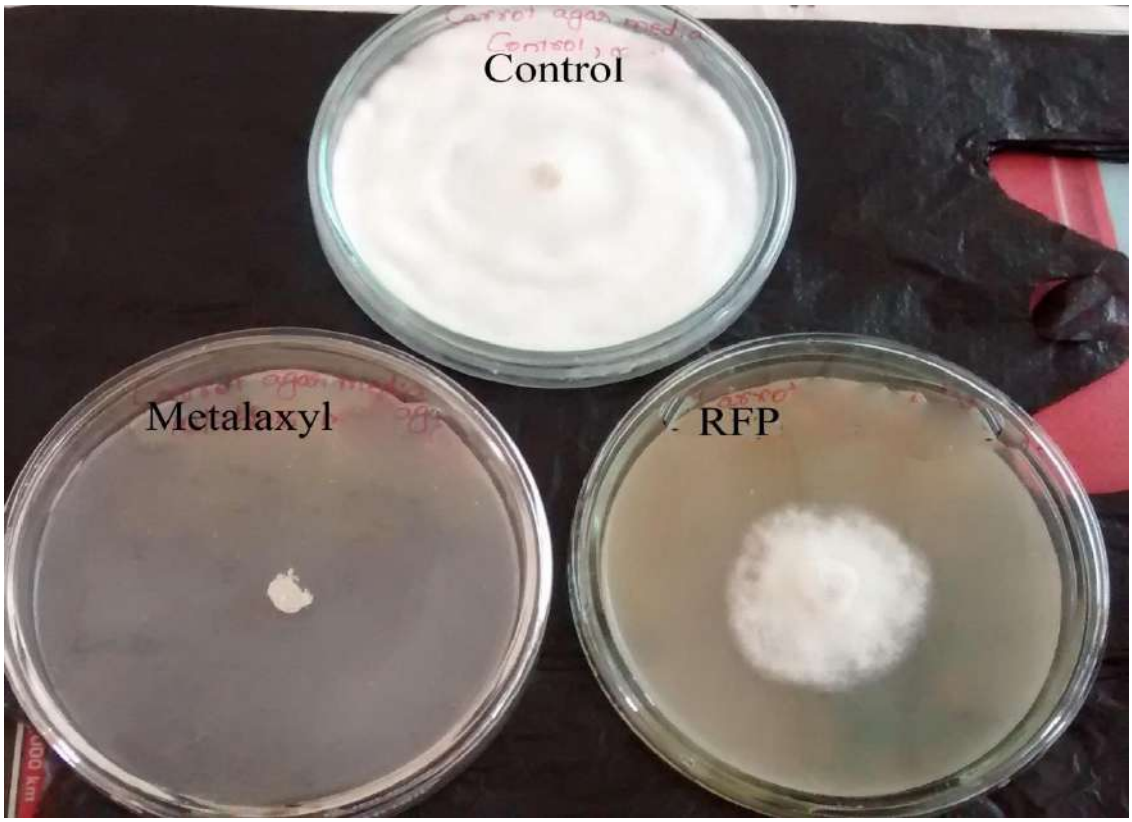


**Fig. 13.** Inhibition of radial mycelia growth of *Phytophthorameadii* by purified RFP**.**

**Table 3.** Antiviral activity of the RFP against *Bm*NPV.

| Treatments | Sets and no. of larvae | Total no. of larvae infected (% Mortality) | Total No of larvae survived (%of survived) |
|---|---|---|---|
| RFP + BmNPV | 3x10 | 6(44%) | 24(80%) |
| BSA+NPV | 3x10 | 30(100%) | 0 (0%) |
| Phosphate buffer (PB) + BmNPV | 3x10 | 30 (100%) | 0 (0%) |
| PB +BSA | 3x10 | 0 | 30 (100%) |
| Phosphate Buffer | 3x10 | 0 | 30 (100%) |
| Control | 3x10 | 0 | 30 (100%) |

### 4.3.4 N-terminal Sequence Analysis

The confirmed purified RFP band from SDS-PAGE was taken for amino acid sequencing using Edman degradation method and the obtained amino acid sequences were, Threonine (T), Glutamine (Q), Threonine (T), Isoleucine (I), Glutamic acid (E), Threonine (T), Aspartic acid (D), Tyrosine (Y), Tryptophan (W), and Valine (V) shown in Fig.14. To understand further its association with protein, using ten amino acids sequences, we have performed a BLAST analysis at NCBI to obtain a homology sequences and it predicted the chlorophyllide-a binding protein *(ChBP)* and showed its 100 per cent homology with *B. mori*. Our results indicated that sequenced 10 amino acids are matched with *ChBP* gene.

## 4.4 Identification and Molecular Characterization of *ChBP* Gene

### 4.4.1 RNA Extraction Analysis

The fifth day old fifth instar silkworm was selected and RNA was isolated and subjected to one percent agarose gel electrophoresis and its purity was confirmed, results shown in the Fig.15.

### 4.4.2 Amplification of Chlorophyll Binding Protein Gene

The cDNA was synthesized and used as the template for the amplification using ChBP gene specific primers and amplified product was analyzed using gel electrophoresis with 500bp DNA molecular weight marker. As seen in the figure there was the PCR

amplified amplicon found to comprise of around 900 bp is presented (Fig. 16).

## 4.5 *ChBP* gene Sequence and Bioinformatics Analysis

### 4.5.1 Sequence Analysis of *ChBP* gene Sequence

The PCR amplified product was sequenced (ABI 3500 XL Genetic Analyser sequencing machine) nucleotides of 911 bp and corresponding of these nucleotides encoding 302 amino acids were shown in Fig. 17. The sequence has been deposited in most accessible public database NCBI with the gene accession number KX186723.

### 4.5.2 *ChBP* Gene Analysis

To obtain a *ChBP* homology in silkworm, BLAST search was performed against to the silkworm genome keeping significant parameter in NCBI and the obtained hits with high sequence similarity with genes gi|507116244, gi|, gi|112883040, gi|827539957 and gi|827539959 *B. mori.* L presented in Fig.18.

### 4.5.3 Domain Analysis

Conserved Domain database at NCBI was used and predicted the domain present in the *ChBP* gene. The query sequence predicted the lipocalin domain. The result revealed that the *ChBP* gene belongs to the lipocalin gene family with the lipocalin-2 domain shown in Table.4 and in Fig.19.

