CHAPTER III

PURIFICATION AND CHARACTERIZATION OF SILKWORM EXCRETORY RED FLUORESCENT PROTEIN (SE-RFP)
PURIFICATION AND CHARACTERIZATION OF SE-RFP

This chapter deals with the purification of red fluorescent protein present in the silkworm excreta. Biochemical, biophysical and proteomical characterization of the purified SE-RFP has also been covered in this chapter.

1. RESULTS

A. PURIFICATION OF SE-RFP

The SE-RFP present in the silkworm excreta was purified as per the procedure described in the 'materials and methods' section, 2.1b. The ammonium sulfate precipitation was used to concentrate the SE-RFP present in silkworm excretory extract and, the protein solution was dialysed against Tris-HCl buffer (10 mM, pH 7.6) for 48 hours at 4°C. Preparative gel filtration chromatography was conducted to obtain SE-RFP in its homogenous state.

The gel filtration profile of dialyzed protein sample is as shown in Figure 3. 1A. The SE-RFP was eluted in a single protein peak. It was indeed a pleasure to watch the red fluorescent band of SE-RFP moving through the gel permeation column under UV-light (Figure 3.1B). The red fluorescent eluates of SE-RFP were pooled and used for further experiments.

Native-PAGE followed by silver nitrate staining of SE-RFP aliquots purified from preparative gel filtration chromatography was carried out. A single distinct band obtained after silver nitrate staining has suggested the homogeneity of the purified SE-RFP (Figure 3. 1C).
B. CHARACTERIZATION OF SE-RFP

a) UV-Visible and fluorescence spectroscopy

The UV-visible absorption spectrum of purified SE-RFP was found to have two main absorption peaks at 280 and 603 nm (Figure 3. 2). The SE-RFP was observed to emit fluorescence at 334 and 619 nm when excited at 280 and 603 nm, respectively (Figure 3. 3A and B, respectively).

b) Molecular mass analysis by gel filtration chromatography

The mass of the purified native SE-RFP was analyzed by gel filtration chromatography on sepharose-6B column calibrated with gel filtration chromatography grade high range molecular weight protein markers (Sigma-Aldrich). Using the calibration curve of log molecular weight against partition coefficient (Kav), the molecular weight of the purified SE-RFP was determined to be about 1100 kDa (Figure 3.4).

c) Treatment of SE-RFP with different salt concentrations and denaturants

Re-chromatography of the purified SE-RFP aliquots in the presence of different salt concentrations (0 to 1M) and denaturants was carried out. Chaotropic agents like urea and guanidinium hydrochloride, a non-ionic detergent- Triton X-100, and, a zwitterionic detergent- CHAPS could not dissociate the SE-RFP as it was eluted in a single peak (Figure 3.5).

Incubation of the purified SE-RFP aliquots separately with different denaturants followed by native-PAGE yielded a single band of SE-RFP observed under UV-light and by coomassie brilliant blue staining (Figure 3.6).
d) Agarose gel electrophoresis

Considering the enormous size of SE-RFP, agarose gel electrophoresis was conducted. Immediately after electrophoresis, the gel was observed under UV-light to locate the red fluorescent protein bands and then stained with coomassie brilliant blue. A single protein band was detected as shown in figure 3.7.

e) Alkaline urea-PAGE

Alkaline urea-PAGE of purified SE-RFP was conducted in presence of 4M urea. In this case also, a single protein band was observed before (under UV-light) and after staining with coomassie brilliant blue (Figure 3.8).

f) 2D-PAGE

Two dimensional electrophoresis was performed to detect the molecular mass and the isoelectric pH of purified SE-RFP. A red fluorescent spot was noticed immediately after the isoelectric focusing of SE-RFP (Figure 3.9A). But after second dimensional SDS-PAGE, there was no red fluorescent band was noticed on the gel. However, A single protein spot was detected after silver nitrate staining corresponding to a pl of about 4.6 and a molecular weight of about 298 kDa (Figure 3.9B). The red fluorescence of the SE-RFP was lost under denaturing conditions employed during the 2D-electrophoresis.

