CHAPTER - I

ISOLATION, PURIFICATION AND CHARACTERIZATION OF RFP
INTRODUCTION

An unknown high molecular weight substance that could inactivate nuclear polyhedrosis virus (*BmNPV*) *in vitro* (Suzuki, 1936; Hayashiya et al., 1968, 1971a) was reported from the digestive juice of *Bombyx mori*. Later this substance was demonstrated to be a protein (Uchida et al., 1984) and possess an antiviral activity in the silkworm *B. mori*.

Studies on antiviral proteins are very scanty. Therefore, the present investigations were undertaken not only to understand the chemistry of RFP but also to initiate the studies on this antiviral protein to pave the way for collecting the basic data for biotechnological application.

In this chapter we report physical chemical and biological characterization of the RFP in order to streamline the procedures for its extraction, purification and estimation so as to demarcate the specificities of the protein. It was reported that RFP was an alkaline protease in the gut juice (Uchida et al., 1984; Funakoshi and Aizawa, 1989). However, it was not detected in the haemolymph (Hayashiya, 1972). Hou and Chiu (1986) suggested that RFP, as a whole, was having more than one midgut proteins and the activity of the purified RFP was attributed to the phospholipase C. However the structure and property, the sequence and the specificity of the protein, and its role as phospholipase C in immune response of the silkworm has not been fully elucidated. Sethuraman et al., (1993) purified the RFP by sephadex G-100 column chromatography and opined that only one peak out
of the three showed high absorbance, both at 280 and 605 nm. They reported a single protein of 65 KD which was different from the one of 28 KD as per Uchida et al., 1984). The same authors conducted the preliminary studies on phospholipase C enzyme activity and found none. Therefore the present studies were undertaken to clarify the view of earlier workers to note whether or not red fluorescent protein is a single protein or a group of proteins. In this chapter, we emphasize the characterization of physical and chemical properties of RFP. And we defer the biological characterization to chapter IV.

MATERIAL AND METHODS

1. Experimental insects and rearing conditions

The experiments were carried out in an aseptic laboratory maintained at 25 ± 2 °C, 65 ± 10 % relative humidity and 12:12 (light: dark) photoperiod. The containers and the instruments were sterilized using 5 % formaldehyde, washed and dried, before use. The conventional rearing procedures were similar to those described by Krishnaswamy (1978) and Sethuraman et al., (1993).

Silkworm Bombyx mori larvae were reared in a hygienically maintained laboratory on fresh mulberry leaves. The 5th instar 3rd day larvae were used for the collection of gut juice. Collected gut juice was stored at 4 °C until it was used for further analysis.
2. **Chemicals**

All the chemicals used for the experiments were of AR grade. They were obtained from Sigma chemical company (St. Louis, MO. USA) except sephadex G-75, which was obtained from Pharmacia Company.

3. **Methods**

   **A. Purification of red fluorescent protein (RFP)**

   The RFP was purified as per Uchida et al., (1984) with few modifications. All procedures were carried out below 4 °C. The collected sample of gut juice was centrifuged at 3,000 rpm to remove undigested mulberry leaves. The supernatant was neutralized to pH 7.0 with 2M acetic acid. To this solution, ammonium sulfate was added to give 40% saturation. After centrifugation at 12,000 rpm for 40 min, the precipitate was collected and suspended it in small amount of deionized water. The suspension was mixed with a half volume of n-butanol for 3 hours and then again centrifuged at 12,000 rpm for 40 min. The lower aqueous layer was collected and cold acetone was added to it to give 50% concentration. The resulting precipitate was collected by centrifugation at 12,000 rpm for 40 min. and dissolved in 40 mM sodium phosphate buffer (pH 7.0) and then dialyzed against the same buffer. The dialyzed fraction was centrifuged at 12,000 rpm for 40 minutes for the third time to remove the insoluble debris. Gelfiltration chromatography of the dialyzed fraction was performed using sephadex G-75 as the matrix, at 4 °C. Separation was accomplished by
passing through gel filtration column (78 cm X 1.5 cm) at a flow rate of 10 ml/hr. Protein peaks were detected by monitoring absorbance at 280 nm on a Hitachi UV-visible spectrophotometer model U-2001. Out of the 5 peaks obtained (Fig. 1.1), the 3 have exhibited fluorescence at 366 nm. These fluorescent fractions were pooled, concentrated by freeze-drying and used as the source of RFP for all experiments.

