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SDS-induced Serine Protease Activity of an Antiviral Red Fluorescent Protein

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***Abstract*—**A rare phenomenon of SDS-induced activation of a latent protease activity associated with the purified silkworm excretory red fluorescent protein (SE-RFP) was noticed. SE-RFP aliquots incubated with SDS for different time intervals indicated that the protein undergoes an obligatory breakdown into a number of subunits which exhibit autoproteolytic (acting upon themselves) and/or heteroproteolytic (acting on other proteins) activities. A strong serine protease activity of SE-RFP subunits on *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) polyhedral protein was detected by zymography technique. A complete inhibition of *Bm*NPV infection to silkworms was observed by the oral administration assay of the SE-RFP. Here, it is proposed that the SE-RFP prevents the initial infection of *Bm*NPV to silkworms by obliterating the polyhedral protein. This is the first report on a silkworm red fluorescent protein that exhibits a protease activity on exposure to SDS. The present studies would help in understanding the antiviral mechanism of silkworm red fluorescent proteins.

Keywords*—Bm*NPV, polyhedra, SE-RFP, SDS-induced protease activity, zymography.

I. Introduction

SDS is a strong surfactant that ruptures functional structure of most of the proteins leading to the loss of their activity completely or partially [1]. In contrast, SDS has also been reported to stimulate the enzymatic activities of several proteins like cystine protease of maize [2], tyrogenase of frog [3] and poly-phenoloxidases of spinach, broad bean, horseshoe crab and spider [4-7]. While, some of the proteins purified from yeast [8, 9] and drosophila [10-12] are reported to undergo proteolytic degradation in presence of SDS.

Red fluorescent proteins (RFPs), a kind of chlorophyll-binding proteins are reported to be present in the silkworm gut-juice/gut-cells [13-21]. RFPs have exhibited significant activity against *Bm*NPV, a most hazardous silkworm infecting virus. However, the anti-*Bm*NPV mechanism of RFPs is not yet understood completely. None of the reported RFPs are known to undergo structural and functional changes in presence of SDS. A few of the purified silkworm mid-gut proteins are reported to possess different enzymatic activities (namely, oxidoreductase, lipase and protease) against *Bm*NPV [22-25]. But no information is available on the induction of activities of these proteins by SDS.

Our recent publications have pioneered the accumulation of information on SE-RFP, the only red fluorescent protein that has been purified from the silkworm excreta, to date [26, 27]. The SE-RFP was found to have an interesting biochemical/physical properties and a significant anti-*Bm*NPV activity [26]. A remarkable broad spectrum bioactivity of SE-RFP against several pathogenic microorganisms has also been reported [27]. But no studies have been reported on the possible anti-microbial mechanism of SE-RFP, to date. In the present work, attempts have been made to discover the antiviral mechanism of SE-RFP. Present investigations have revealed that the purified SE-RFP undergoes an eccentric cleavage yielding several subunits that exhibit an acquired protease activity in presence of SDS.

II. Materials and Methods

A. Insects

The polyvoltine virus resistant strain, namely, Pure Mysore of silkworm *Bombyx mori* larvae were reared on fresh mulberry leaves in a hygienically maintained rearing room. The disease free silkworm layings were obtained from Central Silkworm Germplasm Resource Center, Hosur, Tamil Nadu, India. The excreta of healthy silkworms was collected on 3rd day of 5th instar.

B. Purification of SE-RFP

The SE-RFP was purified as per the procedures described by Matti et al [26, 27]. The silkworm excreta was suspended in 10 mM Tris-HCl saline buffer (1:10 w/v) pH 7.6, and stirred for 10 hours at 1000 rpm under mechanical stirrer at 4°C. The mixture was filtered with cheese cloth and filtrate was centrifuged at 4000 rpm for 30 minutes at 4°C. Solid ammonium sulfate was added to the supernatant to obtain 20% saturation and then centrifuged at 8000 rpm for 30 minutes at 4°C. The precipitate was dissolved in a minimum volume of 5 mM Tris-HCl buffer pH 7.6, and dialyzed against same buffer, for 48 hours. The dialysed protein sample (10 mg/ml) was subjected to the gel filtration chromatography using an 85×2 cm column packed with Sepharose-6B (Sigma). The red fluorescence of SE-RFP band moving through the column was observed under UV-light (366 nm) and the eluates were collected in a fixed volume of 1.5 ml each.

