Article

Double-Stranded RNA-Degrading Enzymes Reduce the Efficiency of RNA Interference in *Plutella xylostella*

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Abstract: DsRNA-degrading enzymes (dsRNases) have been recognized as important factors in reducing RNA interference (RNAi) efficiency in different insect species. However, dsRNases in *Plutella xylostella* are still unknown. We identified the full-length cDNAs of four dsRNases in diamondback moths. In vitro, the recombinant protein of PxdsRNase1 degraded dsRNA completely and PxdsRNase3 cleaved dsRNA without complete degradation. Overall, our findings provided a fundamental basis for understanding the mechanism of dsRNA involvement in the RNAi process and using RNAi to control diamondback moths in the future.

Simple Summary: The efficiency of Lepidoptera RNA interference (RNAi) is highly varied among different species, different periods, and different genes. The stability of dsRNA is one of the important factors. DsRNA-degrading enzymes (dsRNases) are the key factors affecting the stability of dsRNA in insects. The efficiency of RNAi in diamondback moths was low and unstable. Furthermore, in vitro experiments, we found that dsRNA was completely degraded when incubated with the hemolymph or gut fluid of diamondback moths. Therefore, we hypothesized that the efficiency of RNAi in diamondback moths was decreased predominantly due to degradation of dsRNA by dsRNase. In this study, we identified four dsRNases in diamondback moths: PxdsRNase1 was mainly expressed in the hemolymph; and PxdsRNase2 and PxdsRNase3 were mainly expressed in the intestinal tract. PxdsRNase1, PxdNRNase2, and PxdRNase3 were verified to be involved in the RNAi process in diamondback moths. In vitro, the recombinant protein of PxdsRNase1 degraded dsRNA completely and PxdsRNase3 cleaved dsRNA without complete degradation. Overall, our findings provided a fundamental basis for understanding the mechanism of dsRNA involvement in the RNAi process and using RNAi to control diamondback moths in the future.

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PxdsRNases3 were involved in the dsRNA degradation to reduce RNAi efficiency with different mechanisms.

Keywords: dsRNase; RNA interference; diamondback moth; RNA degradation

1. Introduction

The efficiency of Lepidoptera RNAi is highly varied among different species, different periods, and different genes [1]. The dsRNA degradation [2–4] or the inability of dsRNA to enter the cytoplasm [3,5,6] are the main factors affecting RNAi efficiency. Studies have also shown that dsRNA needs to last long enough in the midgut or hemolymph to be absorbed into cells to produce an effective RNAi response [7]. DsRNases are the key factors affecting the stability of dsRNA in insects. The activity of dsRNase has been observed in several insects. In *Bombyx mori*, dsRNase is expressed in the digestive juice and midgut, then secreted into the intestinal cavity for nucleic acid digestion [8,9]. Subsequently, dsRNases are found in more and more insects, such as *Lygus lineolaris* [10], *Manduca sexta* [11], *Acyrthosiphon pisum* [12], *Schistocerca gregaria* [13], and *Spodoptera frugiperda* [14].

In the hemolymph of *M. sexta*, dsRNA is degraded within 1 h, which proves the presence of dsRNase [11]. The RNAi efficiency is increased after the expression of dsRNase in *Locust migratoria* and *S. gregaria* is suppressed [15], verifying that the RNAi efficiency could be affected by dsRNase in insects. Furthermore, in *Anthonomus grandis*, the RNAi sensitivity is increased after dsRNA of nuclease is ingested to suppress the nuclease activity [16]. Interestingly, multiple dsRNases working together could enhance their effect on RNAi efficiency in *Spodoptera litura* [17,18]. The RNAi efficiency is increased after knockdown of RNAi efficiency-related nucleases (REases) in *Ostrinia furnacalis*, and suppressed after up-regulation of REase in *Drosophila melanogaster* [19].

With in vitro experiments, we found that dsRNA was completely degraded when incubated with the hemolymph or gut fluid of *P. xylostella*. Therefore, we hypothesized that the efficiency of RNAi in *P. xylostella* was decreased predominantly due to degradation of dsRNA by dsRNase. To verify this hypothesis, we performed a genome-wide search to identify genes encoding dsRNases in the *P. xylostella* and conducted function analysis of these dsRNases to understand the role of dsRNases in the RNAi process in *P. xylostella*.