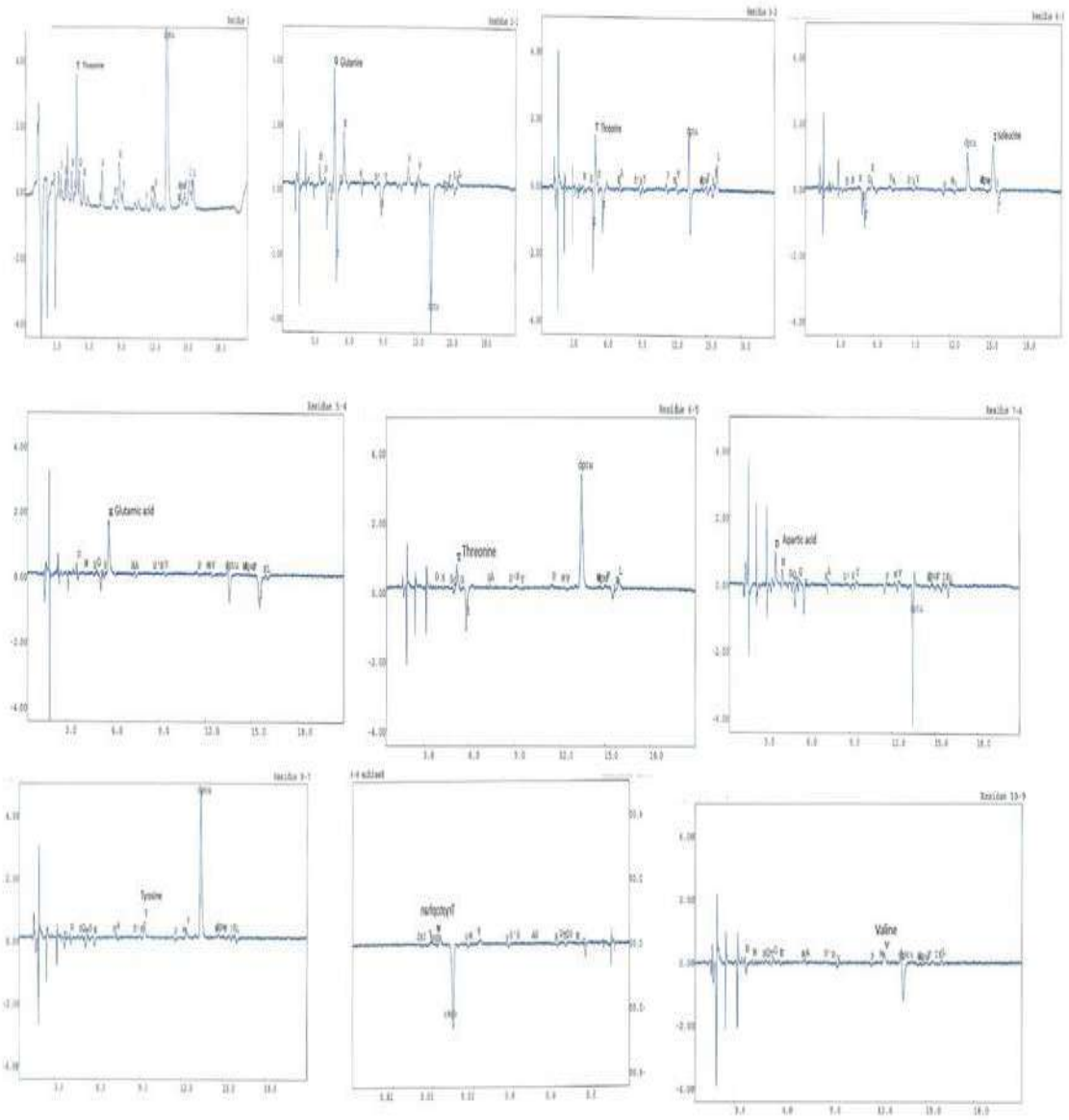


**Fig. 14.** The N-Terminal sequence of 10 amino acids of purified RFP (TQTIETDYWV).

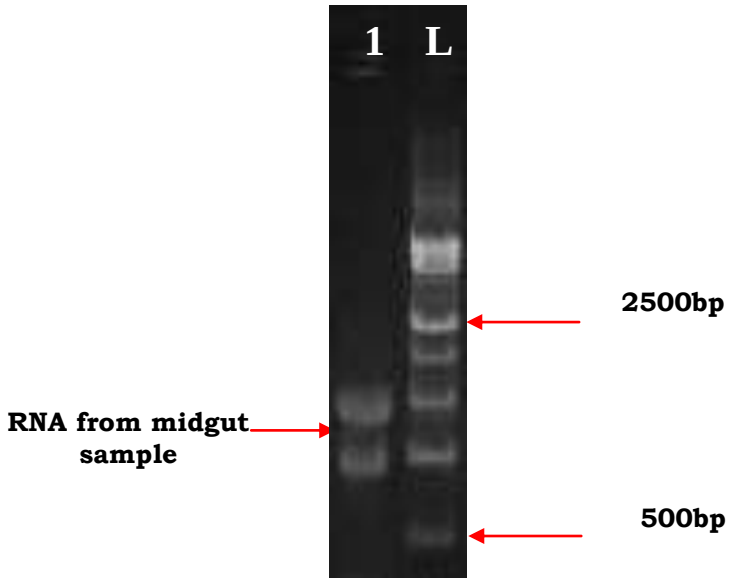


**Fig.15.** Denaturing agarose gel stained with ethidium bromide of total RNA isolated from silkworm *Bombyx mori* midgut.
Lane 1: RNA extracted from midgut sample.
Lane 2: (L) 500bpDNA Ladder

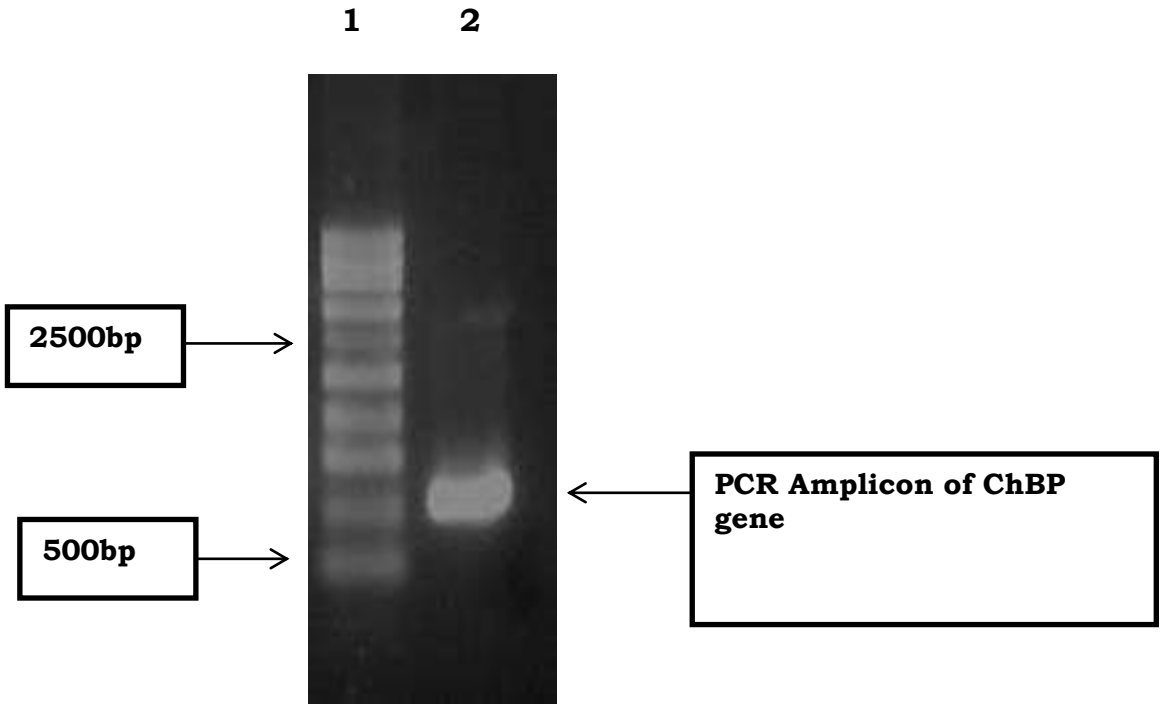


**Fig.16.** Agarose gel stained with EtBr of ChBP gene amplified by colony PCR confirming amplification.
Lane 1: 500bp DNA Ladder
Lane 2: PCR product of amplified ChBP gene.


**Fig.17.** Nucleotide and deduce amino acid sequence of ChBP of silkworm *Bombyx mori* Kolar Gold .

Pink highlight indicates the region (peptide sequence) obtained after N-terminal sequencing. The N-terminal sequence of the novel protein from silkworm midgut juice corresponds to the sequence following signal peptide.

