Characterization of a Lipase From the Silkworm Intestinal Bacterium *Bacillus pumilus* With Antiviral Activity Against *Bombyx mori* (Lepidoptera: Bombycidae) Nucleopolyhedrovirus In Vitro

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Abstract

To investigate whether *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) intestinal microorganisms play a role in the host defense system against viral pathogens, a lipase gene from the silkworm intestinal bacterium *Bacillus pumilus* SW41 was characterized, and antiviral activity of its protein against *B. mori* nucleopolyhedrovirus (BmNPV) was tested. The lipase gene has an open-reading frame of 648 bp, which encodes a 215-amino-acid enzyme with a 34-amino-acid signal peptide. The recombinant lipase (without signal peptide) was expressed and purified by using an *Escherichia coli* BL21 (DE3) expression system. The total enzyme activity of this recombinant lipase reached 27740 U/mg at the optimum temperature of 25°C and optimum pH value of 8.0. The antiviral test showed that a relative high concentration of the recombinant lipase reduced BmNPV infectivity in vitro, which resulted in decreased viral DNA abundance and viral occlusion bodies. Besides, the preincubation method also suggested that the lipase probably directly acting on the budded virions. The results suggest that the lipase from intestinal bacterium *B. pumilus* SW41 is a potential antiviral factor for silkworm against BmNPV.

Key words: *Bombyx mori*, *Bacillus pumilus*, lipase, antiviral activity, *B. mori* nucleopolyhedrovirus

Insect intestines are colonized with diverse microbes, including bacteria, archaea, fungi, and protozoa; however, the dominant flora usually is bacteria (Schloss et al. 2006). The microbial communities and populations in insect intestines are variable, which mainly dependent on the species, developmental stages, food, and environment conditions (Engel and Moran 2013, Yun et al. 2014). These microbes have important biological functions for their hosts. Previous studies showed that insect intestinal microbes may involve in food digestion, nutrient utilization, and even toxin degradation (Piel 2002, Watanabe and Tokuda 2010, Engel and Moran 2013). More interestingly, they may also contribute to host immune systems (Engel and Moran 2013). Dillon et al. (2005) found that the intestinal symbiotic bacteria of the desert locust Schistocerca gregaria (Forsskål 1775) (Orthoptera: Acrididae), including *Pantoea agglomerans* (Beijerinck 1888) (Enterobacteriales: Enterobacteriaceae), *Klebsiella pneumoniae* (Schroeter 1886) (Enterobacteriales: Enterobacteriaceae), *Enterococcus mundtii* (Collins et al. 1986) (Lactobacillales: Enterococcaceae) of *Spodoptera littonalis* (Boisdula 1833) (Lepidoptera: Noctuidae), and *Enterococcus faecalis* (Andrewes and Horder 1906) (Lactobacillales: Enterococcaceae) secreted mupinidin that inhibited colonization of several potential pathogenic bacteria, such as *Enterococcus faecalis* and *E. caseliflavus*. Generally, insect intestinal symbiotic microbes may play an important role in host defense system against pathogens.

The domestic silkworm, *Bombyx mori*, which belongs to lepidopteran, is an oligophagous insect. The natural food of the silkworm is mulberry leaves; however, the leaves of other Moraceae plants, such as *Cudrania tricuspidata*, *Taraxacum officinale*, and *Lactuca sativa* can be substitutions. Despite a monotonous diet and the very simple...
intestinal tract (Engel and Moran 2013), the intestinal microbes of silkworm are abundant, including Enterococcus, Delftia, Pelomonas, Ralstonia, Staphylococcus, Bacillus, Arcobacter, etc (Liang et al. 2014, Sun et al. 2016). However, silkworm intestinal bacterial communities were also affected by forages, developmental stages, and gender (Liang et al. 2014, Sun et al. 2016). More importantly, the silkworm intestinal microbes were significantly changed after pathogen infection. In the silkworms infected with B. mori cytoplasmic polyhedrosis virus (BmCPV), the abundance of Enterococcus and Staphylococcus was increased, but Delftia was decreased (Sun et al. 2016). In addition, using antibiotics eliminating intestinal bacteria would increase the susceptibility of the silkworms to baculovirus and S. marcescens (Rajagopal 2009). It is interesting to study which groups of intestinal microbes might be beneficial for the silkworms to defend against pathogens.