2. Materials and Methods

2.1. Insect Rearing

The *P. xylostella* Fuzhou-sensitive strain (FZss) used in the experiment was maintained at the Institute of Applied Ecology, Fujian Agriculture and Forestry University, Fuzhou, China. The colony was maintained on radish (*Raphanus sativus*) seedlings without exposure to any chemical insecticide at 25 °C with the photoperiod of 16L: 8D and 60–70% relative humidity (RH) in the growth chamber.

The *P. xylostella* sensitive strain (SLss) was reared by the artificial diet [20]. Pupae were collected in a paper cup with a cotton wool containing 10% honey as extra nutrition for adults, and eggs were collected by a parafilm card stained with vegetable powder hung in the cup. The insect was reared at 26 ± 1 °C, 60–80% RH, and the photoperiod of 16L: 8D.

2.2. Isolation and Sequencing of PxdsRNase cDNAs

The *BmdsRNase* (dsRNase of *B. mori*, GenBank ID: NP_001091744.1) was used as a query in tBLASTn to search the genomic database of *P. xylostella* (http://59.79.254.1/DBM/blast.php) (accessed on 24 October 2017)) for obtaining the cDNA sequence of *PxdsRNase*. Total RNA was extracted from the 4th-instar larvae using the RNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer’s instructions. First-strand complementary DNA (cDNA) synthesis was performed from 1 μg of total RNA using the reverse transcription kit (Promega, Madison, WI, USA). PCR was performed with
the cDNA template and gene-specific primers (Table 1) to amplify the full-length cDNA sequence of *PxdRNase* in the following conditions: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 15 s, and 72 °C for 2 min; and 72 °C for 10 min. The PCR product was purified using the E.Z.N.A.™ Gel Extraction kit (Omega, Doraville, GA, USA), inserted into the pESI-Blunt Zero plasmid (Yeasen, Shanghai, China), and then sequenced in two directions by Shangya Biological Company (Fuzhou, China).

### Table 1. Primers used in the experiment.

| Use of Primers | Gene   | Primer Sequence (5′-3′) |
|----------------|--------|-------------------------|
| **cDNA cloning** | *PxdRNase1* | F: ATGCTTCACAAATGGGTTCTAATG<br>R: TCACAGTAGTAATCCAGTGACTTTG |
|                 | *PxdRNase2* | F: ATGATTGTTCGGTGTTGTTGTCGC<br>R: TTAACCTAAAGGCCACATCACGTTAAAAG |
|                 | *PxdRNase3* | F: ATGCTGCGTCCTTGTCTGC<br>R: TTAAGCCAACAGCCCATTAATCCG |
|                 | *PxdRNase4* | F: ATGATTTCACAAAAAATTTTACAG<br>R: TTACACTTTCTTCCGATGCTAG |
| **qPCR primer** | *PxdRNase1* | F: GCCGGAATGTACCCCTCCTGT<br>R: GGACTCAGCAGCCAATCAAC |
|                 | *PxdRNase2* | F: CGGCGTAGAAGATCCTCTGT<br>R: CGAACTCCCTCTCCGCAGCC |
|                 | *PxdRNase3* | F: AAGATGACCCAACTCAGTGCC<br>R: AGCGACACGGCTCCCGAATA |
|                 | *PxdRNase4* | F: GCCCTCCCTACACCGAATC<br>R: CCTTGAAACAGGGCATCT |
|                 | *ExF1* | F: AGACTTGATGTGGTCCGGT<br>R: GTCCAACCTTCTGCCCCTAG |
|                 | *PxCht* | F: GCCGGAATGTACCCCTCCTGT<br>R: GGACTCAGCAGCCAATCAAC |
| **dsRNA primer** | *dsPxdRNase1* | F: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC<br>R: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC |
|                 | *dsPxdRNase2* | F: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC<br>R: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC |
|                 | *dsPxdRNase3* | F: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC<br>R: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC |
|                 | *dsPxdRNase4* | F: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC<br>R: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC |
|                 | *dsPxCht* | F: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC<br>R: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC |
|                 | *dsGFP* | F: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC<br>R: TAATACGACTCCTATAGGTTAGGATGACTCTCAAGCTCACCCCAAC |

Note: The underline sequence represents the sequence of T7 promoter.