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C. Electrophoresis

SDS-PAGE was performed in 7.5% polyacrylamide gel by the method of Laemmli [28]. The SE-RFP aliquots were separately dissolved in sample buffer composed of Tris-HCl buffer (pH 6.8) containing 0.2% SDS, 20% glycerol and 0.001% bromophenol blue and 5% β-mercaptoethanol. The samples were heated at 100^0 C for 5 minutes before loading. The high range molecular weight protein markers (Sigma), namely, myosin (200 kDa), β-galactosidase (116,000), phosphorylase b (97 kDa), Bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) were run in an adjacent lane. In a separate set, SDS-PAGE of SE-RFP aliquots incubated with a fixed concentration of SDS for different time periods was carried out. SE-RFP aliquots (each of 50 µg/25 µl) incubated with 0.2% SDS for 0, 10, 30, 60, 180, 360 minutes followed and subjected to SDS/PAGE. The samples were heated at 100^0 C for 5 minutes before loading onto gel. The high range molecular weight protein markers (Banglore Genei), namely, β-galactosidase (204 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) were run in an adjacent lane. Native-PAGE of SE-RFP aliquots incubated with 0.2% SDS for 4 and 12 hours was also performed.

D. Zymography

The identification of protease activity of SE-RFP subunits was performed by using zymography technique [29]. The SE-RFP aliquots (25 µg each) pre-treated separately with different protease inhibitors, namely, PMSF (5 mM), EDTA (20 mM), aprotinin (0.4µg) and leupeptin (50 µM) were mixed with the standard SDS-PAGE sample buffer (without a reducing agent) and loaded without boiling onto 8% polyacrylamide gel supplemented with 0.2% *Bm*NPV polyhedral protein. Soon after the electrophoresis, gels were washed with 2.5% Triton X-100 for 3 times (20 min each) and incubated for 2 hrs in enzyme assay buffer (25 mM Tris, pH 7.5, 5 mM $CaCl_2$, 0.9% NaCl, 0.05% Na_3N) for the development of enzyme activity bands. After incubation, the gels were stained with 0.05% Coomassie brilliant blue G-250 in a mixture of methanol: acetic acid: water (2.5: 1: 6.5) and de-stained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the blue background. The similar procedure was adopted to test protease activity of fatty-acid treated SE-RFP on *Bm*NPV polyhedrin protein.

E. Preparation of polyhedral

The sources for preparation of polyhedra were the hemolymph, collected shortly before death of experimentally diseased (*Bm*NPV infected at laboratory conditions) fifth instar larvae of the silkworm. The polyhedra were collected from the hemolymph by settling out for a few days in the cold. The sediment containing polyhedral was purified by the procedures described by Ponnuveil *et al* [30].

F. Bioassay of SE-RFP

Oral administration procedure was employed to test the activity of SE-RFP against *Bm*NPV infection to silkworms [26, 17]. Fourth instar NB_4D_2 larvae (*Bm*NPV susceptible race of silkworm) were used in triplicate set (30 × 3). The larvae were fed with a fresh and clean mulberry leaf smeared with 100 µl of SE-RFP solution (2 mg/ml) on one side and 100 µl of *Bm*NPV polyhedral solution (1 x 10^5 polyhedral inclusion bodies/ml) on its opposite side, separately. Positive (treated only with same amount of SE-RFP), negative (treated only with same number of *Bm*NPV polyhedra) and untreated controls of silkworm larvae also were maintained. The larval mortality was recorded twice a day. Each percent survival value was corrected by Abbott's formula [26].

III. Results and Discussions

SDS-induced cleavage of purified SE-RFP

The SE-RFP present in the silkworm excretory extract was purified by ammonium sulfate precipitation followed by gel filtration chromatography. The homogeneity of purified SE-RFP was confirmed by conducting the re-chromatography and electrophoresis under different conditions as described previously [26]. Surprisingly, the purified SE-RFP was observed to undergo an atypical degradation into a number of subunits (~16) in SDS-PAGE. The ladder of SE-RFP subunits in SDS-PAGE is as shown in fig. 1. The breakdown of SE-RFP coupled with release of its chromophoric moieties was presumed to be induced by SDS.