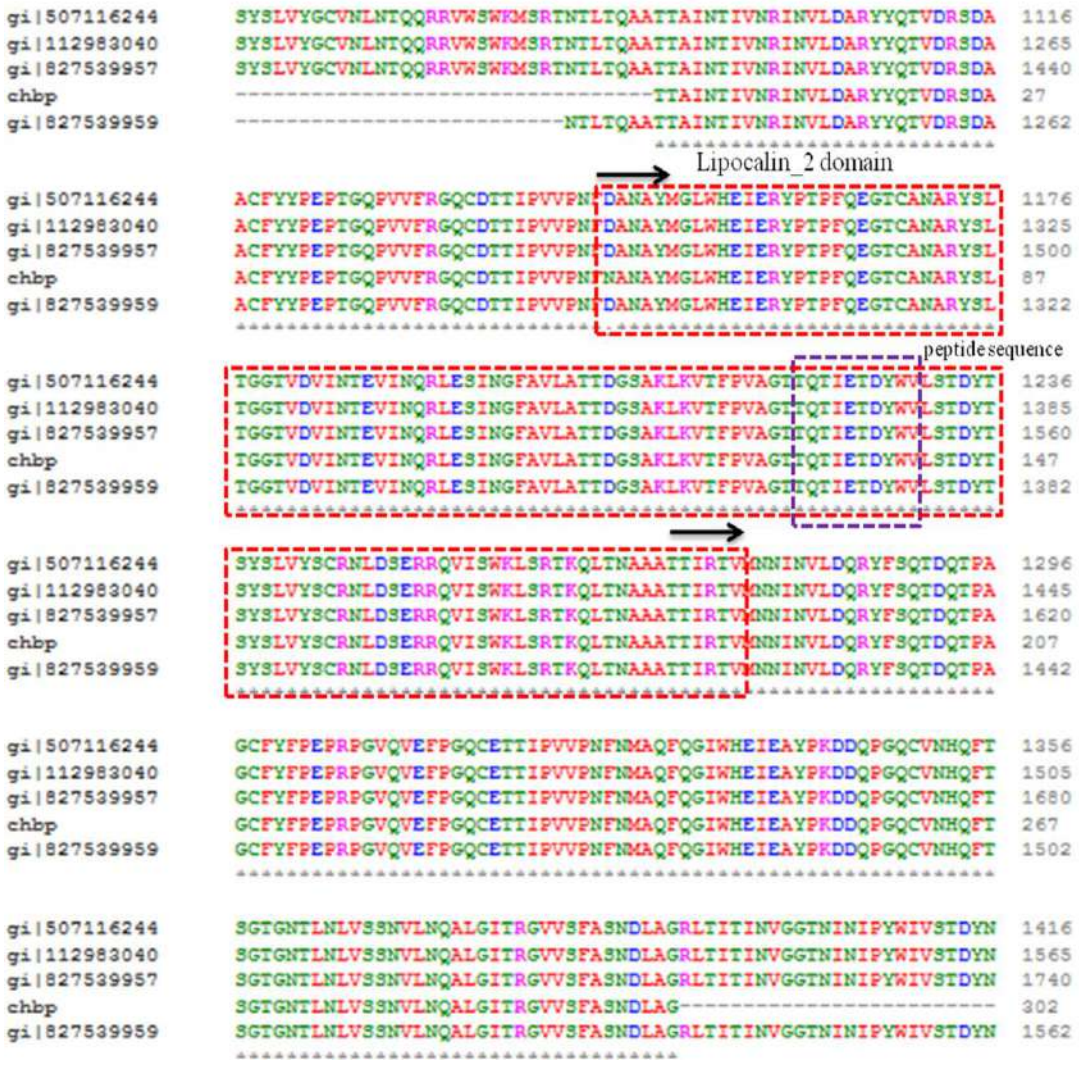


**Fig.18.** Multiple sequence alignment of deduce amino acid sequence ChBP and homologous genes from the silkworm *B. mori.*

Residues shared by several ChBP are indicated with the following symbols: *-denotes identical residues in all sequences, dashes indicate gaps in the alignment. Red dots box indicates starting position to end position of Lipocalin 2 domain, peptide Seq: TQTIETDYWV obtained after N-terminal sequencing.


**Fig.19** Domain Analysis of ChBP gene contains lipocalin protein family in Silkworm Kolar Gold breed.

**Table.4.** List of Protein Domains hits in the NCBI CDD

| **gi\|KX186723 Kolar Gold *Bombyx mori*.** | | | |
|---|---|---|---|
| **Name of the protein** | **Accession** | **Interval** | **E-value** |
| Lipocalin | cl21528 | 56-188 | 3.97e-17 |
| **gi\|827539959 Bombyx mori** | | | |
| | pfam00061 | 760-899 | 9.15 e-12 |
| Lipocalin | pfam00061 | 1118-1259 | 2.18e-07 |
| Lipocalin | pfam00061 | 219-335 | 1.16e-05 |
| Lipocalin | pfam00061 | 580-723 | 7.43e-05 |
| Lipocalin | pfam00061 | 1478-1592 | 1.48e-04 |
| **>gi\|827539957 Bombyx mori** | | | |
| Lipocalin | pfam00061 | 1298-1437 | 3.47e-12 |
| Lipocalin | pfam00061 | 760-899 | 9.77e-12 |
| Lipocalin | pfam00061 | 1118-1261 | 5.15e-06 |
| Lipocalin | pfam00061 | 219-335 | 1.26e-05 |
| Lipocalin | pfam00061 | 580-723 | 7.93e-05 |
| Lipocalin | pfam00061 | 1656-1770 | 1.58e-04 |
| **>gi\|112983040 Bombyx mori** | | | |
| Lipocalin | pfam00061 | 1123-1262 | 3.26e-12 |
| Lipocalin | pfam00061 | 585-724 | 9.16e-12 |
| Lipocalin | pfam00061 | 943-1086 | 4.02e-06 |
| Lipocalin | pfam00061 | 44-160 | 3.14e-05 |
| Lipocalin | pfam00061 | 405-548 | 7.44e-05 |
| Lipocalin | pfam00061 | 1481-1595 | 1.48e-04 |

### 4.5.4 Phylogenetic Analysis

To further understand the *ChBP* gene, we performed multiple sequence alignment of the *ChBP* and it's homologous in other insect genomes and constructed a phylogenetic tree using MEGA tool (Fig.20). The results showed that silkworm *ChBP* homologous found in 24 insect's genome including silkworm and phylogenetic analysis resulted into (Group I,II,III) and among them group III exhibited more diversity. According to the phylogenetic tree, the five *BmChBP*s fell into group I (gi/112983040-gi/827539959 with 98 % identity) followed by second highest homologs was found to Helicoverpa armigera insect with 97 % identity among them.

### 4.5.5 Homology Modelling of a ChBP Gene

The homology model of the ChBP was built based on the crystal structure of Insecticyanin-A from the tobacco hornworm (*Manduca sexta* L) template [1Z24] at 2.60 Å resolution having a QMean Z score of 23.88 and QMean score 4 of 0.5. The modelled residue range was from 15–208. The predicted structure was visualized using Raswin Molecular Graphics Version 2.7.5 (Fig.21).

## 4.6 Effect of Yellow Mulberry Leaves Feeds to Silkworm Larvae on *ChBP* Gene Expression.

The quality of RNA was confirmed by performing 2 % formaldehyde treated agarose gel electrophoresis presented in Fig 22 and used for *ChBP* gene expression analysis. Our results clearly indicates that

*ChBP* gene expression depends on the availability of a chlorophyll pigment which was confirmed in this study when larvae feed with only green leaves (T1) and its expression fold change value of 1.0. The larvae feed with only yellow leaves (T2) the *ChBP* gene expression level decreased with fold change value of 0.0989. However, in the larvae fed with yellow leaves followed by green leaves (T3) the *ChBP* gene expression level decreased to 0.6864 fold presented in Fig. 23.

## 4.7 Evaluation of Feeding Green and Yellow Mulberry Leaves on Growth, Development, Survival and Disease Prevalence.

### 4.7.1 Evaluation of Survival Rate of Silkworms

The survival and mortality rate of silkworms fed with yellow and green leaves of all the three batches, 1st batch (3rd to 5th instar) 2nd batch (4th to 5th instar) and in 3rd batch (5th instar) were observed and results are represented in Table 5. It was observed that more mortality rate in yellow leaves fed silkworm batches then green leaves fed batches.

### 4.7.2 Evaluation of Growth Rate

The growth performance of silk worms of all the 3 batches during the period in which green and yellow mulberry leaves were fed is presented in Table 6 and Fig. 24, 25, 26. The weight of the silk worm when fed with green leaves is significantly higher compared to silkworm fed with yellow leaves, in all the stages and of all the batches, except during 3rd instar of 1st batch and 4th instar of