B. Analytical methods

a. Electrophoresis

(i) Native PAGE (polyacrylamide gel electrophoresis)

Native PAGE of the protein (25-100 μg) was carried out in 12.0 % (w/v) gels using 0.025 M Tris-glycine buffer, pH 8.5 (Davies, 1964) at a constant current of 20 mA per gel. After the run, the gels were stained for the detection of proteins.

(ii) Protein staining

The gels were stained for proteins with Coomassie brilliant blue R-250 (0.1 %) in methanol: acetic acid: water (4:1:5 v/v) for an hour followed by destaining in a solution containing 15 % methanol and 7 % acetic acid.

(iii) Carbohydrate staining

Glycoproteins were detected on the gels as per the procedure of Racusen (1979). All steps were performed at room temperature with gentle shaking.
After electrophoresis, the gels were transferred into a petridish and washed twice with a mixture of 25% isopropanol, 10% acetic acid and 65% distilled water with at least two hours for each wash. Later, the gels were transferred to a solvent mixture, containing 0.2% thymol for 80 min. and then developed in sulfuric acid: ethanol (80:20 v/v) until the opalescent core disappeared and the red coloured zones produced against a pale yellow background became prominent.

(iv) SDS-PAGE (SDS-polyacrylamide gel electrophoresis)

SDS-PAGE of the purified RFP was performed by the method of Laemmli (1970). The experiment was conducted in 12.0% w/v acrylamide gels under reducing (presence of β-mercaptoethanol) and non-reducing conditions (absence of β-mercaptoethanol).

Sample buffer of 0.1 ml containing 40-50 µg of the protein was prepared in a solution having 0.0625 M Tris-HCl buffer (pH 6.8), 2% SDS, 20% glycerol and, 0.001% bromophenol blue as the tracking dye. Samples were heated in boiling water bath for 2-3 min. so that the proteins denatured and bind tightly to the SDS. Tris-glycine buffer pH 8.5 (containing 0.025 M Tris and 0.192 M glycine with 0.1% SDS) was used as the electrode chamber buffer at a constant current strength of 20 mA. Standard Sigma molecular weight protein markers were run along with the protein samples. Electrophoresis was stopped when the tracking dye (bromophenol blue)
reached the bottom of the gel. The protein bands were visualized by silver staining method (Wray et al., 1981) or with Coomassie brilliant blue R-250.

(v) Silver staining

All steps were performed at room temperature, with gentle shaking and thorough water wash after each step. The gels were soaked in 50 % ethanol and 10 % glutaraldehyde for one and twelve hours, respectively. After the removal of glutaraldehyde, the gels were stained with ammonical silver nitrate solution (0.8 g silver nitrate in 100 ml of 0.36 % NaOH containing 1.4 ml of 14.8 M ammonia) for 15 min. Unbound silver was eliminated and the gel developed in 100 ml of the developing solution containing 0.5 ml of 1 % citric acid and 0.05 ml of 37 % formaldehyde in 10 % methanol. The reaction was arrested by the addition of 50 % methanol. The protein bands appeared brown against a clear background (Wray et al., 1981).

b. Molecular weight of RFP

(i) By gelfiltration chromatography (Andrews 1964)

Sephadex G-75 was allowed to swell in excess of water by heating on water bath for 3 to 4 hrs. The gel was then suspended in 0.04 M sodium phosphate buffer of pH 7.0, stirred, allowed to settle and the fine particles were removed by decanting the supernatant. A column (78 cm X 1.5 cm) was packed by pouring a portion of the gel slurry into the column with the outlet closed. The remaining gel slurry was added slowly to the column as
the outlet opened and the gel was packed by gravity flow. The column was equilibrated with 0.04 M sodium phosphate buffer (pH 7.0). Blue dextran was loaded to the column to determine the void volume of the column. The sample was applied to a pre-equilibrated column, calibrated with Sigma molecular weight markers (BSA 66,000, ovalbumin 45,000, carbonic anhydrase 29,000, trypsinogen 24,000, lysozyme 14,000). Fractions of 3 ml size were collected at a flow rate of 10 ml/hr. The eluted protein fractions were monitored at 280 nm in Hitachi UV-visible spectrophotometer model-2001 and the molecular weight of the protein was computed from the calibration curve. All the column operations were carried out at 4 °C. The molecular weight of the protein was calculated from the calibration curve obtained by plotting log molecular weight against partition coefficient (Kav) (Andrews, 1964) (Fig. 1.2).

\[ Kav = \frac{(Ve - Vo)}{(Vt - Vo)} \]

Where

- Ve = elution volume
- Vo = void volume
- Vt = total bed volume of the column.

(ii) By SDS-PAGE.