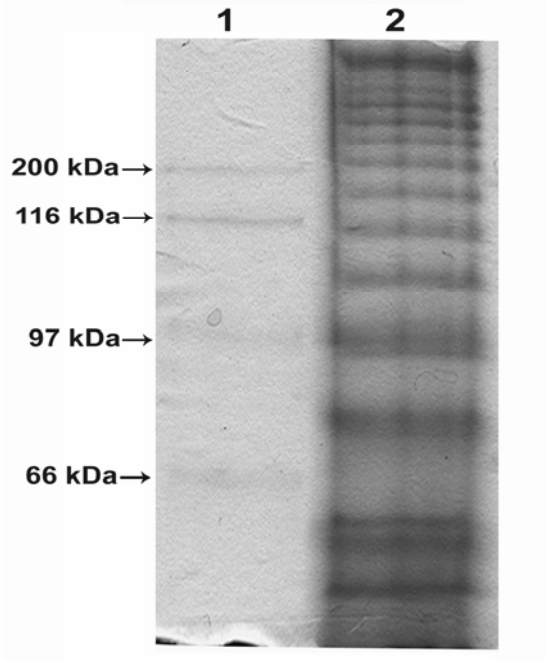

Fig. 1 SDS-PAGE of purified SE-RFP. A ladder of SE-RFP degradation products was observed in lane 2. Lane 1 was loaded with high range molecular weight protein markers.

The approximate molecular masses of degradation products of SE-RFP are as listed in Table I.

TABLE I
MOLECULAR MASSES OF SE-RFP SUBUNITS OBTAINED BY SDS-PAGE[A]

Band number	Standard molecular weight protein markers (in kDa)	SE-RFP proteolytic products (in kDa)
1	200	396
2	116	366
3	97	348
4	66	320
5		295
6		261
7		223
8		169
9		113
10		106
11		095
12		90
13		89
14		58
15		54
16		44

[a] (see Fig. 1)

SDS-induced protease activity of SE-RFP

SDS-PAGE of SE-RFP aliquots incubated in 0.2% SDS for different time intervals clearly indicated that the SE-RFP subunits exhibit SDS-induced protease activity. The high molecular weight protein bands were progressively disappearing in the subsequent lanes with the increase in the time of incubation (Fig. 2).

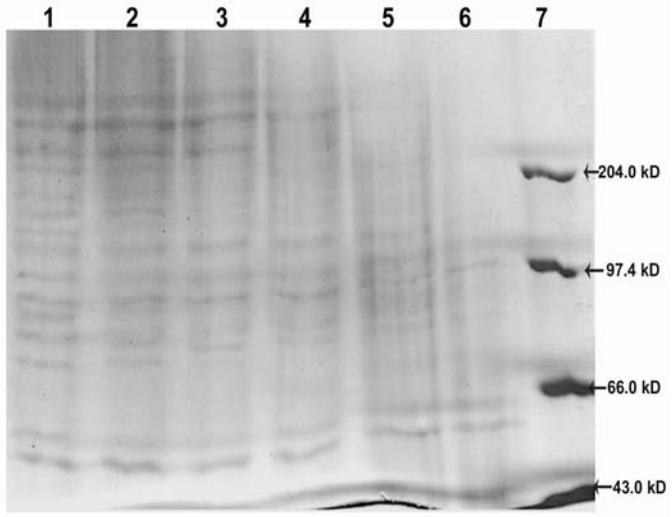

Fig. 2 SDS-PAGE of SE-RFP incubated with SDS for different time periods. SE-RFP aliquots incubated with 0.2% SDS for 0, 10, 30, 60, 180 and 360 minutes were loaded in lane 1, 2, 3, 4, 5 and 6, respectively. Lane 7 was loaded with high range protein molecular weight markers.

No protein bands were detected in native-PAGE when the SE-RFP was incubated with SDS solution for ≥ 10 hours as all the subunits of SE-RFP were found to vanish completely (Fig. 3).

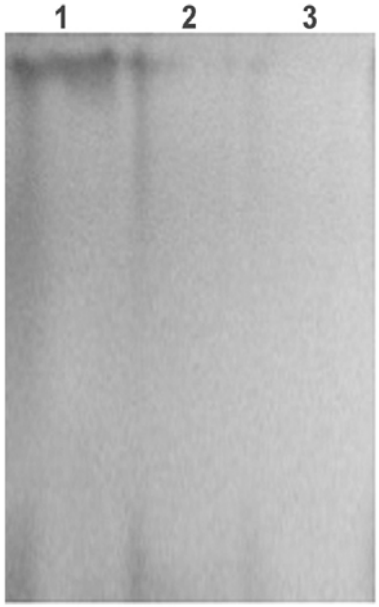

Fig. 3 Native-PAGE of SDS treated SE-RFP. Lane 2 and 3 were loaded with SE-RFP aliquots pre-incubated with 0.2% SDS for 4 and 12 hours, respectively. Lane 1 was loaded with SE-RFP without treating with SDS as a control.

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 ~223-396 kDa) of SE-RFP might have associated with strong protease activity acting on themselves as well as other subunits upon the time.