### 2.3. Amino Acid Sequence Analysis of *PxdRNases*

The cDNA sequences of *PxdRNases* were translated into the amino acid sequence by the online tools available from the ExPaSy website (https://web.expasy.org/translate/ (accessed on 16 November 2017)). Domain architecture and signal peptides were predicted by the SMART domain analysis (http://smart.embl-heidelberg.de/ (accessed on 16 November 2017)) and the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/ (accessed on 16 November 2017)), respectively. Web BLAST tools (https://blast.ncbi.nlm.nih.gov/
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(accessed on 16 November 2017)) were used to analyze the conserved regions, including the endonuclease NS domain, active site, Mg\textsuperscript{2+} binding site, and substrate binding site.

2.4. Determination of Gene Expression of PxdsRNase Genes in Different Tissues and Developmental Stages

For tissue-specific expression of PxdsRNase genes, the total RNA was extracted from the integument, fat body, gut, malpighian tubule, hemolymph, silk and head of 20 4th-instar larvae. For determining the gene expression in different developmental stages, the total RNA was extracted from eggs, 1st, 2nd, 3rd, 4th larvae, pupae and adults. The first-strand cDNA was synthesized from 1 µg of total RNA using the reverse transcription kit (Promega, Madison, WI, USA). PxdsRNase-, PxCht-, EF1-specific primers used for the quantitative PCR (qPCR) are shown in Table 1. Each 20 µL of qPCR mixture consisted of 10 µL SYBR™ Green Real-time PCR Master Mix (Promega, Madison, WI, USA), 2 µL of 10-fold diluted template cDNA, 0.4 µL of 10 µM of each primer, 0.15 µL of ROX and 7.05 µL of deionized water. The PCR reaction conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. A melt curve was developed to confirm the amplification specificity for each qPCR. Three technical replicates of each qPCR and three biological replicates of each treatment were conducted. Gene expression level was analyzed using the \(2^{-\Delta\Delta CT}\) method, and statistical analyses were performed using one-way ANOVA (analysis of variance) followed by the Turkey test (\(p < 0.05\), SPSS software, SPSS Inc. Chicago, IL, USA).

2.5. In Vitro Incubation of dsRNA with Hemolymph/Gut Fluid

Hemolymph was collected with a capillary glass tube from the amputated legs of 30 4th-instar larvae, diluted with 50 µL of PBS, and then centrifuged at 16,000× g for 10 min to remove hemocytes. The supernatant was collected and stored at −20 °C. Meanwhile, guts from 30 larvae of P. xylostella were dissected and collected in cold 1.5 mL tubes with 50 µL of 1× PBS buffer, and centrifuged at 16,000× g for 10 min. The supernatant was collected and stored at −20 °C.

The total protein concentration of the hemolymph or gut fluid was examined using the BCA Protein Quantification kit (Yesea, Shanghai, China), according to the manufacturer’s instruction. Measurements were performed in the Synergy Mx microplate reader (BioTek, Doraville, GA, USA).

For an in vitro incubation assay, 1 µL of dsPxCht solution (containing 120 ng of dsRNA, 564 bp) was mixed with 10 µg of total protein of hemolymph or gut fluid in 1.5 mL microcentrifuge tube and incubated at 28 °C for 0 min, 5 min, 30 min, 60 min, 360 min, and 600 min, separately. Next, 120 ng of dsCht was mixed with 1 µg, 5 µg, 10 µg, 20 µg, and 30 µg of total protein of hemolymph or gut fluid in 1.5-mL microcentrifuge tube and incubated at 28 °C for 5 min. After incubation, these samples were mixed with 1 µL of loading buffer and checked in 1% agarose gel. The gel was visualized using a UV transilluminator (Vilber, Pairs, France) to analyze the integrity of dsRNA.