g) N-terminal sequencing

The protein spot (obtained in 2D-PAGE) electroblotted on PVDF membrane was subjected to N-terminal sequencing. A short query of N-terminal amino acid residues TQTVTKS of SE-RFP has shown significant sequence alignment with some reported Bombyx mori proteins. Among the 68 BLAST HITS on the query of SE-RFP sequence, five proteins having highest sequence alignment are listed in Table 3.1.
Conformational Studies by CD spectroscopy

The results of CD spectral analyses of purified SE-RFP under different conditions are as shown in Figure 3.10. The native SE-RFP is composed of 4.57% \( \alpha \)-helix and 46.69% \( \beta \)-pleated sheet. Conformation of heat denatured SE-RFP (4.62% \( \alpha \)-helix and 46.65% \( \beta \)-pleated sheet) is comparable to that of native SE-RFP. Incubation of SE-RFP with SDS and urea has yielded a notable change in protein conformation. Whereas, the pigment-free protein has showed a distorted CD spectrum.

SDS-induced cleavage of purified SE-RFP

i. SDS-PAGE of SE-RFP

No red fluorescent protein bands were detected on unstained electrophoretogram under UV light after the SDS-PAGE of SE-RFP. But after staining the gel, it was surprising to observe a ladder of protein bands (Figure 3.11). About 20 and 15 subunits of SE-RFP were produced by SDS action in absence and presence of \( \beta \)-mercaptoethanol, respectively (Figure 3.11, lane 2 and 3). It was observed that the tetrapyrrole pigments were detached from the protein and moved ahead of the bromophenol blue dye front in SDS-PAGE of SE-RFP (Figure 3.12 A & B).

ii. Treatment of purified SE-RFP with electrophoretic reagents

The purified SE-RFP aliquots were incubated separately with each electrophoretic reagents (stacking gel buffer of pH 6.8, reservoir buffer of pH 8.3, resolving gel buffer of pH 8.8, 10% glycerol and 0.5% bromophenol blue) and loaded on sepharose-6B column. In each case, a single protein (red fluorescent) peak was obtained. SE-RFP aliquots in the presence of different salt concentrations and various denaturants have also yielded a single protein peak in gel filtration chromatography (Figure 28...
3.5). In contrast, multiple protein peaks were noticed in the gel filtration chromatography profile of the purified SE-RFP incubated with 1% SDS (Figure 3.13). None of the protein peak fractions exhibited red fluorescence under UV-light.

iii. Effect of heat on SE-RFP degradation

To examine the role of heat in the SE-RFP degradation, heating of SE-RFP followed by native-PAGE was conducted. An irreversible loss of fluorescence of SE-RFP solution was occurred upon heating, but the native-PAGE of the same has yielded a single protein band (Figure 3.14). It indicates that the tetapyrroles that confer the red fluorescence property to SE-RFP complex are heat susceptible; however, heat could not cause cleavage of the protein. Enigmatically, the heat denatured (in presence of SDS) SE-RFP solution continues to exhibit fluorescence but protein gets dissociated. It suggests that the tetapyrroles freed from SE-RFP complex are capable of forming a stable structure in combination with SDS that retains their red fluorescence. The released tetapyrrole fraction eluted from unstained gel has showed absorption peaks that are characteristics of chlorophyll derivatives (Figure 3.15).

iv. UV-visible absorption spectra of native and heat denatured SE-RFP (in absence and presence of SDS)

UV-visible spectra of native and heat denatured (in absence and presence of SDS) SE-RFP aliquots are as shown in Figure 3.16. The native SE-RFP has showed two absorption peaks at 280 and 604 nm
The heat denatured (in absence of SDS) SE-RFP has showed protein peak (279 nm) but there was no tetrapyrrole absorption peak around 604 nm region. While, heat denatured (in presence of SDS) SE-RFP showed a moderate absorption at 604 nm but a low absorption at 276 nm.

