CHAPTER VI

ANTI-MICROBIAL MECHANISM OF
ACTION OF SE-RFP
 Chapter 6

ANTI-MICROBIAL MECHANISM OF ACTION OF SE-RFP

A remarkable broad spectrum antimicrobial activity of SE-RFP has been elucidated in previous chapter. In the present chapter, attempts have been made to discover the possible mechanisms of anti-\(BmNPV\) activity of SE-RFP.

1. RESULTS

a) SDS-INDUCED PROTEASE ACTIVITY

i. SDS-induced cleavage of purified SE-RFP

The homogeneity of the SE-RFP purified from excreta of silkworm \(Bombyx mori\) (Pure Mysore strain) was confirmed by conducting the re-chromatography and electrophoresis under different conditions as described in chapter III, section 1A. Surprisingly, the purified SE-RFP was observed to undergo an atypical degradation into a number of subunits in SDS-PAGE. The ladder of SE-RFP subunits in SDS-PAGE is as shown in figure 6.1. The approximate molecular masses of degradation products of SE-RFP are as listed in Table 6.1.

ii. SDS-induced protease activity of SE-RFP

SDS-PAGE of SE-RFP aliquots incubated in 0.2% SDS for different time intervals clearly indicated the SDS-induced protease activity of SE-RFP subunits (Figure 6.2). The high molecular weight protein bands were progressively disappeared in the subsequent lanes with the increase in the time of incubation (Figure 6.2). A graph obtained by plotting total number of protein bands (SDS-induced degradation
products of SE-RFP) versus time of incubation of SE-RFP with 0.2% SDS solution is shown in Figure 6.3.

No protein bands were detected when the SE-RFP was incubated with SDS solution for 10 hours prior to native-PAGE. An intact band of SE-RFP untreated with SDS was detected in lane 1 (Figure 6.4).

iii. Zymography of SDS-treated SE-RFP

The zymography technique was used to detect the SDS-induced protease activity of SE-RFP on *BmNPV* polyhedral protein. High molecular weight subunits of SE-RFP were observed to exhibit a strong protease activity on polyhedral protein (Figure 6.5). The SDS-induced protease activity was occurred even in presence of specific protease inhibitors like EDTA, aprotinin and leupeptin. However, the action of SE-RFP subunits was remarkably inhibited by PMSF.

iv. Zymography of fatty acid treated-SE-RFP

The zymography of linolenic acid-treated SE-RFP was performed by using the *BmNPV* polyhedral protein as substrate. The clear zone at the top position of the electrophoretogram was observed due to serine protease activity of high molecular weight subunits (of 261, 295 & 320 kDa) of SE-RFP on polyhedral protein (Figure 6.6). PMSF was observed to inhibit the fatty acid-induced protease activity of SE-RFP subunits.

v. N-terminal sequencing of protease band

One prominent SE-RFP subunit (of ~295 kDa) which has SDS and fatty acid-induced serine protease activity (Figure 6.5 and 6.6) was subjected to N-terminal sequencing. The obtained short queries of amino acid residues EPTVIELP have showed significant alignment with the sequences of some reported *Bombyx mori*
proteins listed in Table 6.2. Among the matched proteins, the chlorophyllide $a$
binding protein is a red fluorescent protein purified from silkworm mid-gut.\textsuperscript{16}

\noindent b) NUCLEASE ACTIVITY OF SE-RFP

As the SE-RFP was found to be a broad spectrum antimicrobial protein, it was
suspected to have additional mechanism(s) other than the SDS or fatty acid induced
protease action. The action of SE-RFP on genomic DNA of \textit{BmNPV} and $\lambda$-phage
DNA (used as a standard DNA) has been studies in this section.

A strong nuclease action of SE-RFP on \textit{BmNPV} genomic DNA and $\lambda$-phase
dNA was noticed by employing the agarose gel electrophoresis (Figure 6.7 A and B).
The gel pictures clearly indicated the presence of nuclease activity of the SE-RFP.
The DNA treated with SE-RFP was degraded; hence, no bands were appeared in lane
2 (Figure 6.7, A and B). Distinct bands of control DNA (untreated with SE-RFP)
were visualized in lane 1 (Figure 6.7, A and B).

2. DISCUSSIONS

Though there are several reports on anti-\textit{BmNPV} activity of RFPs, the
antiviral mechanism of RFPs is still incompletely understood. The possible
mechanisms of anti-\textit{BmNPV} activity of purified SE-RFP have been elucidated in the
present chapter. Interestingly, a rare phenomenon of SDS-induced cleavage followed
by the activation of latent protease activity has been observed here.