2.6. RNAi Response after dsRNA Injection or Oral Delivery

DsRNAs were prepared in vitro by the T7 RiboMAX Express RNAi System (Promega, USA). Primers (Table 1) for dsRNA synthesis of PxCht (564 bp), GFP (417 bp) and PxdsRNases (407 bp of PxdsRNases1, 476 bp of PxdsRNases2, 458 bp of PxdsRNases3, and 412 bp of PxdsRNases4) were designed using the NCBI web service (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (accessed on 8 May 2018)). The templates containing the T7 promoter sequence at both ends were synthesized through PCR by 2× Taq Master Mix (Vazyme, Nanjing, China). The PCR was performed at the condition: 94 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were purified using the E.Z.N.A™ Gel Extraction Kit (Omega, Doraville, GA, USA). About 2 µg of each purified PCR product was used to synthesize dsRNA, dsRNA was dissolved with 20 µL of nuclease-free water, and the final concentration of dsRNA was adjusted to 4.0 µg/µL.
To compare the RNAi responses induced by dsRNA between injection and oral delivery, *PxCht* involved in the molting process was selected as the RNAi target gene. DsGFP, dsPxdsRNase, the mixture of dsGFP and dsPxCht, and the mixture of dsPxCht and dsPxdsRNase were separately injected into the internode of abdomen of one 4th-instar larva of FZs at the amount of 600 ng by a microsyringe and separately fed to the 100 larvae at the amount of 3 µg. All the treated larvae were reared in the same condition, as previously described. The total RNA of 5 larvae were extracted for each treatment, and RT-qPCR was performed with the primers listed in Table 1 to determine the expression of *PxCht* and *PxdsRNase* using *EF1* as the reference gene. Three replications were performed for each treatment. The Turkey test after one-way ANOVA was used for determining differences in RNAi efficiency among different treatments.

### 2.7. Heterologous Expression of PxdsRNases

The codon-optimized cDNA sequences of *PxdsRNase1*, *PxdsRNase2*, and *PxdsRNase3* without the signal peptide and with 6× His tags attached at its 3′ end, were individually inserted at *NdeI/HindIII* in PET-30a(+) vector by the Genscript Biotech Corporation (Nanjing, China). The constructed plasmids were individually transformed into *E. coli* DH5α competent cells, and the subsequent cells were cultured at 37 °C for 14 h. Positive clones were picked and verified by PCR and sequencing.

The recombinant plasmid extracted from the transformed *E. coli* DH5α was transformed into *E. coli* BL21 (DE3). Then, the dsRNases were expressed by the method of He et al. [21], and extracted by the ultrasonically crushing method [22]. The extracted proteins were identified by SDS-PAGE and Western blot.

### 2.8. Determination of PxdsRNase Activity

The protein concentration was determined as the method described in 2.5. To test the dsRNA-degrading activity of PxdsRNase, 1 µg dsRNA dissolved in 5 µL of nuclease-free water was added to 20 µL of recombinant enzyme solution of PxdsRNase1 (1 µg, 2 µg, 3 µg, 4 µg, and 5 µg), PxdsRNase2 (5 µg, 10 µg, 15 µg, 20 µg, and 30 µg), or PxdsRNase3 (1 µg, 2 µg, 3 µg, 4 µg, and 5 µg), and then incubated at 28 °C for 15 min. After incubation, the samples were examined as the method described in Section 2.5.

### 3. Results

#### 3.1. Identification of DsRNases in *P. xylostella*

Four cDNA sequences putatively encoding *PxdsRNase1* (GenBank: MZ517187), *PxdsRNase2* (GenBank: MZ517188), *PxdsRNase3* (GenBank: MZ517189), and *PxdsRNase4* (GenBank: MZ517190) were identified from *P. xylostella* genome. The cDNA sequences were cloned by RT-PCR and sequenced. The ORFs of these four genes were of 1212 bp, 1359 bp, 1350 bp and 939 bp, encoding 403, 451, 449 and 312 amino acids, respectively. The domain analyses showed that all enzymes contained an endonuclease NS domain and a signal peptide, except PxdsRNase4 which did not have a signal peptide (Figure 1A). Alignment of the endonuclease domains of PxdsRNases indicated that their amino acid sequences were of high identity, with six active sites, three substrate binding sites, and an Mg$^{2+}$ binding site (Figure 1B).
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Figure 2. Relative expression levels of PxdsRNase genes. (A) Relative expression levels of Pxd