v. Effect of SDS concentration on SE-RFP degradation

To evaluate the effect of SDS concentration on SE-RFP degradation, the purified SE-RFP was incubated with different concentrations of SDS then subjected to native-PAGE. A moving front of detached tetrapyrroles was noticed during the electrophoresis under UV-light (Figure 3.17A & B). Electrophoretic mobility of released tetrapyrroles was directly proportional to the SDS concentration (Figure 3.18).

vi. MALDI-TOF-MS analysis of SE-RFP band(s) obtained from native-PAGE and SDS-PAGE

The purified SE-RFP has yielded a single band in native-PAGE and a ladder of bands in SDS-PAGE. The SE-RFP band eluted from native-PAGE was digested with trypsin and subjected to MALDI-TOF-MS analysis. The peptide mass fingerprinting spectrum of SE-RFP is as shown in Fig. 3.19. The SE-RFP could be a new protein as none of the proteins present in the search engine (MASCOT) database have shown significant Protein scores (Protein score is $-10 \times \log (P)$, where ‘$P$’ is the probability that the observed match is a random event).

Among the degradation products of SE-RFP obtained in SDS-PAGE, eight prominent protein bands (Figure 3.11, lane 3) were eluted and
subjected to trypsin-digestion followed by MALDI-TOF-MS analysis. Interestingly, these protein bands were found to have comparable peptide compositions as their PMF spectra (Fig 3. 19, labeled as F1, F2, F3, F4, F5, F6, F7 and F8) contain several common m/z values around 1622, 1625, 1773, 1892, 1993, 2203, 2530, 2699 and 3142.

None of the proteins present in the search engine (MASCOT) database have shown significant ‘protein scores’. Hence, the SE-RFP and its degradation products could be a set of new proteins, not reported in the literature, to date.

vii. Urea antagonizes SDS action on the SE-RFP

Amazingly, a single band was obtained when SE-RFP was pre-incubated with urea prior to SDS-PAGE (Figure 3. 20).

2. DISCUSSION

Several RFPs have been purified from silkworm gut juice and midgut cells having significant anti-viral activity, but hundreds of silkworm larvae to be sacrificed for the purification of these protein. Biotechnological processes are not yet established successfully to produce a quantitative amount of antimicrobial RFPs which could have shunned the scarification of silkworm larvae. Silkworm excreta is enormously available as a waste product of sericulture industry. Hence, we were interested to examine the presence of antimicrobial RFP (s) in the excreta of silkworms, if any. Our attempts were fruitful and we have purified a unique red fluorescent protein present in silkworm excreta.
In the present work, an intact tetrapyrrole-bound SE-RFP was purified to its homogeneity by ammonium sulfate saturation (20% w/v) followed by gel filtration chromatography of silkworm excretory extract (Figure 3.1).

The absorption and fluorescence spectral results of SE-RFP (Figure 3.2 and 3.3) are comparable to that of the silkworm gut-juice RFP. But the SE-RFP is different from silkworm gut-juice/midgut cell RFPs in having a very high molecular mass and a unique biochemical composition.

It was suspected that the SE-RFP may be a multiple protein–pigment complex as it has a very huge molecular weight of about 1100 kDa. Re-chromatography of the purified SE-RFP in presence of a high salt concentration (upto 1M) and several denaturants could not dissociate the giant SE-RFP (Figure 3.5). An increase in the absorbance (at 280 nm) of gel filtration eluates of SE-RFP was attributed to the increased solubility of the protein in presence of salt and detergents (Figure 3.5). Native-PAGE of the SE-RFP aliquots incubated with different denaturants has also failed to dissociate the SE-RFP into the components, if any (figure 3.6).