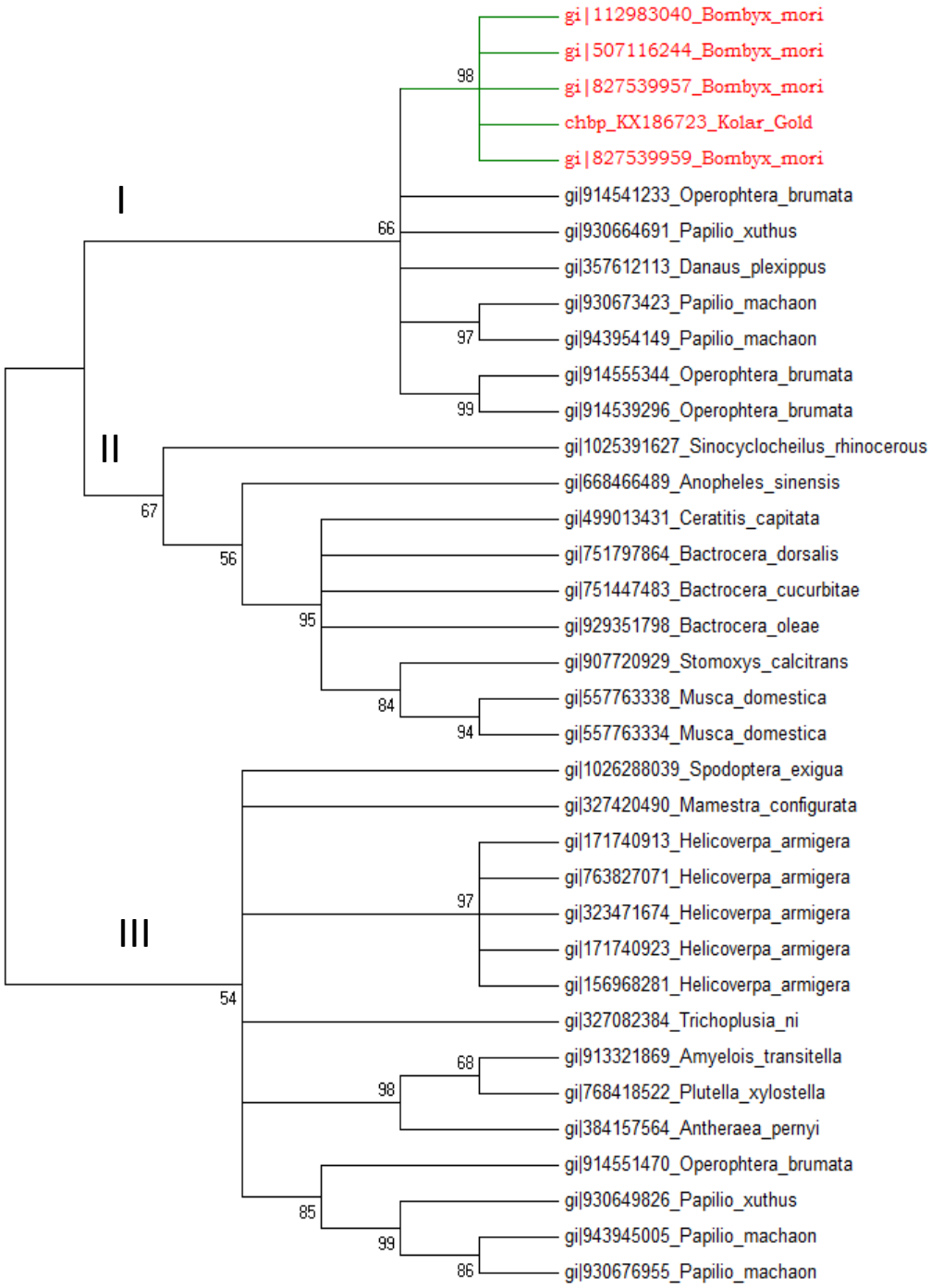


**Fig.20.** Phylogenetic tree of ChBP gene Silkworm *B.mori.* Kolar G

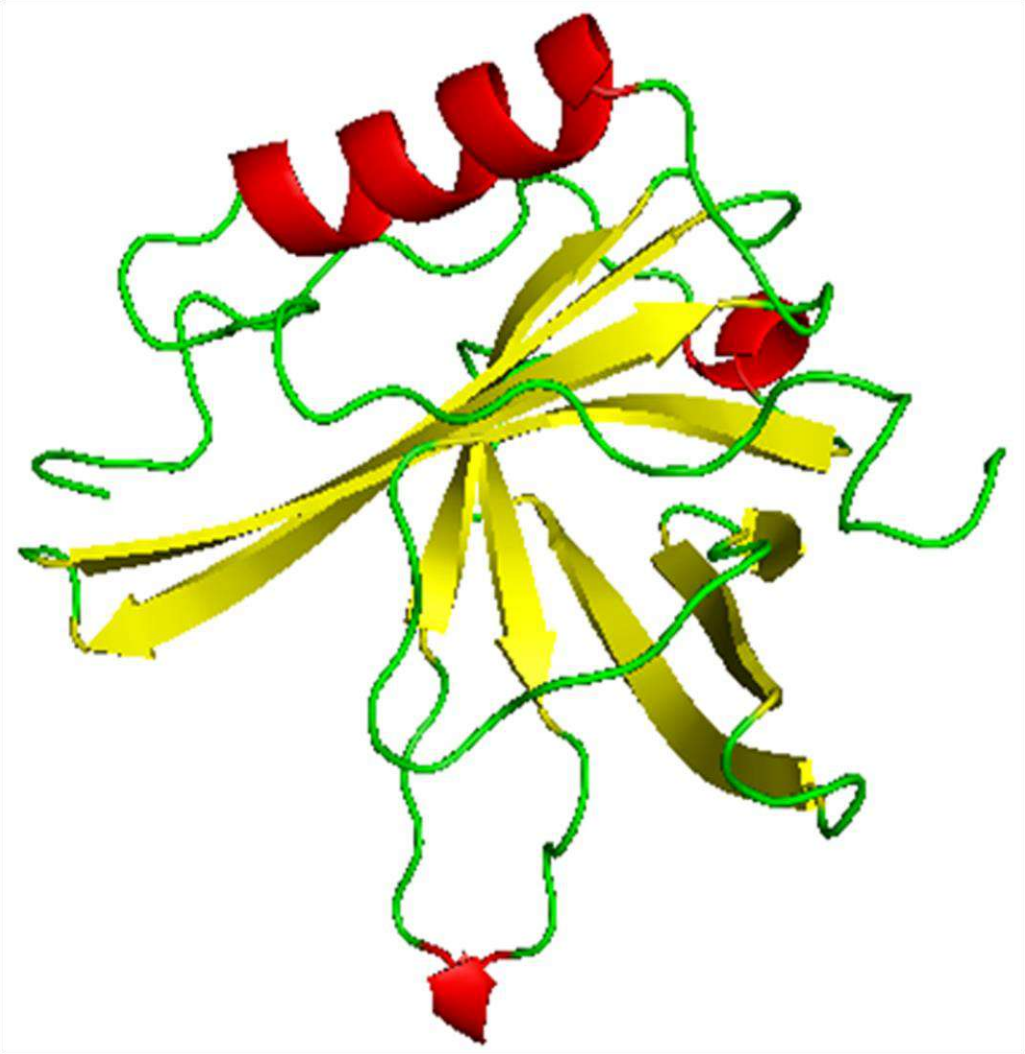

**Fig. 21.** Homology Modeling for the *ChBP* gene using SWISS MODEL at expasy.org.

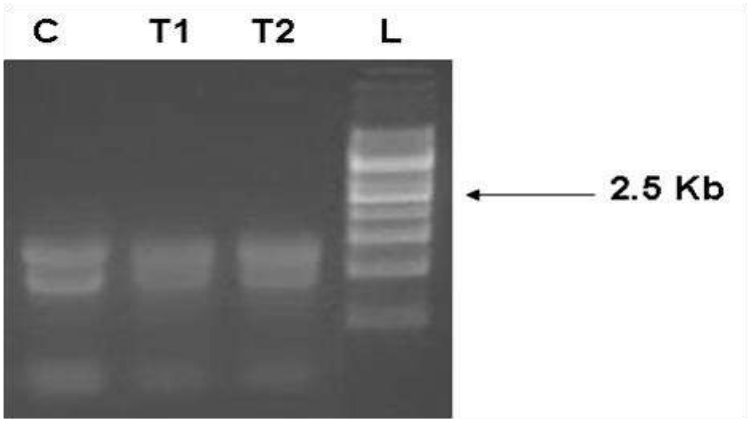


**Fig. 22. Formaldehyde treated agarose gel electrophoresis**

C- only green leaves fed silkworm larvae.

T1- only yellow leaves fed silkworm larvae.

T2- Two-day yellow leaves and three days green leaves fed silkworm larvae.