SDS is a strong surfactant that ruptures functional structure of most of the
proteins leading to the loss of their activity completely or partially.\textsuperscript{90} In contrast, SDS
has also been reported to stimulate the enzymatic activities of several proteins like
cystine protease of maize,\textsuperscript{91} tyrogeanse of frog\textsuperscript{92} and poly-phenoloxidases of spinach,
broad bean, horseshoe crab and spider.\textsuperscript{93-96} While, some of the proteins purified from
yeast\textsuperscript{97-98} and drosophila\textsuperscript{69-70,99} are reported to undergo proteolytic degradation in presence of SDS. However, none of the reported RFPs are known to undergo atypical cleavage in presence of SDS. Also, no information is available on the SDS-induced enzymatic activities of silkworm RFPs so far in the literature.

The protease activity associated with the SE-RFP could have been activated by SDS-treatment prior to electrophoresis. Particularly, the high molecular weight subunits (in the range of \textasciitilde 223-396 kDa) of SE-RFP might have associated with strong protease activity acting on themselves as well as on other subunits upon the time (Fig. 6.2 and 6.3). Hence, the disappearance of protein bands was due to SDS-induced autoproteolytic and/or heteroproteolytic activity exhibited by SE-RFP subunits.

The SE-RFP was unable to hydrolyse the substrate (BmNPV polyhedral protein) when it was incubated with PMSF prior to SDS-treatment followed by SDS-PAGE. Hence, it was concluded that the SE-RFP subunits are associated with the serine protease activity.

A silkworm digestive enzyme (BmSP-2) having serine protease activity was purified and suggested to be a potential antiviral factor against BmNPV at the initial site of viral infection.\textsuperscript{54} However, the SE-RFP differs from BmSP-2 in having a latent serine protease activity, huge molecular mass and a unique biochemical composition. A good many polypeptide chains may acquire protease activity by refolding during or after denaturation either alone or in combination with a polar/nonpolar component such as SDS.\textsuperscript{69-70} Other detergents like urea, guanidinium hydrochloride, CHAPS and Triton X-100 were unable to activate the associated latent protease activity of SE-RFP.
How the latent protease activity of SE-RFP gets activated in the body of insects? This obvious question was struck our mind as the SDS is not a physiological molecule of insect system.

The body of silkworm would contain plenty of the fatty acids (structurally related to SDS) as the silkworm feeds exclusively on mulberry leaves. Fatty acids are structurally comparable to the SDS. Linolenic acid is the main fatty acid present in silkworm body that has been supplied from dietary leaves.\(^{100}\) Hence, in the current studies, SE-RFP was treated with linolenic acid to test the induction of protease activity. The linolenic acid also induced the latent protease activity associated with the SE-RFP subunits (Figure 6.6).

Hence, the fatty acids might be involved in bringing the action of SE-RFP on viral protein, \(\textit{in vivo}\). Mitchel \textit{et al.} suggested that the fatty acids could play a role \(\textit{in vivo}\) similar to SDS \(\textit{in vitro}\).\(^{69-70}\) The enzymatic activities of spinach proteosome and rat protinases were known to be induced by SDS and fatty acids.\(^{101-102}\)

The SE-RFP subunit having SDS or fatty acid induced serine protease activity was found to have a significant homology with chlorophyllide \(\alpha\) binding protein purified from silkworm midgut cells. A silkworm midgut membrane protein, P252 (which can bind to chlorophyllide to form a RFP) also has significant homology with chlorophyllide a binding protein.\(^{18}\) Therefore, SE-RFP also may be synthesized in the midgut cells of the insect. The titin-like protein, titin-2 and titin are silkworm muscle proteins.\(^{57,103}\) Note that the SE-RFP spot obtained by 2D-PAGE was observed to have significant homology with the titin and titin-like protein, described in chapter 3, section 3.1B.g)
The life cycle of *BmNPV* has two phenotypically different but genetically identical forms, namely, occluded virus (Polyhedra or polyhedron derived virus) and a non-occluded virus. Both of these forms are pathogenic to the insects and perform a different role during pathogenesis. The polyhedra serve to transmit the infection from one insect to another, while free virions are responsible for systemic infection of the host. Virus particles when multiplied in host body, they become embedded in a protein matrix forming the inclusion bodies called polyhedra. The polyhedra are hexahedrons which consist of 3-5% viral particles, the rest is being contributed by the protein. This polyhedral protein coat provides an extraordinary resistance to occluded virus when they are released into the environment upon the death and decomposition of infected insects. Polyhedral protein coat also preserves the infectivity of virions for several years. Of the two *BmNPV* phenotypes the polyhedras are the main infectious elements for horizontal transmission through the midgut of a susceptible host.