Taking into the consideration of enormous size of the SE-RFP, it was subjected to agarose gel electrophoresis in presence of 6M urea and 0.1% SDS (Figure 3.7). Again, a single red fluorescent band of SE-RFP was noticed. These results suggest that the protein could be a tight aggregate with tetrapyrrole moieties buried deeply within the complex. The molecular mass of SE-RFP in 2D-PAGE (under denaturing conditions) was about 298 kDa which is very smaller as compared to the mass of native SE-RFP (as determined by gel filtration chromatography). Hence, it indicates that the native SE-RFP is a huge multimeric protein. As the red fluorescence of the SE-RFP was lost under the denaturing conditions during two dimensional SDS-PAGE, the tetrapyrrole pigments were presumed to be non-
covalently bound to the protein. A good fluorescence quantum yield of 0.86 was exhibited by SE-RFP as it represents a photosensitive protein–pigment complex.

N-terminal sequencing analysis of SE-RFP spot (obtained from 2D-PAGE) showed significant sequence alignment with Titin, a multidomain muscle protein of silkworm. Based on the huge molecular mass and sequence homology, the SE-RFP would be a Titin-type protein. But there are no reports in the literature on Titin having tetrapyrrole binding sites. Among the different proteins having significant sequence alignment with the SE-RFP sequence query, cytochrome P450 CYP4M9 is a phorphyrin–protein complex. However, the SE-RFP sequence query did not produce significant alignment with the sequence of a reported silkworm red fluorescent protein (Polycalin) available in the database search engine. The red fluorescence of the Polycalin was retained even after 2D electrophoresis as the chlorophyllide a is covalently bound to the protein, whereas, SE-RFP was non-fluorescent as the tetrapyrroles were detached from the protein under similar conditions. The SE-RFP also differs from a Bombyx mori midgut membrane protein P252 in having free N-terminal amino acid residue.

In contrary to the native-PAGE results, the tetrapyrrole pigments were detached from the protein and moved ahead of the bromophenol blue dye front in SDS-PAGE of SE-RFP. Hence, no red fluorescent protein bands were detected on unstained electrophoretogram under UV light. But after staining the gel, it was surprising to observe a ladder of protein bands of SE-RFP (Figure 3.11). About 20 and 15 subunits of SE-RFP in absence and presence of β-mercaptoethanol, respectively, were detected (Figure 3.11, lane 2 and 3, respectively). It seems, SDS opens up the complex structure of SE-RFP and makes the avail of the interiorly positioned disulfide bonds to β-mercaptoethanol, hence, the combined action of SDS
and β-mercaptoethanol on SE-RFP has yielded higher number of degradation products.

Initially, we had suspected that the degradation of SE-RFP in SDS-PAGE was due to contaminant protein/s having protease activity, which might have been copurified along with the SE-RFP. But the presence of contaminant protein/s was ruled out as there no additional protein band/s other than SE-RFP were detected in native-PAGE (Figure 3.1C and Figure 3.6). To ensure the homogeneity of purified SE-RFP, we also conducted alkaline urea-PAGE that again resulted into a single SE-RFP band (Figure 3.8).

Even if, the breakdown of SE-RFP is due to a protease action, there seems to be some polypeptide chains in SE-RFP which have a latent protease-like activity. These polypeptides might be activated by SDS leading to the self-degradation of the protein complex. A good many polypeptide chains in a protein may have refolding potential during or after denaturation either alone or in combination with a polar/nonpolar component such as SDS, to yield a product with protease activity.\textsuperscript{69} Some proteins undergo SDS-induced structural changes to acquire a stable protease conformation.\textsuperscript{69,70} But the SE-RFP was continued to degrade in the SDS-PAGE even in presence of several specific protease inhibitors, namely, PMSF, EDTA, aprotinin, leupeptin, pepstatin, bestatin and E-64 (Table 3.2).\textsuperscript{71} The futile attempts to stop the SDS-induced cleavage of SE-RFP by using several specific protease inhibitors suggested that the protein cleavage is not due to a protease action.