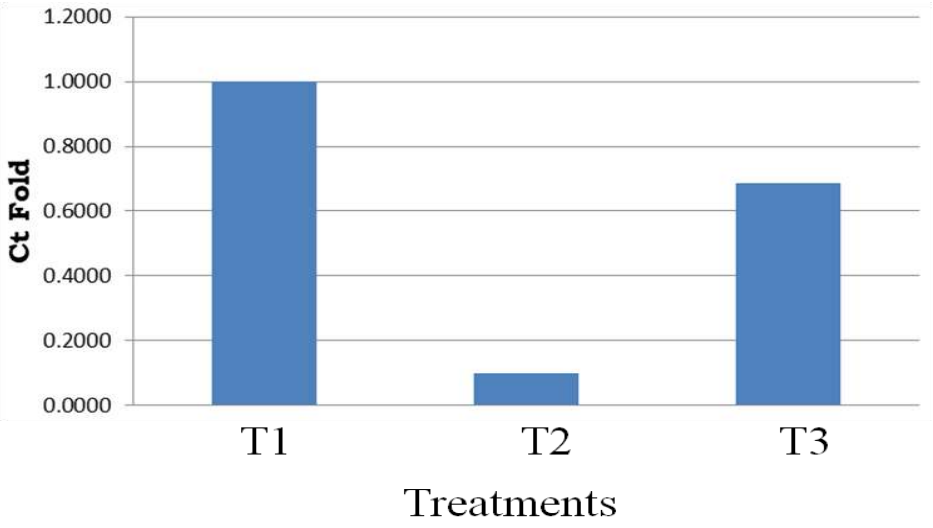


**Fig.23. ChBP gene expression analysis of silkworms fed with yellow leaves and control.**

**T1** - only green leaves fed silkworm larvae.

**T2 -** only yellow leaves fed silkworm larvae.

**T3 -** Two-day yellow leaves and three days green leaves fed silkworm larvae.

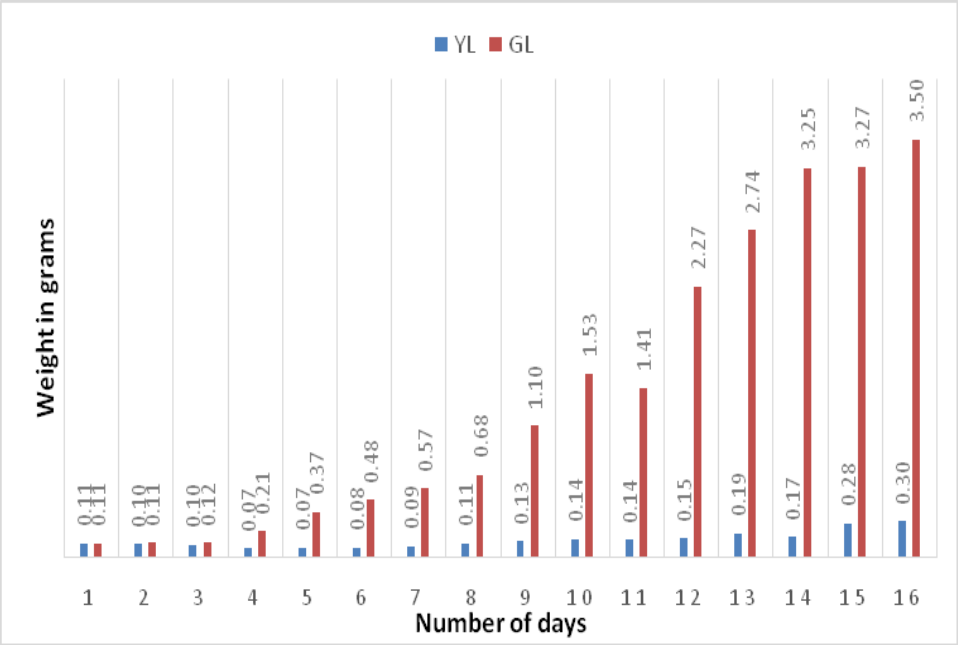


**Fig.24.**Weight of 3rd to 5th instar silk worm fed with yellow & green leaves.

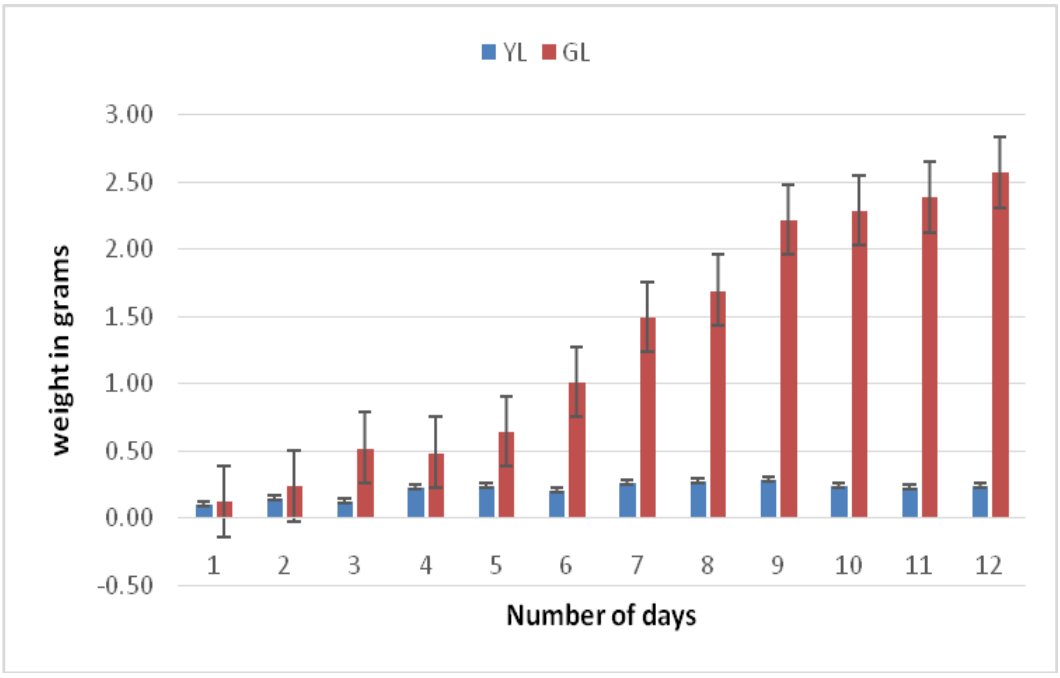


**Fig.25.** Weight of 4th to 5th instar silk worm fed with yellow & green leaves.

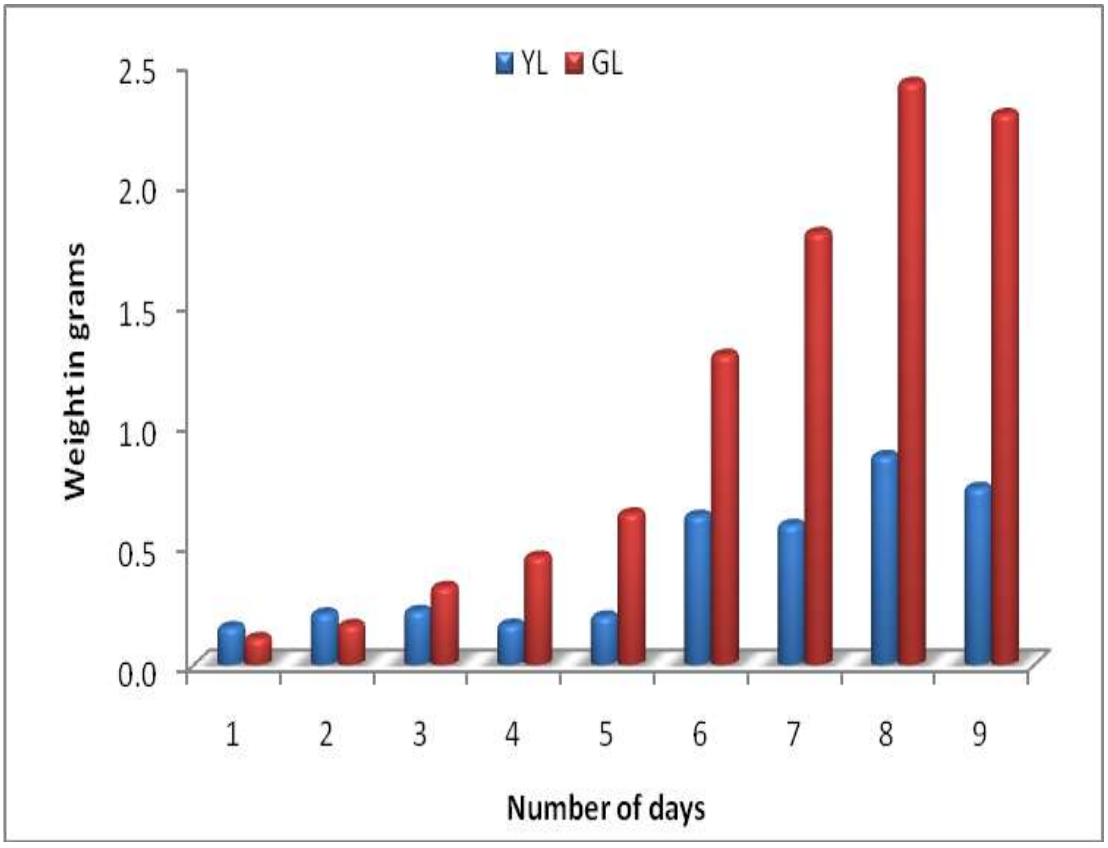


**Fig.26.** Weight of 5th instar silk worm fed with yellow & green leaves.

**Table.5.** Percentage (%) of survival rate while treatment with yellow and green leaves

| Batch/Instar | Mulberry leaves | Total no worms/3rep | % of larvae survived | % of mortality |
|---|---|---|---|---|
| 1st /3rd -5th | Green leaf | 90 | 95.5 | 4.44 |
| | Yellow leaf | 90 | 0 | 100 |
| 2nd /4th-5th | Green leaf | 90 | 94.44 | 5.55 |
| | Yellow leaf | 90 | 11.11 | 88.88 |
| 3rd / 5th Instar only | Green leaf | 90 | 96.66 | 3.33 |
| | Yellow leaf | 90 | 55.55 | 44.44 |