BmNPV (B. mori nucleopolyhedrovirus) is an enveloped double-chain DNA virus that belongs to Baculoviridae, which is one of the most common pathogens of silkworm. As other baculovirus, BmNPV has two phenotype, the budded virions (BVs) and the occlusion-derived virions (ODVs). BVs mainly cause systemic infection from cell to cell within tissues (Blissard and Theilmann 2018). The orally infected ODVs were embedded in the polyhedron, mainly spread among insect individuals (Blissard and Theilmann 2018). The infectivity of ODVs is nearly 10,000-fold higher than that of BVs in midgut epithelial cells in vivo, but is nearly 1,000-fold less than that of BVs in cultured cells in vitro (Rohrmann 2013). The different composition of nucleocapsids and envelope proteins of BVs and ODVs has been indicated by proteomic studies of purified virions (Wang et al. 2010). Despite that, the lipid components of the envelope in two kinds of virions are partially similar to each other on some level (Braunagel and Summers 1994, Blissard and Theilmann 2018). During the infection cycle, the polyhedrons first enter into the digestive tract and are immediately broken down by the alkaline digestive juice, thus releasing the ODVs. The ODVs then reach the first defensive line (the midgut and its contents) before establishing effective infection in the midgut epithelial cells. In fact, the midgut protects silkworm from BmNPV infection through different mechanisms, such as secretion of antiviral agents, apoptosis, and physical barrier. The digestive juice of silkworm larvae contains several antiviral proteins against BmNPV, including B. mori lipase Bmlipase-1 (Ponnuvet et al. 2003), serine protease BmSP-2 (Nakazawa et al. 2004), B. mori NADPH oxidoreductase BmNOX (Selot et al. 2007), and red fluorescent proteins (Sunagar et al. 2011). Even if midgut epithelial cells are infected, the virus-containing cells can be removed by high-frequency apoptosis. To avoid virions from directly entering into the hemolymph, the complete midgut basilar membranes serve as a physical barrier with the pore size of only 15 nm, which is usually smaller than viral particles. Before infecting midgut epithelial cells or entering into hemolymph, the free ODVs will unavoidably meet with the intestinal microbes and their products. Do these microbes play a role in host defense to BmNPV infection?

To explore the relationship between intestinal microbes and BmNPV susceptibility of silkworm larvae, our lab utilized different forages to modify intestinal microbial communities in previous studies (Feng et al. 2011), and found that silkworms that fed on Caudania tricuspidata leaves were much susceptible to BmNPV than those that fed on mulberry leaves. This phenomenon could be partially ascribed to the very different intestinal microbial communities and populations caused by the two forages (Xiang et al. 2010). Furthermore, a significant pattern by which different lipase-producing intestinal microbes from the silkworms fed on different forages was noticed (Feng et al. 2011). Nine lipase-producing strains were obtained from the silkworm larvae that fed on mulberry leaves; however, only one strain existed in the intestine of the silkworm fed with C. tricuspidata leaves (Feng et al. 2011). Do bacterial lipases play a role in host defense against BmNPV infection? In this research, the intestinal bacterium Bacillus pumilus sw41 from mulberry-fed silkworms, which produced the most abundant lipase with the highest enzymatic activity, was analyzed. We have cloned the bacterial lipase gene Bplipase, expressed it by using the prokaryotic expression system, purified the recombinant protein, and evaluated the antiviral activity of this lipase to BmNPV in vitro.

Materials and Methods

Bacterial Strain and Plasmids

A lipase-producing bacterial strain (named B. pumilus SW41) was isolated by using the TIAnamp Bacteria DNA Kit (Tiangen, China) according to the manufacturer’s protocol and used as the template. Primers (BP-LIP1: 5′-GAACATAGAAGTGATCGATTATTAAAG-3′, BP-LIP2: 5′-GAGAGATCCATATGAGTTGATTC-3′) (Kim et al. 2002) were used to clone the complete sequence of the B. pumilus SW41 lipase gene. To express the Bplipase in E. coli, the forward primer Lip-F (5′-CGGCCGTCCGCTCGCGATATCCCGGT-3′, the restriction site of BamHI is underlined) and the reverse primer Lip-R (5′-CGCGCTCGATATGAGTTGATTC-3′, the restriction site of Xhol is underlined), based on the open-reading frame (ORF) of B. pumilus SW41 lipase, were used to amplify the lipase gene, and the signal peptide was removed. The PCR steps were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 50 s, 53°C for 50 s, and 72°C for 1 min, and a final extension of 10 min at 72°C. The PCR products were cloned into the pMD19-T simple vector after their recovery using a DNA gel extraction kit (Tiangen, China) according to the manufacturer’s protocol and used as the template. Primers (BP-LIP1: 5′-GAACATAGAAGTGATCGATTATTAAAG-3′, BP-LIP2: 5′-GAGAGATCCATATGAGTTGATTC-3′) were designed, and the constructed plasmids were sequenced in two directions by GenScript Bio Co., Ltd (Nanjing, China).