The observations of a single band in native-PAGE and numerous bands in SDS-PAGE of SE-RFP are the evidence for the belief of SE-RFP degradation is caused by SDS. Although, the notion of the SE-RFP degradation due to an artefact in the SDS-PAGE system or due to any electrophoretic agent (other than SDS) was ruled
out by conducting gel filtration chromatography. The purified SE-RFP aliquots were incubated separately with each electrophoretic reagents (stacking gel buffer of pH 6.8, reservoir buffer of pH 8.3, resolving gel buffer of pH 8.8, 10% glycerol and 0.5% bromophenol blue) and loaded on sepharose-6B column. In each case, a single protein (red fluorescent) peak was obtained. SE-RFP aliquots in presence of different salt concentrations and various denaturants have also yielded a single protein peak in gel filtration chromatography. In contrast, multiple protein peaks were noticed in the gel filtration chromatography profile of the purified SE-RFP incubated with 1% SDS (Figure 3.13). None of the protein peak fractions found to have red fluorescence under UV-light. Therefore, SDS appeared to be the only agent that is responsible for the breakdown of SE-RFP and release of its tetrapyrroles.

As per the routine practice of SDS-PAGE, the SE-RFP samples were heated for 5 min at 100°C before loading onto the gel. To examine the role of heat in the SE-RFP degradation, heating of SE-RFP followed by native-PAGE was conducted. An irreversible loss of fluorescence of SE-RFP solution was occurred upon heating, but the native-PAGE of the same has yielded a single protein band (Figure 3.14). It indicated that the tetrapyrroles which confer the red fluorescence property to SE-RFP complex are heat susceptible; however, heat could not cause cleavage of the protein. Enigmatically, the heat denatured (in presence of SDS) SE-RFP solution continues to exhibit fluorescence but protein gets dissociated. It suggests that the tetrapyrroles freed from SE-RFP complex are capable of forming a stable structure in combination with SDS that retains their red fluorescence. The released tetrapyrrole faction eluted from unstained gel has showed absorption peaks that are characteristics of chlorophyll derivatives (Figure 3.14). In the succeeding chapter-IV, we have reported the
presence of chlorophyll derivatives, namely, pyropheoporbide \(a\) and pheophytin \(a\) were associated with the SE-RFP complex.

UV-visible absorption spectra of native and heat denatured (in absence and presence of SDS) SE-RFP aliquots are as shown in Figure 3.16. The native SE-RFP has shown two absorption peaks at 280 and 604 nm (attributed to protein and pigment components of SE-RFP, respectively). The heat denatured (in absence of SDS) SE-RFP has shown protein peak (279 nm) but there was no absorption around 604 nm region attributed to chlorophyll derivatives. While, heat denatured (in presence of SDS) SE-RFP showed a moderate absorption at 604 nm but a low absorption at 276 nm. It indicated the endurance of pigment nature and degradation of protein part in the SE-RFP complex in presence of SDS.

To evaluate the effect of SDS concentration on SE-RFP degradation, the purified SE-RFP was incubated with different concentrations of SDS then subjected to Native-PAGE. A moving front of detached tetrapyrroles was noticed during the electrophoresis under UV-light (Figure 3.17A & B). Electrophoretic mobility of released tetrapyrroles was directly proportional to the SDS concentration (Figure 18). It suggested that the tetrapyrroles get detached from the SE-RFP complex more readily with the higher concentrations of SDS. These tetrapyrroles moves faster in the electric field through polyacrylamide matrix as they posses relatively higher charge density and lower molecular mass as compared to proteins.

Eight prominent protein bands among the degradation products of SE-RFP (see Figure 3.11, lane 3) were eluted and subjected to trypsin-digestion followed by MALDI-TOF-MS analysis. None of the proteins present in the search engine (MASCOT) database have shown significant ‘protein scores’ (Protein score is \(-10\log (P)\), where ‘\(P\)’ is the probability that the observed match is a random event).
Hence, the SE-RFP and its degradation products could be a set of new proteins, not reported in the literature, to date.

Interestingly, these protein bands were found to have comparable peptide compositions as their PMF spectra (Figure 3.19) contain several common m/z values. It indicates that the protein bands in SDS-PAGE might be the degradation products of a single giant protein, SE-RFP. Mass spectrometric analysis has been used in a few studies on proteolysis/autoproteolysis of proteins. Here, for the first time we have used MALDI-TOF-MS to elucidate the SDS-induced degradation of an insect exogenous protein, SE-RFP.