**Table. 6.** Comparison of means: Growth performance of silkworm fed with yellow & green leaves.

| Batch | Instars | Yellow leaves | Green leaves | 't' statistic | Pr<t |
|---|---|---|---|---|---|
| 1st batch | 3rd | 0.10 | 0.14 | -1.62 | 0.18 |
| | 4th | 0.09 | 0.53 | -6.58 | 0.01 |
| | 5th | 0.19 | 2.38 | -6.56 | 0.00 |
| | 3rd -5th | 0.14 | 1.36 | -3.90 | 0.00 |
| 2nd batch | 4th | 0.16 | 0.35 | -1.86 | 0.14 |
| | 5th | 0.25 | 1.79 | -6.23 | 0.00 |
| | 4th -5th | 0.22 | 1.31 | -4.14 | 0.00 |
| 3rd batch | 5th | 0.42 | 1.05 | -1.98 | 0.08 |

2nd batch. The weight of silkworms fed with green leaves increased gradually as the age of larvae increased in all the batches compared to that of yellow leaves fed worms. The silkworm fed with yellow leaves was not survived at the end of fifth instar and not able to form cocoons.

# DISCUSSION

For the present study the silkworm *Bombyx mori* L (Kolar Gold) breed was selected and examined for the RFP identification, purification, its biochemical characterisation, molecular characterization and to measure its expression levels. The results of the study on "**Biochemical Characterization of Antiviral Red Florescent Protein (RFP) Isolated from Silkworm *Bombyx mori* L.** are discussed.

## 5.1 Isolation and Purification of Red Fluorescent Protein (RFP) from the Silkworm

The midgut fluid collected from the silkworm, RFP was purified by GFC, three peaks were obtained, and one elution profile (Fractions 30-60) has shown maximum absorption at 280 nm and glow red fluorescence under UV light, while monitoring under UV light its elution was made easy to collect its fraction. Its red fluorescence is may be due to binding of chlorophyllide-a, the prosthetic group of the chlorophyll released from the action of Chlorophyllase. Similar findings have been reported by the earlier studies too (Sunagar et al., and 2011: Suresh et al., 2007: Sethuraman et al., 1993: Hou and Chiu, 1986: Uchida et al., 1984).

The electrophoretic profile of the purified RFP protein by Native-PAGE showed one RFP band under UV light and in SDS-PAGE an RFP

band that corresponds to 40 kDa as shown. Many earlier researchers reported that, the RFP present in silkworm *Bombyx mori* ranged from 14 kDa to 1100 kDa (Asish et al., 2014: Sunagar et al., 2011: Matti et al., 2009: Suresh et al., 2007: Sethuraman et al., 1993: Hou and Chiu, 1986: Uchida et al., 1984). These varied molecular weight RFPs present in the digestive juice of the silkworm exhibits anti NPV protection (Yao et al., 2009: Pandian et al., 2008: Mauchamp et al., 2006: Nakazawa et al., 2004: Ponnuvel, 2003: Sethuraman et al., 1993). Similarly, the molecular weight of RFP found in silkworm excreta ranged 25 to 1,100 kDa (Hiraki et al., 1997: Neelgund et al., 2007: Matti et al., 2009). Aizawa et al. 1962 first reported the antiviral activity of RFP against *Bm*NPV. It was reported that RFP could be obtained *in vitro* from the midgut juice and bind to Chlorophyllide-a, a prosthetic group of chlorophyll (Chl)- a (Hayashiya et al., 1976).

The polypeptides of the midgut juice of silkworm may bind to tetra pyrrole pigment moiety of chlorophyll. The multivoltine and bivoltine silkworm races posses many RFPs with different molecular weights (Sunagar et al., 2011) which supports the fact that the protein isolated in this study is perhaps a novel RFP of Kolar Gold silkworm.

Sunagar et al. (2007) studied on the midgut juice of the Pure Mysore race and showed three heterogenic RFPs with different molecular weights with tetra pyrrole moieties.

The RFP eluted from the SDS-PAGE was subjected to MALDI-TOF-MS analysis. The molecular weight yields 40 kDa in agreement with Sunagar et al. (2008), they also identified antiNPV RFPs eluted from the PAGE and subjected to trypsin digestion followed by MALDI-TOF-MS analysis. The RFP-PM1 yielded molecular weight values of 51 kDa and 43 kDa.

The purified RFP was subjected to N-terminal sequence analysis and obtained the TQTIETDYWV amino acid sequence. The sequence analysis at publically available database search showed its homology with the silkworm Chlorophyllide-a Binding Protein (*ChBP*) and is belonging to the lipocalin family, known to be involved in many physiological functions like transportation of various hydrophobic molecules like bilins, retinoid, lipids and steroid hormones, and also one of the component of pigmentation colour of the larva ubiquitously present in both prokaryotes and eukaryotes (Grzyb et al., 2006).

## 5.2 Antimicrobial Activity of RFP Protein

India is the second largest producer of silk and created a global attraction. In India silk industry suffers a loss upto 30-40 % is mainly because of disease caused by virus, bacteria and fungi, etc. The large proportion of which is attributed to *Bm*NPV and minor forms of bacterial flacherie, septicaemia and toxaemia caused by bacterial diseases (Ponnuvel et al., 2003).

To combat various microbial infections *Bombyx mori* evolved a genetic resistance or tolerance. *Bm*NPV belongs to Bacuolviridea family, a double stranded virus infect silkworm mainly by oral infection (Jiang et al., 2014).

The viral particles are embedded in proteinaceous occlusions of the larval midgut, by the action of alkaline pH of the gut, virus particles released into the larval mid gut. During infection the insects may activate expression of certain antiviral resistance genes (Nobiron et al., 2003).

In the present investigation a novel secretory protein present in the midgut juice bound to chlorophyllide-a, a prosthetic group of chlorophyll that acquire a red fluorescence property is purified. Many RFP proteins were characterised in the silkworm midgut and faecal matter have shown antimicrobial properties (Aiazwa, 1962, Haysha et al., 1969: Matti et al., 2009: Patnaik et al., 2012: Neelgund et al., 2007: Sunagar et al., 2011).

Earlier researchers reported the antibacterial property of RFP which is mainly attributed to the chlorophyll binding to *ChBP* (Aizwa, 1962: Hayashia, 1978: Ponnuvel et al., 2003: Nakazawa et al., 2004).

The purified RFP exhibited against antibacterial activity against *E. coli and B. Subtilis.* The MIC of the purified RFP concentrations is high in 50 µl compared with 25 µl against bacterial strains *E. coli* and *B. subtilis*, the earlier reports also showed inhibitory characters of RFP.

Patnaik et al.(2012).SE-RFP showed the MIC value of the purified protein was low against most of the bacterial strains tested at 2.5 mg, 5 mg and 10 mg of the purified protein was sufficient to inhibit considerable proliferation of *P. entomophila* and *A. hydrophila, B. subtilis and S. marcescens, S. enterica* and *P. rhodesiae* respectively. The SE-RFP reported earlier also showed growth inhibition of the pathogenic bacterial strains and the MIC values were in the range of 7.5–25 mg/ml. The SE-RFP activity was high against bacterial strains viz. *S. aureus, K. pneumoniae* and *E. coli* (Matti et al., 2009). In another report of purification of 35 kDa proteins from faecal extract, antibacterial activity was observed at 50 µg and the MIC against clinical strains viz. *S. hemolyticus, S. aureus, S. typhi, P. aeruginosa, B. subtilis* and *P. aeruginosa* were observed at 30 mg.

The 252 kDa isolated from silkworm Bombyx mori bind to 1Aa, Cry 1Ab and Cry 1Ac toxins of B. thuringiensis and also proved the antibacterial property of midgut membrane protein P252 (Pandian et al., 2008) . They believed that the antimicrobial property may be due to the generation of stable radicals.

The purified RFP exhibits antifungal activity also, the RFP showed that antifungal activity against fungus *Phytophthora meadii.* The results of the earlier studies reported the (Patnaik et al., 2012) antifungal assays of the purified proteins, they also indicated high antifungal activity against fungal strains viz. *F. solani* and *F.*

*oxysporum*. Antifungal assay for SE-RFP showed a good activity against *C. albicans* and *A. flavus*, whereas activity was low against *A. Niger* as observed from the MIC values.

### 5.3 Antiviral Activity of RFP

The treatment of NPV with RFP significantly reduced the mortality (80%) compared to the control (100%) in the bioassay studies in an agreement with the earlier reports (Suresh et al., 2007: Funakoshi and Aizawa, 1989: Sethuraman et al., 1993).