Expression of the Lipase Gene in E. coli

The expression vector plasmid pET-28a was used for gene expression in E. coli in this study. After digestion by BamHI/Xhol, the Bplipase gene was recloned and ligated with the pET-28a vector, which had been digested by the same restriction endonuclease (Fig. 1). The positive clones were selected by restriction enzyme digestion and then confirmed by sequencing. The recombinant plasmid pET28a-Bplipase was transformed into E. coli BL21 (DE3) (Invitrogen, Carlsbad, USA). All primers were designed, and the constructed plasmids were sequenced in two directions by GenScript Bio Co., Ltd (Nanjing, China).
0.1 mM when the absorbance at 600 nm was approximately 0.6. After being cultivated at 20°C for 12 h, the strains were harvested by centrifugation (8000 × g, 15 min), and the crude enzyme was then collected by centrifugation (12,500 × g, 10 min) after being disrupted with ultrasound. The negative control was prepared by the same method without IPTG induction.

Purification of the Recombinant BpLipase Enzyme and SDS–PAGE Analysis

The N-terminally attached His-tag lipase was purified using an immobilized metal ion affinity chromatography column (GenScript, China). The crude enzyme preparation was loaded onto a Ni-nitrotriacetate (Ni-NTA) column previously equilibrated with native binding buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). The column was washed with 20 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0) to remove protein impurities. Then, the bound target protein was eluted with the elution buffer (50 mM NaH2PO4, 300 mM NaCl, 100 mM imidazole, pH 8.0). Finally, a desalting column with a molecular weight cut-off of 3 kDa (Millipore) was used to remove the salts and concentrate the target protein. The purification procedure was carried out at 4°C. The recombinant BpLipase was prepared for further experiments in this study.

For the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) procedure, 5× loading buffer (250 mM Tris–HCl (pH 6.8), 10% (W/V) SDS, 0.5% (W/V) bromophenol blue (BPB), 50% (W/V) glycerol, and 5% (W/V) β-mercaptoethanol (2-ME)) were added to the protein sample. The crude extract and the pure enzyme samples were boiled for 10 min and subjected to SDS–PAGE with a 13.5% (W/V) SDS polyacrylamide gel. Electrophoresis was performed on the Mini-protean Tetra system (Bio-Rad), and gels were stained with Coomassie Brilliant Blue R250, destained, and analyzed.

Activity Assay of Recombinant Lipase and Enzymatic Properties

The enzyme activity of recombinant lipase was detected by using Rhodamine B agar plate method (the nutrient agar plate contained olive oil (3%, V/V) and rhodamine B (0.001%, W/V)). In brief, 10 µl of recombinant BpLipase was added on sterilized filter paper discs of 6 mm diameter, and then incubated at 30°C for 24 h before detection under UV-light. Moreover, lipase activity was measured by using para-nitrophenyl palmitate (pNPP) as the substrate according to the classic method with slight modification as follows (Winkler and Stuckmann 1979, Ma et al. 2015). A pNPP reaction mixture consisted of 100 µl of the pNPP solution (pNPP dissolved in isopropanol, 3 mg/ml) and 2.8 ml of Tris–HCl buffer (50 mM, pH 8.0). Then, 2.9 ml of freshly prepared substrate solution was preincubated at 30°C for 5 min. The reaction mixture was mixed with 100 µl of recombinant lipase solution and incubated at 30°C for 5 min, and the reaction was terminated by being placed on ice. The absorbance at 405 nm was measured against that of the negative control (hyperthermia-inactivated recombinant BpLipase) by using a microplate reader (Gene Co., Ltd, China). Specific activity of recombinant BpLipase was defined as 1 mg of enzyme required to release 1 µmol of p-NP from the substrate (pNPP) per minute.