PAGE in the presence of SDS was carried out to determine the molecular weight of purified RFP by the method of Laemmli (1970). The experimental detail of SDS-PAGE has been described in previous section.
The standard molecular weight marker proteins from Sigma Company (Serum albumin 67,000, Ovalbumin 45,000, Carbonic anhydrase 31,000, Trypsin inhibitor 20,000 and Lysozyme 14,000) were used. The molecular weight of the RFP was computed using gel documentation and analysis system (Alphalmagaer™ 1220) that has built-in programme to plot the calibration curve of log of molecular weight verses relative mobility of the standard molecular weight protein markers. And the calibration curve of log molecular weight against the migrated proteins (distance in centimeters) was also plotted manually (Fig.1.3).

(iii) Protein estimation

Protein content in the purified RFPs was estimated by the method of Lowry et al., (1951) using BSA as the standard. To 10 μl of purified RFP preparation, 5 ml of alkaline copper reagent (50 ml of 2 % Na₂CO₃ in 0.1 N NaOH mixed with 1 ml of 0.5 % CuSO₄ in 1 % sodium potassium tartrate) was added and mixed thoroughly. After 10 min incubation at room temperature, 0.5 ml of 1 N Folin's reagent was added and the mixture vortexed. The blue colour developed after 30 min. incubation in dark and OD was measured against blank at 660 nm using Elico-157 colorimeter.
RESULTS

1. Gelfiltration chromatography of the RFP

The gelfiltration of partially purified RFP preparation was carried out in dark, yielded five protein peaks (Fig. 1.1). Out of which 3 were red fluorescent (peaks 2, 3 & 4) at 366 nm. The peak fractions were separately pooled, concentrated and used to determine the activity in vitro and in vivo.

2. Molecular weight determination of the purified RFP

Two techniques were used to determine the molecular mass of the purified RFP.

a. Molecular sieve chromatography on sephadex G-75 column (Andrews, 1964)

Using the calibration curve of log molecular weight against partition coefficient (Kav), the molecular weight of the purified RFPs i.e. peaks 2, 3 and 4 (Fig. 1.1) were determined to be about 68, 46 and 27 KD respectively (Fig. 1.2)

b. By SDS-PAGE

(i) Using Gel Documentation and Analysis System

On computing with Gel Documentation and Analysis System, the molecular weight of RFP (peak 3, Fig. 1.1) is determined to be 46 KD.
Fig. 1.1. Gelfiltration pattern of RFPs

Gelfiltration chromatography was performed using sephadex G-75 column (78 cm X 1.5 cm) at a flow rate of 10 ml/hr at 4 °C. Protein was eluted with 40 mM sodium phosphate buffer (pH 7.0) and 5 peaks were obtained. The peaks 2, 3 and, 4 exhibit red fluorescence and were found to have bioactivity.
Figure 1.1

Absorbance at 280nm vs. Fraction Numbers

1 2 3 4 5
Fig. 1.2. Calibration curve for the determination of molecular weight of RFP by gel filtration chromatography using sephadex G-75 column (78 cm X 1.5 cm). Standard molecular weight protein markers used were

1. Lysozyme : 14,000 KD
2. Trypsinogen : 24,000 KD
3. Carbonic anhydrase : 31,000 KD
4. Ovalbumin : 45,000 KD
5. Bovine serum albumin : 67,000 KD
(ii) Using calibration curve

Using the calibration curve of log of molecular weight verses relative mobility of the standard molecular weight protein markers, the molecular weight of RFP is determined to be about 46 KD (Fig. 1.3).

3. Chemical characterization of the purified RFP

a. Electrophoresis results

(i) Native PAGE

The 12 % native polyacrylamide gel electrophoresis of purified RFP yielded single protein band (Fig. 1.4).

(ii) SDS-PAGE

The SDS-PAGE analysis of peak 3 (Fig. 1.1) displayed only one protein band under reducing (presence of β-mercaptoethanol) and non-reducing conditions (absence of β-mercaptoethanol) with the molecular mass of about 46 KD (Fig. 1.5).

b. Detection of glycoproteins

The glycoprotein staining of the purified RFPs on native-PAGE indicated only one red fluorescent protein (band C, Fig. 2.9) out of the three RFPs is a glycoprotein.

c. UV-Visible spectroscopic results

The partially purified RFP preparation has shown absorbance maxima at 666, 622, 535, 506 and 410 nm. (Fig. 1.6).
Fig. 1.3. Calibration curve for the determination of molecular weight of the RFP by SDS-PAGE using 12.0% polyacrylamide gel. Standard molecular weight proteins were

1. Lysozyme : 14,000 KD
2. Trypsin inhibitor : 20,000 KD
3. Carbonic anhydrase : 31,000 KD
4. Ovalbumin : 45,000 KD
5. Bovine serum albumin : 67,000 KD
Fig 1.3

Relative mobility in cm vs log molecular weight
RFP sample was subjected to native PAGE employing 12.0% polyacrylamide gel using 0.025 M Tris-glycine buffer at pH 8.5 and stained with Coomassie brilliant blue R-250 (0.1%)
Fig. 1.5. SDS-PAGE was carried out employing 12.0% polyacrylamide gel. (stained with coomassie brilliant blue R-250).