The prediction of secondary structures by CD spectral analysis (Figure 3.10) suggested that the most of conformation of the native SE-RFP is made up of β-pleated sheet (46.69%). Conformation of heat denatured SE-RFP was comparable to that of native SE-RFP indicating heat could not cause protein breakdown. Incubation of SE-RFP with SDS has yielded a notable increase in α-helical and decrease in β-pleated conformation of the SE-RFP. But there was slight decrease in α-helical but increase in β-pleated sheet conformation when SE-RFP was incubated with urea. It suggests that urea has the antagonistic effect on the action of SDS on SE-RFP. Whereas, the pigment-free protein has shown a distorted CD spectrum that strongly indicates that the removal of tetrapyrrroles from the SE-RFP complex leaves behind an unstable conformation leading to the collapse of the protein into number of subunits. Hence, SDS-induced detachment of tetrapyrrroles from the SE-RFP complex could be the actual reason for protein breakdown observed in SDS-PAGE.

In some of the reports, covalently bound chlorophyll derivatives were found to remain with the protein after electrophoresis in presence of SDS. But in case of SE-RFP, tetrapyrrroles were detached from the protein in SDS-PAGE. Hence, the
tetrapyrroles in SE-RFP complex could have non-covalently bound to the protein part by forming secondary bonds. These interactions seemed to be somewhat sturdy as the denaturants like urea, guanidine hydrochloride, CHAPS and Triton X-100 were unable to disrupt them. Hence, these denaturants could not cause the release of tetrapyrroles as well as dissociation of protein. The bonds between the protein and tetrapyrroles in the SE-RFP complex were disrupted by SDS which could have the ability to form equally strong or stronger bonds with the groups holding the entire conformation together. Most denaturation changes consist of changes in secondary bonds like ionic bonds, hydrophobic interactions, hydrogen bonds, Van der Waals forces and dipole-dipole bonds.

Astonishingly, only a single band was obtained in SDS-PAGE when SE-RFP was pre-incubated with urea (Figure 3.20), hence, no proteolysis of SE-RFP has occurred in presence of urea. Urea being a protein denaturant one can expect it to be synergistic to the effect of any other denaturant on a protein. Contrary to this presumption, urea treated SE-RFP has exhibited resistance to the action of SDS. To the best of our literature knowledge, this is the first report on the inhibition of SDS-induced degradation of a protein by urea.

How does the presence of urea with the SE-RFP makes it resistant to the SDS-induced proteolysis? Based on our present experimental results and literature, we have strived to answer this question as follows-

Urea can remove the water molecules from the functional domain of a protein which renders the potential hydrogen bonding sites to be free leading to the change in the conformation and loss of biological activity. Whereas, SDS would provide the proteins with the structural changes needed to fix them in a stable 'protease
conformation’, besides facilitating the cleavage of the substrate by extensive
denaturation. 69-70

SDS was incapable of inducing proteolytic activity of particular peptides of
SE-RFP may associated with potential protease activity either due to the
conformational changes that were brought about by urea or, access of these latent
proteases to SDS was physically blocked by the urea. SDS and urea have exhibited
opposite actions on the conformation of SE-RFP (Figure 3. 10). It may be the SDS
could not cause a conformational change required to breakdown of the SE-RFP in
presence of urea.

The structures of proteins-SDS aggregates and, the mechanisms of their
formation have not been established unambiguously. However, extensive
investigations on SDS-protein interactions have led to the emergence of several
models.76-82 These studies suggest that the denaturation process occurs by
encapsulation of protein by SDS (or vice versa) leading to the formation of SDS-
protein aggregates. In present case, the presence of urea might be declining the access
of SE-RFP to SDS that prevents the formation of SE-RFP-SDS aggregate which could
have a potential to split over into numerous degradation products.