sRNase genes in different stages; (B) Relative expression levels of PxdsRNase genes in different tissues o

of the fourth instar larvae. EF1 was used as the reference gene. One-way ANOVA was conducted to
calculate the statistical significance, followed by the Turkey test. Different letters on the bars represent
significant differences (p < 0.05) among different samples.
3.3. DsRNA Degradation by the Proteins Extracted from the Gut and Hemolymph of Larvae

DsRNA of 120 ng was totally degraded by 10 µg of total proteins extracted from the gut or hemolymph in 6 h at 28 °C (Figure 3A,B), indicating that the enzymes in the gut or hemolymph could degrade the dsRNA. The total proteins of 30 µg extracted from the gut and 20 µg of total proteins extracted from the hemolymph degraded 120 ng of dsRNA in 15 min at 28 °C, individually, which indicated that the hemolymph proteins might have a higher ability to degrade dsRNA than the gut proteins (Figure 3C,D).

![Figure 3](image_url)

**Figure 3.** Degradation of dsRNA by the total proteins extracted from the gut or hemolymph at 28 °C. (A) Incubation of 120 ng dsRNA with 10 µg of total gut proteins for different times. (B) Incubation of 120 ng dsRNA with 10 µg of total hemolymph proteins for different times. (C) Incubation of 120 ng dsRNA with 1–30 µg of total gut proteins for 5 min. (D) Incubation of 120 ng dsRNA with 1–30 µg of total hemolymph proteins for 5 min.

3.4. Effects of PxdsRNase Suppression on RNAi Efficiency

In the injection experiment, the expression levels of *PxdsRNase1* were significantly suppressed by dsRNA at 36 h and 48 h, *PxdsRNase2* at 24 h and 36 h, *PxdsRNase3* at 24 h, and *PxdsRNase4* at 48 h (Figure 4). Co-injection of ds*PxdsRNase1/dsPxdsRNase2/dsPxdsRNase3* + ds*PxCht* (1/2/3 + C) caused significant reduction in the transcript levels of *PxCht* compared with dsGFP + ds*PxCht* (G + C) (Figure 5A–C), but not with ds*PxdsRNase4* + ds*PxCht* (4 + C) (Figure 5D). Therefore, RNAi efficiency in *P. xylostella* was improved after suppression of *PxdsRNase1*, *PxdsRNase2*, or *PxdsRNase3* by dsRNA injection.

In the feeding experiment, the expression level of *PxdsRNase1* in the larvae showed a significant reduction at 60 h post-feeding on ds*PxdsRNase1* (Figure 6A), as well as *PxdsRNase2* (Figure 6B). Interestingly, there was no suppression observed by ds*PxdsRNase3* (Figure 6C) and ds*PxdsRNase4* (Figure 6D). The expression level of *PxCht* in the larvae feeding on ds*PxdsRNase1 + dsPxCht* (1 + C) was significantly lower than that in the larvae feeding on dsGFP + ds*PxCht* (G + C), and the same case for ds*PxdsRNase2 + dsPxCht* (2 + C) and ds*PxdsRNase4 + dsPxCht* (4 + C) (Figure 7A). The expression level of *PxCht* in the larvae feeding on ds*PxdsRNase3 + dsPxCht* (3 + C) was not changed compared with that in the larvae feeding on ds*GFP + dsPxCht* (G + C) (Figure 7A). The expression of *PxdsRNase1* was observed to be significantly suppressed by ds*PxdsRNase4* (Figure 7B). Therefore, oral administration of ds*PxdsRNase4* might increase the RNAi efficiency by suppressing *PxdsRNase1*. The above results indicated that the RNAi efficiency in *P. xylostella* was enhanced after suppression of *PxdsRNase1* and *PxdsRNase2*. 
**Figure 4.** Effects of dsRNA injection on the expression levels of *PxdsRNases*. The fourth instar larvae were injected with 600 ng of different dsPxdsRNases or dsGFP, and the transcription levels of *PxdsRNase* genes were detected by RT-qPCR at different times. EF1 was used as the reference gene for normalization. Statistical analyses were performed using one-way ANOVA followed by the Turkey test. (A) *PxdsRNase1*; (B) *PxdsRNase2*; (C) *PxdsRNase3*; (D) *PxdsRNase4*. (*, *p* < 0.05; **, *p* < 0.01).