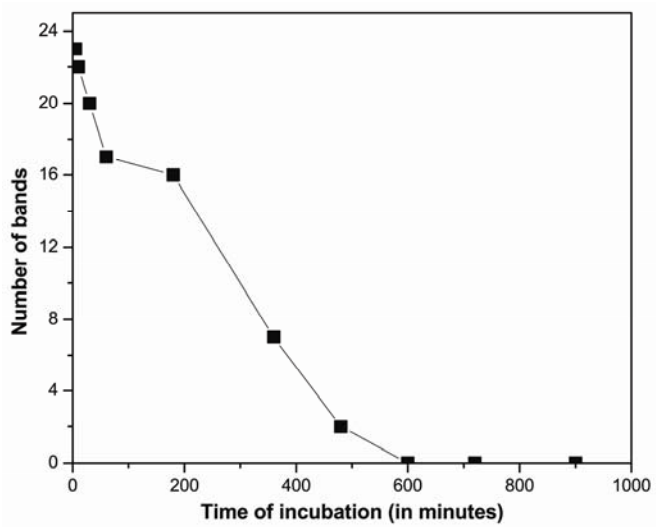

Fig. 4 Progress of SDS-induced proteolysis of SE-RFP as a function of time. Graph was obtained by plotting the time periods of incubation of SE-RFP with 0.2% SDS solution against the number of protein bands obtained in SDS-PAGE.

Hence, the disappearance of protein bands was due to SDS-induced autoproteolytic/heteroproteolytic activity exhibited by SE-RFP subunits. A graph obtained by plotting total number of protein bands (SDS-induced proteolytic products of SE-RFP) versus time of incubation of SE-RFP with 0.2% SDS solution is shown in Fig. 4.

Zymography of SDS-treated SE-RFP

The zymography technique was used to detect the SDS-induced protease activity of SE-RFP on *Bm*NPV polyhedral protein. High molecular weight subunits of SE-RFP were observed to exhibit a strong protease activity on polyhedral protein (Fig. 5). However, the action of SE-RFP subunits was remarkably inhibited by phenylmethylsulfonyl fluoride (PMSF).

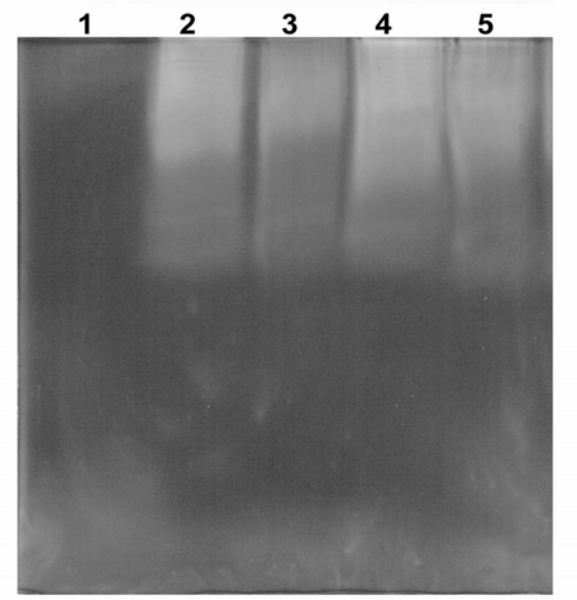

Fig. 5 Zymography of SDS-treated SE-RFP. The SE-RFP aliquots pre-treated separately with different specific protease inhibitors, namely, PMSF (5 mM), EDTA (20 mM), aprotinin (0.4µg) and leupeptin (50 µM) were mixed with the standard SDS-PAGE sample buffer and loaded under non-denaturing conditions onto 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.

The SE-RFP was unable to hydrolyse the substrate (*Bm*NPV polyhedral protein) when it was pre-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 *Bm*NPV at the initial site of viral infection [24].

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 [10, 11]. Other detergents like urea, guanidinium hydrochloride, CHAPS and Triton X-100 were unable to activate the associated latent protease activity of SE-RFP.

Zymography of fatty acid treated-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.