Lane-1. Standard molecular weight protein markers.

1. Lysozyme : 14,000 KD
2. Trypsin inhibitor : 20,000 KD
3. Carbonic anhydrase : 31,000 KD
4. Ovaalbumin : 45,000 KD
5. Bovine serum albumin : 67,000 KD

Lanes – 2 and 3. Peak 2 fractions were treated under reducing and non-reducing conditions, respectively.
Fig 1.6. Visible absorbance spectrum of the partially purified RFPs.

Absorbance spectrum of the partially purified RFPs was taken in sodium phosphate buffer at pH 7.0. RFPs preparation has shown the absorbance maxima at 666, 622, 535, 506 and 410 nm.
DISCUSSION

Uchida et al., (1984) have purified an RFP of molecular weight of 27 KD to 28 KD from digestive juice of the silkworm B. mori. The red fluorescent protein (RFP), which can inactivate NPV, was reported to have a molecular weight of 65 KD (Sethuraman et al., 1993). Hiraki et al., (1996) have observed that the antiviral protein (AVP) isolated from faeces was found to suppress the production of only the enveloped viruses like, HVJ, HSV, HIV, etc. It was also found to effectively inhibit them, possibly by blocking the synthesis of viral specific genes. Lectin like substance was purified from silkworm faeces with a molecular weight of 60 KD. It was known to inactivate quail myoblast - Rous sarcoma virus (QM-RSV) cells (Hirayama et al., 1993). Antiviral substances from silkworm faeces were purified; the molecular weights of these substances were found to be 25 KD and 14 KD (Hiraki et al., 1997). The SDS-PAGE analysis of RFP resulted in single protein band of molecular weight 46 KD (Fig. 1.5). The SDS-PAGE gel was subjected to carbohydrate staining procedures. Of the three RFP forms, only the protein disc corresponding to RFP-C was found to be a pigmented glycoprotein complex as opposed to the RFP-A and B which were only the pigmented-protein complexes. With these observations, it is clear that the RFP isolated here is different from the proteins obtained by Uchida et al., (1984) and Sethuraman et al., (1993).
The precipitation was observed when RFP and NPV were mixed and incubated. This recognition and subsequent binding of RFP to NPV is probably due to surface interaction between RFP and NPV. Uchida et al., (1984) have isolated a protein from gut juice of silkworm larvae, which can precipitate NPV in vitro. The NPV inhibition by protein was thought to be due to disruption of the viral envelope. However, the mechanism of interaction of RFP with NPV needs a detailed study before it can be proposed.

**CONCLUSION**

1. The purification of RFP from silkworm gut juice was carried out using the biochemical techniques like ammonium sulphate precipitation, acetone fractionation, dialysis, lyophilization and gelfiltration chromatography with minor modifications of the procedure developed by Uchida et al., (1984).

2. The molecular weight of the purified RFP was determined by gelfiltration chromatography using calibrated sephadex G-75 column. From the calibration curve, molecular weight of RFP was found to be 46 KD. Purified RFP obtained from the gelfiltration chromatography was found to be pure by electrophoretic analysis. Native PAGE of the purified RFP resulted in a single protein band.
3. The molecular weight of the purified RFP was also determined by SDS-PAGE, which has revealed only one protein band of molecular weight 46 KD.

4. Reducing and nonreducing SDS-PAGE (presence and absence of β-mercaptoethanol, respectively) of the purified RFP yielded single protein band of molecular weight 46 KD suggesting that the native protein is a monomer.

5. The electrophoresed gel was examined for the presence of carbohydrate containing proteins by the thymol sulfuric acid method. Of the three RFP bands, only band – C was found to be a glycoprotein.

6. RFP has shown the absorbance maxima at 410, 622 and 674 nm indicating the presence of tetrapyrrole pigment derivative(s) in RFP. Thus the RFP is a pigmented (containing a porphyrin derivative) protein.