To investigate the optimum temperature of this lipase, the recombinant BpLipase was incubated at different temperatures (5–70°C). Reactions determining the thermostability of the enzyme were carried out by preincubating at different temperatures (5–70°C) for 5 min in 50 mM Tris–HCl buffer (pH 8.0) with pNPP as a substrate. Then, the lipase activity of the samples was determined by measuring their optical density at 405 nm.

To study the effect of pH on lipase activity, the recombinant BpLipase was examined after 5 min of preincubation at 25°C in different buffers ranging from pH 3.0 to 12.0 with pNPP as a substrate. Reactions determining the thermostability of the enzyme were carried out by preincubating at different temperatures (5–70°C) for 5 min in 50 mM Tris–HCl buffer (pH 8.0) with pNPP as a substrate. The activities were tested by the standard method mentioned earlier.

The experiments were repeated three times, and the data were subjected to one-way ANOVA analysis.

Testing Antiviral Activity Against BmNPV

Antiviral activity was measured in triplicate according to the procedure of Selot’s report with a little modification (Selot et al. 2007). The BVs of BmNPV (multiplicity of infection, MOI = 5) were prepared for further experiments. The antiviral activity of recombinant lipase was evaluated on BmNPV-infected BmN cells, which the BVs were pretreated with recombinant BpLipase or water. In brief, 100 µl of BVs and 1 mg of recombinant BpLipase were homogenized in fresh TC-100 cell-medium in a sterile tube (a total volume of 1 ml) and incubated at 28°C for 24 h before detection under UV-light. Moreover, lipase activity was measured by using para-nitrophenyl palmitate (pNPP) as the substrate according to the classic method with slight modification as follows (Winkler and Stuckmann 1979, Ma et al. 2015). A pNPP reaction mixture consisted of 100 µl of the pNPP solution (pNPP dissolved in isopropanol, 3 mg/ml) and 2.8 ml of Tris–HCl buffer (50 mM, pH 8.0). Then, 2.9 ml of freshly prepared substrate solution was preincubated at 30°C for 5 min. The reaction mixture was mixed with 100 µl of recombinant lipase solution and incubated at 30°C for 5 min, and the reaction was terminated by being placed on ice. The absorbance at 405 nm was measured against that of the negative control (hyperthermia-inactivated recombinant BpLipase) by using a microplate reader (Gene Co., Ltd, China). Specific activity of recombinant BpLipase was defined as 1 mg of enzyme required to release 1 µmol of p-NP from the substrate (pNPP) per minute.

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further experiments. BmN cells were seeded in six-well culture plates and allowed to attach at 28°C. The cells in each well were infected with water-pretreated and recombinant lipase (1 mg)-pretreated BVs, respectively. Then, the BVs in the supernatant were removed by replacing the medium with fresh medium after 2 h of incubation. The cells were continuously cultured at 28°C for 3 to 4 days to analyze the viral infection process. The occlusion body (OB)-forming cells, which were designated as cells with at least a single OB inside, were directly counted under a microscope. The experiments were repeated three times, and morphological changes were continuously photographed.

To confirm the antiviral activity of recombinant lipase, the relative expression levels of the viral DNA were evaluated by the capsid gene gp41 of BmNPV via quantitative real-time PCR (qPCR) assays. The B. mori glyceraldehyde-3-phosphate dehydrogenase gene, BmGAPDH, was served as an internal control. The total DNA of BmNPV infected BmN cells was prepared by using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer’s protocol. The primers used in the qPCR assays were GP41-F: 5'-CGTACTTAGTATACTGCGCCG-3', GP41-R: 5'-AGTCGAGTCGTCGGTGTTGCAAT-3', and GAPDH-F: 5'-CATTCCGCCGTTCCTGTTGCAAT-3', and GAPDH-R: 5'-GCTGCCCTCCTGGACCTTTGAC-3'. The qPCRs were prepared with a Sybr Green Qpcr Mix (Zoonbio biotechnology, China) according to the manufacturer’s instructions. The reactions were carried out in the Lightcycle K System (BIOER, China). The thermal cycling profile consisted of an initial denaturation at 94°C for 30 s and 45 cycles at 94°C for 10 s, 60°C for 12 s, and 72°C for 30 s. All assays were repeated three times. Relative expression levels were calculated using the 2^−ΔΔCt method, and the data were subjected to Student’s t-test.