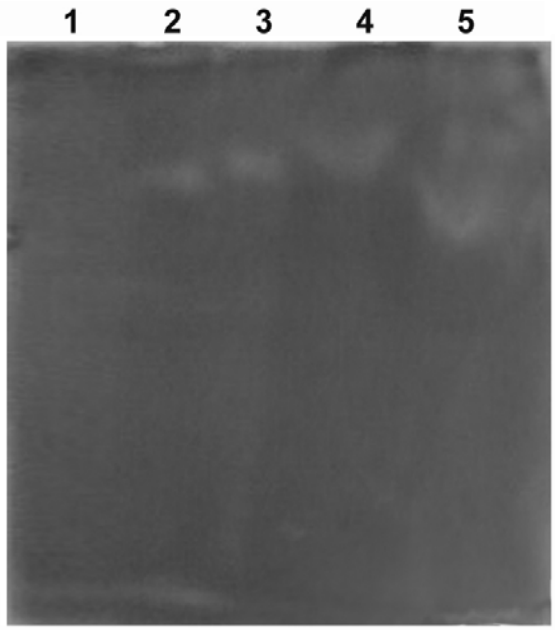

Fig. 6 Zymography of fatty acid-treated SE-RFP. The SE-RFP aliquots pre-treated separately with different specific protease inhibitors, namely, PMSF (5 mM), EDTA (20 mM), aprotinin (0.4µg) and leupeptin (50 µM) were mixed with the standard SDS-PAGE sample buffer and loaded under non-denaturing conditions 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.

The body of silkworm would contain plenty of the fatty acids (structurally related to SDS) as the silkworm feeds exclusively on mulberry leaves. Linolenic acid is the main fatty acid present in silkworm body that has been supplied from dietary leaves [31]. Hence, in the current studies, induction of protease activity of SE-RFP by linolenic acid was tested. Consequently, the linolenic acid was observed to activate the latent serine protease activity of SE-RFP.

The zymography of linolenic acid-treated SE-RFP was performed by using the *Bm*NPV 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 (Fig. 6). Hence, the fatty acids might be involved in bringing the action of SE-RFP on viral protein, *in vivo*. Mitchel *et al.* suggested that the fatty acids could play a role *in vivo* similar to SDS *in vitro* [11]. The enzymatic activities of spinach proteosome and rat

protinases were known to be induced by SDS and fatty acids [32, 33].

Oral administration assay of SE-RFP against BmNPV polyhedra

The results of oral administration assay of SE-RFP against *Bm*NPV-polyhedra are shown in Table II. Hundred percent survival of the silkworm larvae fed with *Bm*NPV-polyhedra along with SE-RFP was noticed. The silkworm larvae were found to remain healthy on feeding with SE-RFP (positive control) indicating the SE-RFP has no insecticidal activity. Whereas, 100% mortality of silkworm larvae was observed when they were fed with *Bm*NPV-polyhedra (negative controls).

TABLE II
ORAL ADMINISTRATION ASSAY OF SE-RFP

Sl. No.	Treated groups	No. of silkworms treated	Percent mortality	Percent survival
1	Untreated control	30 X 3	04	96
2	SE-RFP (+ ve control)	30 X 3	00	100
3	*Bm*NPV polyhedra (- ve control)	30 X 3	98	02
4	*Bm*NPV polyhedra + SE-RFP	30 X 3	00	100

The life cycle of *Bm*NPV has two phenotypically different but genetically identical forms, namely, occluded virus (Polyhedra or polyhedron derived virus) and a non-occluded virus [24]. Both of the 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 [34]. 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 [30]. 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 [34]. Polyhedral protein coat also preserves the infectivity of virions for several years [34].

Oral administration assay of SE-RFP and some gut-juice RFPs has resulted into a significant activity against *Bm*NPV non-occluded virus [26, 17]. Of the two *Bm*NPV phenotypes the polyhedras are the main infectious elements for horizontal transmission through the midgut of a susceptible host [30, 34]. Hence, in the present case, *Bm*NPV polyhedra (polyhedron derived virus) were used for the SE-RFP bioassay experiment. 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. However, the free virions released from polyhedra into the hemocoel could possibly penetrate the intestinal cells to bring the infection. Astonishingly, the SE-RFP was observed to wipe out the free virions as it is also associated with a strong DNase activity (data not shown). Therefore, the SE-RFP could be an efficient anti-*Bm*NPV agent which has an action on the protein as well as DNA of the virus.

IV. CONCLUSIONS

SDS cleaves SE-RFP yielding several protein subunits that possess auto/heteroproteolytic activity. The SE-RFP has exhibited a SDS-induced serine protease activity on polyhedral coat protein of *Bm*NPV. Linolenic acid was found to be effective in mimicing the SDS effect on SE-RFP in producing protease activity. Successful efforts are made to have some understanding the complex mechanism of anti-*Bm*NPV mechanism. The oral administration of SE-RFP to silkworms yielded 100% resistance against *Bm*NPV infection. The SE-RFP might be inactivating the ingested polyhedra at the initial infection site before they propagate in the midgut and spread into hemocoel of the insect. These results have encouraged us to initiate exploiting in-field application of SE-RFP to prevent the nucleopolyhedrovirus disease to silkworms.

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