A single protein spot obtained in 2D-electrophoresis of purified SE-RFP has
provided an experimental evidence for the inhibition of SDS-induced proteolysis of
SE-RFP by urea (Figure 3.20). Note that the SE-RFP was solubilised in rehydration
buffer containing 8 M urea before IEF. The isoelectrically focused SE-RFP was
subsequently soaked in solution-1 and 2 containing 6 M urea prior to SDS-PAGE.39-40
Hence, the protein could have acquired the resistance to SDS-induced proteolysis
because of its pre-treatment with urea. Observation of the degradation of a well
characterized silkworm midgut RFP (Polycalin) in 2D-PAGE was may be due to
SDS-induced proteolysis. But, it was limited (only two protein spots around 105 kDa) may be because of the obvious presence of the urea in 2D-PAGE system.

The present observations would expand the horizon of understanding the interaction between proteins and denaturant(s) as their molecular mechanisms of the interaction are still incompletely understood, to date.
FIGURES AND TABLES
Figure 3.1. Purification of SE-RFP by gel filtration chromatography. (A) Gel filtration profiles of SE-RFP. Absorbance of each eluate was measured both at 280 nm (red) and 410 nm (green). (B) Red fluorescent SE-RFP band moving through the column as observed under UV-light. (C) Native-PAGE of red fluorescent eluate yielded a single protein band as detected under UV-light (red fluorescent) (1) and, after silver nitrate staining (2).
Figure 3.2. UV–Visible Absorption Spectrum of Purified SE-RFP.
Two main absorption peaks at 280 and 603 nm were observed.

Figure 3.3. Fluorescence Emission Spectra of Purified SE-RFP. Emission peak was noticed at 334 nm when excited at 280 nm (A). Emission peak at 619 nm was observed upon exciting at 603 nm (B).
Fig. 3. Molecular weight determination of SE-RFP by Gel filtration chromatography using sepharose-6B column. (A) Gel filtration chromatography profile of SE-RFP. (B) Calibration curve of log molecular weights of standard molecular weight protein markers against partition coefficient (Kav).
Figure 3.5. Gel filtration profiles of SE-RFP in presence of different salt concentrations and denaturants. In each case, SE-RFP was eluted in a single peak.
Figure 3.6. Native-PAGE of SE-RFP. (A) Lane 1 was loaded with native SE-RFP; Lane 2, 3, 4 and 5 were loaded with SE-RFP aliquots denatured with urea, guanidinium hydrochloride, Triton X-100 and CHAPS, respectively. A single band of SE-RFP was detected in each lane when unstained electrophoretogram was observed under UV-light and, (B) the same gel stained with coomassie brilliant blue.
Figure 3.7. Agarose gel electrophoresis of purified SE-RFP. A red fluorescent band of SE-RFP (A) was observed under UV-light (366 nm), Gel stained with commassie brilliant blue showing SE-RFP band (B).

Figure 3.8. Urea-PAGE of purified SE-RFP. (A) A single band of SE-RFP was noticed when unstained electrophoretogram was observed under UV-light and, (B) after staining with coommassie brilliant blue.
Figure 3.9. 2D-PAGE of purified SE-RFP. (A). A red fluorescent zone was observed on the IPG strip (under UV light) after isoelectric focusing of SE-RFP. (B) The SE-RFP spot with a molecular weight of ~298 kDa and a pi of ~4.6 was detected by silver nitrate staining. Molecular mass of molecular weight protein markers separated in adjacent lane are indicated with arrows.
Fig 3.10. Conformational studies of SE-RFP. Far-UV CD spectra of
native (N), heat denatured (H), urea treated (U), SDS treated (S) and
pigment free (F) SE-RFP were recorded at 20°C, separately.
Figure 3.11. SDS-PAGE of SE-RFP. Degradation profile of SE-RFP in absence and presence of β-mercaptoethanol (lane 2 and 3, respectively). High range protein molecular weight markers were loaded in lane 1. To avoid contamination, in-between lanes were left empty. Eight prominent bands (shown with arrows, lane 3) were selected for MALDI-TOF-MS analysis.