**Figure 5.** RNAi efficiency of *PxCht* after RNAi of *PxdsRNase* by dsRNA injection. RNAi efficiency of *PxCht* in the fourth instar larvae after injection with 1200 ng of dsGFP, a mixture of ds *PxCht* and dsGFP, or a mixture of dsPxdsRNase and dsPxCht, was evaluated by RT-qPCR, and EF1 was used as the reference gene for normalization. Statistical analyses were performed using one-way ANOVA followed by the Turkey test. G, dsGFP; C, dsPxCht; 1, dsPxdsRNase1; 2, dsPxdsRNase2; 3, dsPxdsRNase3; and 4, dsPxdsRNase4. (A) *PxdsRNase1*; (B) *PxdsRNase2*; (C) *PxdsRNase3*; (D) *PxdsRNase4*. (*, *p* < 0.05; **, *p* < 0.01).
Figure 6. Effects of uptake dsRNA on relative expression levels of PxsdsRNase genes in the larvae. Three μg of dsPxdsRNase (dsPxdsRNase1, dsPxdsRNase2, dsPxdsRNase3 or dsPxdsRNase4) or dsGFP were fed to the fourth instar larvae, and the transcription levels of PxsdsRNase genes were measured by RT-qPCR. EF1 was used as the reference gene for normalization. Statistical analyses were performed using one-way ANOVA followed by the Turkey test. (A) PxsdsRNase1; (B) PxsdsRNase2; (C) PxsdsRNase3; (D) PxsdsRNase4. (*, p < 0.05; **, p < 0.01).

Figure 7. RNAi efficiency of PxCht after RNAi of PxdsRNase by oral dsRNA. (A) Expression level of PxCht in the fourth instar larva 60 h after oral administration of 1200 ng of dsRNA of GFP, a mixture of dsGFP and dsPxCh (G + C) or a mixture of dsPxdsRNases and dsPxCh (N + C) was evaluated by RT-qPCR, and EF1 was used as the reference gene for normalization. Statistical analyses were performed using one-way ANOVA followed by the Turkey test. G, dsGFP; C, dsPxCh; N, dsPxdsRNase1/ dsPxdsRNase2/ dsPxdsRNase3/ dsPxdsRNase4. (B) PxsdsRNase1 relative expression level in the fourth instar larvae 60 h after oral administration of dsPxdsRNase4. (*, p < 0.05; **, p < 0.01).

3.5. Enzymatic Activities of PxdsRNases

The recombinant proteins, His-PxsdsRNase1, His-PxsdsRNase2, and His-PxsdsRNase3, were successfully expressed in the prokaryotic expression system with the estimated molecular masses of 43 kDa, 50 kDa, and 51 kDa, respectively (Figure 8), which were consistent with the expected protein sizes determined by the sequences. PxsdsRNase1 showed a high dsRNase activity to degrade dsPxCh (Figure 9A), PxsdsRNase2 had no effect.
on dsRNA (Figure 9B), and PxdsRNase3 cleaved dsRNA without complete degradation (Figure 9C).

Figure 8. Western blot analysis of recombinant PxdsRNase proteins. Electrophoresis was performed in 12% SDS-PAGE gel. The separated protein was transferred to membrane (100 V for 100 min). After blocking with 5% w/v BSA (Solarbio, Beijing, China), each protein was incubated with His monoclonal antibody (GenScript, Nanjing, China), separately. The EstinTM L1 staining kit (GenScript, Nanjing, China) was used for signal generation. The arrows point to the target proteins. (A) PxdsRNase1; (B) PxdsRNase2; (C) PxdsRNase3.

Figure 9. Degradation of dsRNA by recombinant PxdsRNases. M, marker. (A) PxdsRNase1; (B) PxdsRNase2; (C) PxdsRNase3.