In the present case, action of SE-RFP on polyhedral protein as well as the genomic DNA of *BmNPV* has been observed. The polyhedral protein coat was presumed to be destroyed by the protease action of SE-RFP. The released virions might lose their infectivity (partially or completely) due to their exposure to local environment. The SE-RFP was observed to wipe out the free virions as it has strong DNase activity. Though the protease activity of SE-RFP is SDS or fatty acid dependent, but its nuclease activity can operate independently, which does not require any pre-activation. Therefore, the SE-RFP could be an efficient anti-*BmNPV* agent which has an action on the protein as well as DNA of the virus.
FIGURES AND TABLES
Figure 6.1. SDS-PAGE of purified SE-RFP. A ladder of SE-RFP degradation products was observed in lane 2. Lane 1 was loaded with high range molecular weight protein markers.

Figure 6.2. SDS-PAGE of SE-RFP incubated with SDS for different time periods. SE-RFP aliquots incubated with 0.2% SDS for 0, 10, 30, 60, 180 and 360 minutes were loaded in lane 1, 2, 3, 4, 5 and 6, respectively. Lane 7 was loaded with high range protein molecular weight markers.
Figure 6.3. Progress of SDS-induced proteolysis of SE-RFP as a function of time. Graph was obtained by plotting the time periods of incubation of SE-RFP with 0.2% SDS solution against the number of protein bands obtained in SDS-PAGE.
Figure 6.4. Native-PAGE of SDS treated SE-RFP. Lane 2 and 3 were loaded with SE-RFP aliquots pre-incubated with 0.2% SDS for 4 and 12 hours, respectively. Lane 1 was loaded with SE-RFP without treating with SDS as a control.
Figure 6.5. Zymography of SDS-treated SE-RFP. The SE-RFP aliquots pre-treated separately with different specific protease inhibitors, namely, PMSF (5 mM), EDTA (20 mM), aprotinin (0.4 μg) and leupeptin (50 μM) were mixed with the standard SDS-PAGE sample buffer and loaded under non-denaturing conditions onto lane 1, 2, 3 and 4. Lane 5 was loaded with SE-RFP dissolved in SDS sample buffer without the pre-treatment of any protease inhibitor. Serine protease activity of high molecular weight subunits of SE-RFP on polyhedral protein was noticed.
Figure 6.6. Zymography of fatty acid-treated SE-RFP. The SE-RFP aliquots pre-treated separately with different specific protease inhibitors, namely, PMSF (5 mM), EDTA (20 mM), aprotinin (0.4 μg) and leupeptin (50 μM) were mixed with the standard SDS-PAGE sample buffer and loaded under non-denaturing conditions onto lane 1, 2, 3 and 4. Lane 5 was loaded with SE-RFP dissolved in SDS sample buffer without the pre-treatment of any protease inhibitor. High molecular weight subunits of SE-RFP were observed to exhibit a serine protease activity of on polyhedral protein was observed.
Figure. 6.7. DNase activity of SE-RFP. Agarose gel electrophoresis of BmNPV genomic DNA (A) and λ-phage DNA (B). Samples in the lane 2 were treated with SE-RFP prior to electrophoresis.
Table 6.1. Approximate molecular weights of SE-RFP subunits obtained in SDS-PAGE.

<table>
<thead>
<tr>
<th>Band number</th>
<th>Standard molecular weight protein markers (in kDa)</th>
<th>SE-RFP proteolytic products (in kDa)</th>
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<tbody>
<tr>
<td>1</td>
<td>200</td>
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<tr>
<td>2</td>
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Table 6.2. N-terminal sequencing analysis of SE-RFP subunit having SDS or fatty acid-induced protease activity.

<table>
<thead>
<tr>
<th>Reported <em>B. mori</em> proteins showing significant alignment</th>
<th>Identities</th>
<th>Positives</th>
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<tr>
<td>Coiled coil domain containing 12-like protein</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>Chlorophyllide a binding protein precursor</td>
<td>6/7 (85%)</td>
<td>6/7 (85%)</td>
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<td>Titin-like protein</td>
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<td>7/8 (87%)</td>
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<td>Titin 2</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
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<tr>
<td>Titin</td>
<td>5/8 (62%)</td>
<td>7/8 (87%)</td>
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</table>