Figure 3.12. SDS-PAGE of SE-RFP. (A) A red fluorescent front (f) of the tetrapyrroles (detached from protein) moving ahead of the bromophenol blue dye front (b) was observed under UV light. (B) The detached tetrapyrroles were appeared as a greenish front in visible light.
Figure 3.13. Gel filtration chromatography profile of SDS-treated SE-RFP. The absorption of eluted fraction was taken at 280 nm. The SE-RFP aliquot was incubated with 1% SDS solution prior to loading on gel filtration column packed with sepharose-6B.

Figure 3.14. Native-PAGE of native and heat denatured SE-RFP. Lane 1 was loaded with SERFP without heating. Another aliquot of SE-RFP heated for 5 min at 100°C and loaded on Lane 2. A single protein band was observed in each lane.
Figure 3.15. UV-absorption spectra of tetrapyrrole pigment mixture eluted from the gel after electrophoresis. Main absorption peaks in the region of 409 and 667 nm which are the characteristics of chlorophyll derivatives were observed.

Figure 3.16. Absorption spectra of SE-RFP in different conditions. Two main absorption peaks at 280 nm and 603 nm were observed for native SE-RFP. Heat denatured SE-RFP has showed a peak at 278 nm. SDS treated SE-RFP has absorption maxima at 603 and 276 nm.
Figure 3.17. Native-PAGE of SE-RFP incubated with different SDS concentrations. (A) SE-RFP aliquots were incubated with 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.0% of SDS. (B) SE-RFP aliquots were incubated with 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 10% and 0.0% of SDS. The samples were heated at 100°C for 2 minutes prior to loading onto gel. Movement of red fluorescent tetrapyrroles during electrophoresis was observed under UV-light. No fluorescent band was noticed in lane 7(A) due to effect of heat on SE-RFP. SE-RFP exhibited red fluorescence when it was untreated with SDS and loaded without heating as in lane 7 (B).

Figure 3.18. Effect of SDS concentration on electrophoretic mobility of tetrapyrrole released from SE-RFP. Graph of different SDS concentrations against electrophoretic mobility of tetrapyrroles was plotted.
Figure 3.19 (A). MALDI-TOF-MS analysis of SE-RFP. Peptide mass fingerprinting spectra of SE-RFP band obtained in native-PAGE (see inset figure).
Figure 3.19 (B). MALDI-TOF-MS analysis of SE-RFP. Peptide mass fingerprinting spectra of prominent SE-RFP subunits (labeled as F1, F2, F3, F4, F5, F6, F7 and F8) selected from SDS-PAGE (see Figure 3.11, lane 3). Several common m/z peaks were noticed in above spectra.
Figure 3. 20. SDS-PAGE of urea treated SE-RFP. A single protein band was observed.
Table 3.1 Proteins (in NCBI databases) showing significant sequence alignments with SE-RFP

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Table 3.2. Effect of specific protease inhibitors on SDS-induced proteolysis of SE-RFP

<table>
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<th>Protease inhibitor</th>
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<th>Inhibition of SDS-induced cleavage</th>
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<tr>
<td>Aprotinin</td>
<td>Inhibits serine proteases, plasmin, kallikrein, trypsin, chymotrypsin and urokinase.</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Bestatin-HCl</td>
<td>Inhibits Metalloprotease, cell surface aminopeptidases, leucine aminopeptidase.</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>Reversible inhibitor of chymotrypsin-like serine and some cysteine proteases.</td>
<td>No inhibition</td>
</tr>
<tr>
<td>E-64</td>
<td>Non-competitive irreversible inhibitor of papain and other cysteine proteases.</td>
<td>No inhibition</td>
</tr>
<tr>
<td>EDTA-Na2</td>
<td>Reversible inhibitor of metalloproteases.</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Leupeptin-hemisulfate</td>
<td>Inhibitor of serine and cysteine proteases, phospholipase D and C in rat hepatocytes.</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Reversible inhibitor of aspartic proteases like pepsin, cathepsin D, chymosin, rennin.</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

* Information was collected at: http://www.sciencegateway.org/resources/protease.htm