Statistical analyses were performed using Sigma Plot software, version 13.0 (Systat Software GmbH, Erkrath, Germany). Each experiment was repeated three times, and each experiment included three replicates.

Results

Cloning and Analysis of the Lipase Gene from B. pumilus

The complete sequence of the lipase gene BpLipase from B. pumilus SW41 was amplified by primers BP-LIP1/BP-LIP2. Then, the gene was successfully subcloned into a pMD19-T simple vector and confirmed by sequencing. The sequence was deposited in the GenBank database with the accession number KJ013340 (protein sequence accession number AHM24990). Sequence analysis revealed that the nucleotide sequence showed 43.8 mol% G+C content and that the lipase gene ORF was 648 bp long and encoded a polypeptide of 215 amino acid residues. The signal peptide was predicted by using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP). The results indicated that 34 amino acids were in the N-terminal region and the peptide bond between the 34th and 35th amino acids was cleaved by signal peptidase. Thus, the mature enzyme contained 181 amino acid residues. The molecular weight of BpLipase was estimated to be 22.95 kDa (19.17 kDa without the signal peptide), and the isoelectric point (pI) value was calculated as 9.95 (9.58 without the signal peptide) by using the ExPASy compute pl/Mw program algorithm (http://web.expasy.org/protparam/).

Expression and Purification of the Recombinant BpLipase

The BpLipase gene (without signal peptide) was amplified by the primers Lip-F/Lip-R. To achieve high-level expression of the SW41 lipase gene, a pET28a(+) plasmid, containing a strong T7 promoter, was used as the expression vector. The recombinant strain (BL21-pET28a-BpLipase) was constructed and grown in LB medium supplemented with appropriate antibiotic to express the recombinant protein.

When the induction temperature was 37, 30, or 25°C, the recombinant protein was detected in both precipitate and cell-free supernatant but showed low activity in the latter. To avoid inclusion bodies, lower concentrations of IPTG, lower temperatures, and lower agitation rates have been used in expression studies (Lee et al. 2003). When a lower induction temperature (20°C) was used, the SDS–PAGE results showed that the most of the expressed recombinant protein was soluble and intracellular. There was no further accumulation of the recombinant protein when the cells were fermented for more than 12 h. Therefore, we chose 12 h as the culture time for harvesting the recombinant strain BL21-pET28a-BpLipase. The optimal expression conditions of the shake-flask culture were 0.1 mM IPTG, 12 h inductions, 100 rpm shaking, and 20°C.

After Ni-NTA purification, desalting and concentrating procedures, the purified recombinant lipase migrated as a single band on SDS–PAGE with a molecular mass of approximately 23.0 kDa, which was the same as the calculated value (Fig. 2A).

![Fig. 2. Purification and enzyme activity of recombinant BpLipase. (A) SDS–PAGE of recombinant BpLipase. Lane M, standard marker proteins; lane 1, the supernatant of fermented bacteria disrupted ultrasonically after induction for 12 h; Lane 2, the BpLipase after Ni-NTA purification; Lane 3, purified recombinant BpLipase after desalting and concentration. The sizes of the protein marker are shown on the left side. The black arrow represents the band of recombinant BpLipase. (B) Orange fluorescence on rhodamine B plates under ultraviolet light (left is the negative control without recombinant BpLipase).](image-url)
The Enzyme Activity of the Recombinant BpLipase

The enzyme activity was determined using a rhodamine B plate. After a 24 h incubation on the rhodamine B plate at 30°C, significant orange fluorescence was observed around a paper disc under UV light (Fig. 2B), which indicated significant lipase activity. The enzymatic activity of recombinant lipase was also measured by using a microplate reader with pNPP as the substrate.

The effect of temperature on lipase activity is shown in Fig. 3A. The optimum temperature of the recombinant BpLipase is 25°C (pH 8.0), and the enzyme maintained more than 85% activity in the range of 15–30°C (Fig. 3A).

The effect of pH on enzyme activity was also examined. The optimum pH value of the recombinant BpLipase was 8.0. In addition, the recombinant BpLipase retained more than 60% of its maximal activity in the pH range from 7.0 to 11.0 (Fig. 3B). No lipase activity was detected at pH values 3.0 and 12.0.