4. Discussion

DsRNases are widely distributed in different organisms. The number of dsRNases varies from one to five among insects, such as, four dsRNases in L. migratoria and S. gregaria [13,15], three dsRNases in A. grandis [16], five dsRNases in S. litura and T. castaneum [18,23]. In P. xylostella, 4 dsRNases, PxdsRNase1, PxdsRNase2, PxdsRNase3 and PxdsRNase4, were identified. There is still no evidence to demonstrate that more dsRNases cause lower RNAi efficiency in one species. PxdsRNases shared similar conserved domains of endonuclease NS domain active site and substrate binding site with dsRNases in B. mori, L. migratoria, and S. gregaria [8,13,15].

In P. xylostella, dsRNases were mainly expressed in the intestinal tract and hemolymph. DsRNases were mainly expressed in the head and intestine in T. castaneum [23], and in the intestines and salivary glands in Halyomorpha halys [24]. Therefore, dsRNase expression is
inconsistent in different insects. The ability of total protein of hemolymph to degrade dsRNA was higher than that of the intestinal juice in *P. xylostella*. Similar cases are reported in Lepidoptera and Coleoptera [25]. Expression of dsRNases, both in the intestinal tract and hemolymph, might be the reason why the injection of dsRNA is more effective than feeding dsRNA for RNAi in insects [2,25–27].

Not all dsRNases are always involved in the RNAi process in insects. PxdsRNase1/ PxdsRNase2/ PxdsRNase3 affected RNAi efficiency, and PxdsRNase4 without signal peptide had no effect on RNAi efficiency, neither through injection or oral intake of dsRNA. Similar case happens in *S. litura*, where four dsRNases could degrade dsRNA and the other one without signal peptide is not active [18]. Only LmdsRNase2 decreased the RNAi efficiency in *L. migratoria* [15], only dsRNase2 in *S. gregaria* [13,28], and only dsRNase3 in *Cylus puncticollis* [29]. Therefore, the involvement of dsRNases in insects is species-specific. In addition to dsRNases, an RNAi efficiency-related nuclease (REase) is found to degrade dsRNA and suppress RNAi response in the Asian corn borer, *Ostrinia furnacalis* [19]. Uncovering the molecular mechanisms behind the phenomenon that some factors inhibit the initiation of RNAi responses is imperative to apply RNAi for the control of pests [30–32]. In our research, dsPxdsRNase4 did not suppress the expression *PxdsRNase4*, but *PxdsRNase1* in oral experiment. This phenomenon is also found in the Asian migratory locust [13]. It is not clear whether similar situation will occur in other species and the mechanism behind this phenomenon is also uncovered.

PxdsRNase1 degraded dsRNA rapidly, PxdsRNase3 cleaved dsRNA without complete degradation, and PxdsRNase2 could not degrade dsRNA. DsRNase activity depends on the pH of the working environment [13,15]. LmdsRNase1 could degrade dsRNA efficiently under the pH of 5, and could be intensively suppressed at the physiological pH of hemolymph (7.0), leading to the long-term stability of dsRNA in the hemolymph [15]. In addition, the length of dsRNA could also affect dsRNase’s activity [7]. Therefore, the effect of factors, including pH and the length of dsRNA on the activity of PxdsRNase, need to be investigated in the future to understand the stability of dsRNA in *P. xylostella*. In the meantime, new techniques, such as nanoparticles [33,34], transfection reagents [35], and dsRNA encapsulation using bacteria [36] are currently under the investigation by many groups for using RNAi technology as a new method for pest control. It is promising for RNAi-based pest control after the mechanism of dsRNase is explored.

5. Conclusions

We found four PxdsRNases, and three of them, PxdsRNase1, PxdsRNase2, and PxdsRNase3 were verified to be involved in the RNAi process in *P. xylostella*. In vitro, the recombinant protein of PxdsRNase1 degraded dsRNA completely, and PxdsRNase3 cleaved dsRNA without complete degradation. This study provided a fundamental basis for understanding the mechanism of dsRNase involvement in the RNAi process and using RNAi to control *P. xylostella* in the future.

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