The mode of action of the RFP on BmNPV has not been understood completely. Several reports suggest that the protein to be an enzyme like a protease (Datta et al., 2000: Funakoshi and Aizawa, 1989). The RFP is also thought to have phospho lipase activity (Hou and Chiu, 1986). Uchida et al. (1984) have opined that protein has both virus precipitating and inactivating properties.

The isolated protein (RFP) had precipitation and neutralization characteristics on the virus and it was also thought to have properties similar to serum antibody produced against viruses in vertebrates. However, since these proteins were found to destroy the virus and act on the nucleocapsid, the protein was thought to be more an enzyme than a protein similar to mammalian immunoglobulin. Mukai et al. (1969), suggest the RFP to be an enzyme an intermediary constituent of oxide reduction system in or not in relation to digestion mechanism of the silkworm larvae. Funakoshi and Aizawa (1989) isolated three

fractions fI, fII and fIII from silkworm gut extract, of these fIII is found to have the highest antiviral activity against the non-occluded BmNPV appeared to be a protease, while f1 which was probably the RFP effective in inactivating the occluded BmNPV or the polyhedral.

The BmPNV protein also inactivated NPV of *Antographacalifornica* (AcNPV) Bromo Mosaic Virus (BMV) of wheat and New Castle Disease Virus (NDV) of vertebrates, as reported by Uchida et al. (1984). A virus inactivating protein L4-1 with molecular weight of 530 has been isolated from silkworm faeces (Hirayama et al., 1983), and (Hiraki et al., 1997) which inactivates enveloped viruses like HJV, Herpes Simplex Virus type I (HSV) and Human Immuno-Deficiency Virus (HIV) (Hiraki et al., 1996).

From the above studies we can conclude that RFP has antiviral activity and it is an important antiviral protein present in the digestive juice of silkworm.

### 5.4 *ChBP* Gene and Sequence Analysis

*In-silico* analysis of *ChBP* gene revealed many insights at sequence and structure level. The *ChBP* homology search of Kolar Gold sequence predicted the homologous with varied sequence length for genes of *ChBP* (till date) with more than 90 per cent sequence similarity to the *B. mori* genome.

The domain analysis of *ChBP* sequence predicted lipocalin domain found in the position from 71-202 amino acids with total length of 140

amino acids and belongs to the Lipocalin super family. The other *ChBP* sequences predicted similar lipocalin domain with varied locations but with fixed length (140 amino acids) of domains. The domains analysis of the presently available *B. mori* full length *ChBP* genes (4 *ChBP* genes) in public domain revealed the varied highly conserved, similar length lipocalin domain and belongs to the lipocalin super family.

*ChBP* of Kolar Gold revealed that its structure contains eight-stranded anti parallel beta sheet, these pattern of domains found in lipocalin and are known to be involved in transport, mediation of cell homeostasis and immune response respectively (Flower 1996). Earlier work on B Mori carried out by Muschamp et al., (2006) revealed the complete gene sequences which posses 45.5 kb of nucleotide bases that contains 46 exons and deduced amino acids 2721. He has shown pentadecalin lipocalin domain and attributed as Chlorophyllide Binding Protein (*ChBP*). In the present study the *ChBP* phylogenetic analysis revealed its homologous feature among the other group of insects.

### 5.5 Effect of Yellow Fed Leaves on Expression of ChBP gene

Induction and expression of ChBP gene may depend upon the availability of a green chlorophyll pigment and its prosthetic group chlorophyllide-α. Previous reports shows that the purified RFP's from

silkworms are mainly fluorescence because of bound prosthetic group chlorophyllide-α.

Based on the N terminal sequences of the purified RFP from the Kolar Gold has identified and confirmed that it belongs to the lipocalin protein family.

The silkworm, *B. mori* midgut secretory protein Chlorophyllide-a binding protein exhibits red fluorescence was identified in the midgut epithelial cells of silkworm (Chen et al., 2014).

The specific chlorophyll derivative has conferred the red fluorescence property to the RFPs purified from the silkworm (Nakazawa et al., 2004: Mauchamp et al., 2006: Sunagar et al., 2011). In 2006, ChBP was identified from the silkworm midgut and its red fluorescence was retained even after 2D electrophoresis as Chlorophyllide-α is covalently bound to the protein (Mauchamp et al., 2006).

The N-terminal sequence of the purified RFP provide us the base to identify the gene associated with it, *in silico* analysis predicted the presence of intact ten amino acids and highly conserved *ChBP* of *B. mori*, supported by *ChBP* gene expression analysis confirmed by Chen et al 2014. However none of the earlier studies have reported on feeding effect of mulberry green or yellow leaves on the expression level of *ChBP*. To move towards the conclusion the present investigation confirms the induction of *ChBP* gene require green

pigment (chlorophyll-a) of mulberry leaves, further it shows the 10 fold down regulation of *ChBP* in yellow leaves fed silkworm.

Different isoforms of chlorophyllide binding protein may be expressed in silkworm and among them one may be purified RFP.

## 5.6 Evaluation of Feeding Green and Yellow Mulberry Leaves on Growth, Development, Survival and Disease Prevalence.

The present study confirmed that the yellow leaf fed silkworms growth rate, nutrition content and also their survival rate were comparatively less over green mulberry fed worms. The yellow leaves treated worms were susceptible to infectious diseases and died, so the results have shown a decrease in the biochemical parameters and growth rate in yellow leaf fed worms i.e similar to the earlier studies (Siraj et al., 2007) which have also reported the same results with BmDNV1 infection in CSR2 breed silkworm. The cent percent mortality was observed in the silkworms that were fed with yellow mulberry leaves.

## FINDINGS AND CONCLUSIONS

In this chapter, the important findings, conclusion and future perspective of the work have been summarized. Current research work entitled "**Biochemical Characterization of Antiviral Red Fluorescent Protein (RFP) Isolated from Silkworm *Bombyx mori* L.”**.

India is the second largest producer of silk, next to China. India produces a variety of silks, declining its production due to primarily by diseases infection by *B. mori* Nucleo Polyhedrosis Virus (BmNPV). There are three types of silkworm races which are classified based on voltinism viz. univoltine, bivoltine and polyvoltine. From 1960 onwards, many improved polyvoltine breeds were developed and have been reared for the production of silk. Kolar Gold a hybrid Polyvoltine breed developed by crossing CSR2 with Pure Mysore breed (CSR2 X PM). This breed has been regularly reared because of its high yield in all seasons, tolerance to diseases, yield good quality silk and also the oblong shape of its cocoons attracted the rearing farmers and silk industry. In Karnataka, particularly in Kolar and Chikkaballapur Districts, Kolar Gold silkworm breed has been regularly rearing in all the seasons. Because of the acceptance of this breed by the farming community, its natural viral tolerance and also it is vital to improve the breeds by developing new hybrids which

results in enriching the economy of the farming community and leads to self-sustainable in the production of silk and boost the country's economy.

The main natural viral tolerance acquired by silkworm is mainly due to the expression of Red Fluorescent Protein (RFP) in the Midgut. To understand its tolerance, the midgut juice was collected from Kolar Gold silkworm larvae, its RFP protein was purified by applying biochemical techniques such as GFC, exhibits red fluorescence, subjected to native PAGE and confirms its red fluorescence, its molecular weight determined by SDS-PAGE that was 40 kDa. The results found were also match up the fluorescence of the purified various RFPs present in silkworms which were reported by previous research reports.

The purified RFP was further subjected to find out its N-terminal sequences by Edman degradation, found 10 amino acids TQTIETDYWV. The obtained peptide sequence was subjected to publicly available databases search, it hits a group of proteins belonging to *ChBP* and by that time the same was awaited. Further, applied bioinformatics approaches unearth the related genes and proteins it belongs, made us to raise the question does it belongs to the red fluorescence proteins and/or *ChBP* lipocalin family?

Based on the N-terminal RFP sequences, bio-informatically predicted the *B. Mori ChBP* genes, the gene was identified and

interpreted and designed the forward primer 5'-GCTGGAAGATGAGCAGAACGAACAC-3' and reverse primer 5-GTTGTTATAATCGGTGCTCACGATCCAG-3' Henceforth, the mature silkworm larval midgut was dissected and utilized for total RNA extraction and was used for cDNA synthesis using Oligo (dT) primer. By using the above said primers, the *ChBP* gene was amplified. The PCR amplified amplicon was sequenced and the obtained sequences were searched against NCBI database using BLAST search tool to obtain the relevance of genes sequences. The obtained sequence hits speculate the amplified amplicon nucleotide sequence belonging to the *ChBP*, a member of the lipocalin family.

Lipocalin proteins ambiguously distributed from bacteria to human and many of them are involved in various physiological functions and exhibit vast diversity at the amino acid sequences.

The PCR amplified amplicon contain the nucleotide "ACACAAACAATCGAAACTGACTACTGGTTTT" sequence corresponds to the 10 N-terminal amino acid sequences "TQTIETDYWV". By applying conserved domain database at NCBI using Kolar Gold *ChBP* sequences, it predicted domain of lipocalin with 140 amino acids and highly conserved with eight beta barrel anti parallel sheet of other B. mori *ChBP* genes and it belongs to the protein lipocalin super family.

Comprehensive literature reports revealed the bound chlorophyll derivative conferred the red fluorescence property to the silkworm

RFPs. The purified RFP also showed antiviral, antibacterial and antifungal activity.

The present work was intended to find out does the chlorophyll induces the expression of RFPs/ChBP-Lipocalin gene family? To answer this, fifth instar silkworm Kolar Gold larvae were fed with mulberry green leaves, mulberry yellow leaves (senescence), yellow leaves followed by green leaves, the results found that induction of *ChBP* requires a green pigment (Chlorophyll) and it is confirmed in the silkworm larvae fed with green leaves and low level expression in silkworm fed with only yellow leaves which indicates the importance of green chlorophyll pigment in induction of *ChBP* gene.

The quality of mulberry leaves fed to silkworm confine on growing larvae and prone to bacterial, viral infections and also quality of the silk it produces. This was also demonstrated that, yellow fed silkworms were very much susceptible to bacterial and viral infections. Disease prevalence minimized in silkworms grown up with green mulberry leaves. One side of the green mulberry leaves were smeared with purified RFP and other side of the same leaves were smeared with *Bm*NPV and fed them to silkworms results in tolerance to *Bm*NPV also.

In conclusion, the RFP present in the Kolar Gold silkworm larvae confirmed and showed its anti-pathogenic properties. Determined RFP's molecular weight and its N-terminal sequences. The purified

RFP's N-terminal sequences are highly conserved and found in *ChBP* gene, a lipocalin with highly conserved domain with eight beta barrel sheet. For up-regulation of *ChBP* expression requires green chlorophyll pigment and down-regulated by yellow pigment of mulberry.

## Future Line of Work

1) Development of RFP formulations for management of BmNPV infection in silkworm rearing.

2) RFP protein and its N-terminal amino acid sequences may be used a marker protein for the identification of Kolar Gold breed.

3) Complete amplification of *ChBP* gene may provide its physiological role, its molecular evolution is also helpful in the development of specific gene probe that will be useful in molecular genetics and identification of new silkworm breed generated by its crossing.

4) Molecular evolution relationship of *ChBP* gene sequence may help for further complete amplification and its gene, its molecular characterization and also construction of phylogenetic tree.

5) Role of *ChBP* gene and its involvement in viral resistance may be proved in *in-vitro* or *in-vivo* model.

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# APPENDIX

## ❖ Preparation of Dialysis Tube

The dialysis membrane molecular cut-off ranges 14,000 kDa (sigma Aldrich) was cut into required length, boiled in a beaker for 10 min in equilibration buffer (2% $Na_2Co_3$). The tube was thoroughly rinsed with distilled water and their boiled for 10 min in 1 mM EDTA and again rinsed with distilled water. The tubes are then stored at $4^0$C in the storage solution (50% Ethanol) until use.

## ❖ Solutions and Buffers for Poly Acryl Amide Gel Electrophoresis (PAGE)

**Note:** The composition of solutions and buffers are same for native and SDS PAGE but in native PAGE, SDS, β-mercaptoethanol is absent.

- **30% (w/v) acrylamide stock solution**: 29.2 g of acrylamide and

  0.8 g of N, N' methylene bis-acrylamide was dissolved in autoclaved double distilled water and the volume was made up to 100 ml and stored in amber coloured bottle at 4° c.
- **10% (w/v) SDS:** 10.0 g Sodium Dodecyl Sulphate was dissolved in autoclaved double distilled water with gentle stirring and the volume was made to 100 ml and stored at 4°C.

- **10% (w/v) Ammonium Per-Sulphate (APS):** 100 mg APS was dissolved in 1.0 ml of autoclaved double distilled water with gentle stirring and stored at 4°C.
- **TEMED:** 100% (N, N, N', N' tetramethyle ethylene diamine).
- **Resolving gel buffer (1.5 M Tris HCl, pH 8.8):** 12.0 g Tris-base was dissolved in 80.0 ml distilled water, pH adjusted to 8.8 with 1N HCl, the volume was made to 100 ml with distilled water and stored at 4°C.
- **Stacking gel buffer (1 M Tris HCl, pH 6.8):** 2.0 g Tris-base dissolved in 60 ml distilled water, pH adjusted to 6.8 with 1 N HCl, the volume was made to 100 ml with distilled water and stored at 4°C.
- **Order of mixing the gel components:** water, Acrylamide-bisacylamide solution, Tris buffer solution, Sodium Dodecyl Sulphate solution, TEMED, Ammonium Per-Sulphate solution.

**i) Resolving (separating) gel composition (12 %)**

| Reagents | Quantity used for 10 ml |
|---|---|
| Distilled water | 3.300 ml |
| 1 .5 M Tris (pH 8.8) | 2.500 ml |
| 10 % SDS | 0.100 ml |
| 10 % APS | 0.100 ml |
| 30%Acrylamide mixture | 4.000 ml |
| TEMED | 0.080 ml |

**ii. Stacking gel preparation (5 %)**

| Reagents | Quantity used for 5 ml |
|---|---|
| Distilled water | 2.500 ml |
| 1 M Tris (pH 6.8) | 1.300 ml |
| 10 % SDS | 0.120 ml |
| 10%APS | 0.120 ml |
| 30 % Acrylamide mixture | 0.880 ml |
| TEMED | 0.050 ml |

- **Sample buffer**

  - 0.5M Tris-HCl (pH 6.8) - 0.5ml
  - β –mercapto ethanol -0.2ml
  - 10% (w/v) SDS -0.8ml
  - 1% (w/v) bromophenol blue -0.2ml
  - Glycerol -0.4ml

  Add Distilled water to make up the volume 4ml.

- **Running Buffer (5X $P^H$ 8.3)**

  - Tris-base 15.0 g
  - Glycine 72.0 g
  - SDS 5.0 g

  These were dissolved in distilled water, pH adjusted to 8.3, volume made up to 1000 ml with distilled water and stored at

4°C. The buffer was warmed to 37°C before use. This buffer was diluted at 1:4 (v/v) with distilled water.

- **Protein staining solution**

  - Coomassie brilliant blue (CBB) R-250 : 100 mg
  - Methanol : 45 ml
  - Distilled water : 45 ml
  - Glacial acetic acid : 10 ml

- **Protein distaining Solution**

  - Methanol : 45 ml
  - Distilled water : 45 ml
  - Glacial acetic acid : 10 ml

❖ **Total RNA isolation and gel electrophoresis buffers Materials**

Distilled water, micro centrifuge tubes Pestle and Mortar, (DEPC treated), 2 ml collection tube, Micro centrifuge, Water bath, Chloroform: isoamylalcohol mixture (24:1), Agarose, 37% Formaldehyde (v/v), Iso-propanol, Extraction buffer, Wash buffer, Elution buffer, Liquid nitrogen.

- **DEPC water preparation and treatment**

  - Added 1ml of diethyl pyro carbonate (DEPC) to 1000ml distilled water (0.1%)

- Mixed well and set at room temperature for 1 hour
- Autoclave
- Cooled to room temperature prior to use
- Glass ware and plastic ware were treated with DEPC water, rinsed and incubated overnight at room temperature and then dried and autoclaved for further use.

➢ **10X MOPS**

0.2 M MOPS (sodium salt), 0.05 M sodium acetate, 0.01 M EDTA in DEPC treated $H_2O$. Adjust the pH to 7.0 with NaOH, store at room temperature in the dark. Do not autoclave.

➢ **10X RNA Loading Buffer**

50% glycerol,1mM EDTA,0.25% (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanole.

➢ **50X TAE buffer, pH 8.0**

- Tris Buffer : 242 gm
- Glacial acetic acid : 57.1 ml
- EDTA : 93 gm

Volume was made up to 1000 ml using distilled water

- Working concentration 1X TAE (2ml of 50X TAE/ 100 ml)

- **Sample buffer (6X):**

  - 0.25% Bromophenol Blue.
  - Xylene Cyanol FF.
  - 30% Glycerol in $H_2O$.