The specific activity of the recombinant BpLipase was calculated as 277.40 U/mg at temperature and pH values of 25°C and 8.0, respectively.

Antiviral Activity Against BmNPV

Because BmNPV is a lytic virus, the viral infected cells will be finally fulfilled with OBs which were then released along with cell lysis. Thus, the antiviral activity of the recombinant BpLipase against BmNPV can be directly appraised by the number of OB-forming cells. The infectivity of BmNPV, which was treated with 1 mg of the recombinant BpLipase, was compared with that of water-pretreated BVs. The results indicated that BmN cells inoculated with the recombinant-BpLipase-pretreated BVs exhibited normal morphological characteristics and that the control group inoculated with water-pretreated BVs was lysed within 72 h (Fig. 4A). The number of OB-forming cells (%) with and without the recombinant BpLipase pretreatment was counted by the direct count method from photomicrographs at 24, 48, and 72 hpi, respectively. The results showed that the percentage of OB-forming cells in the experimental group was as high as 87.5% at 24 hpi; in contrast, 73.9% of OB-forming cells were present in the control group (Fig. 4B). The percentage of OB-forming cells was as high as 47.5 and 99.2% at 48 hpi in the treated experimental group and the control group, respectively; the percentage of OB-forming cells was as high as 67.2 and 100% at 72 hpi in the treated experimental group and the control group, respectively (Fig. 4B).

Based on enzymatic function, the recombinant BpLipase might destroy viral integrity, reducing the infectivity of BmNPV. The reduced infectivity of BVs led to a lower abundance of viral DNA in a certain period within the host cells. To investigate the amount of viral DNA in BmN cells infected with the recombinant-BpLipase-pretreated and water-pretreated virus, the viral nucleocapsid gene gp41 was analyzed by using qPCR (Fig. 4C). The results showed that very little viral DNA was detected in both the experimental group and the control group at 12 hpi. At 24 hpi, the onset of viral DNA replication in the control group was detected, with a DNA abundance 211.6-fold greater than that of the internal control; very few viral DNA copies were detected in the treated experimental group (Fig. 4C). From 24 to 72 hpi, a sustained increase in viral DNA abundance was detected in both groups, but viral DNA replication in the experimental group showed a delayed onset that occurred in 48 hpi, with a DNA abundance 362.34-fold greater than that of the internal control, which was 43% of that of the control group (387.28-fold) (Fig. 4C). The number of viral DNA copies in the experimental group was as low as 18.2, 43.2, and 60.0% of that in the control group at 24, 48, and 72 hpi, respectively (Fig. 4C). At 96 hpi, the decreased amount of viral DNA in control group might be ascribed to the packing of virions into OBs which was in agreement with previous report (Zhou et al. 2016). The results showed that viral DNA replication was partly inhibited in the group in which the BVs were pretreated with the recombinant BpLipase. In other words, the recombinant BpLipase reduced the infectivity of BVs on BmN cells.

Discussion

Insect intestinal microbes are a benefit to host defense systems (Engel and Moran 2013); however, very few reports are focussed on microbes–virus interactions. In this study, we explored the potential interactions between a silkworm intestinal bacterium and host viral pathogen through in vitro experiments. We found that the lipase produced by silkworm intestinal bacterium showed certain antiviral activity against BmNPV in vitro. Previous reports indicated that a lipase BmLipase-1 from midgut of silkworm showed significant antiviral effect against BmNPV (Ponnuvel et al. 2003); however, the detailed mechanism still remained unclear. Despite that, it was accepted as an antiviral factor in silkworm breeding (Jiang et al. 2014). Based on the enzymatic pattern, the antiviral modes of action of B. pumilus Bplipase might be similar to that of silkworm BmLipase-1, because they both belonged to the ‘true’ lipase group (EC 3.1.1.3) according to Arpigny and Jaeger’s report (1999). In the present study, to avoid the unpredictable effects affected by
bacterial strains, we used the recombinant lipase and preincubated with BmNPV. The results showed that the infectivity of the recombinant-BpLipase-pretreated BmNPV was significantly decreased, which in other words, the recombinant BpLipase might directly act on BmNPV and have a certain antiviral effect. Our study indicated that silkworm intestinal microbes may play a role in defending BmNPV infection.

Animal intestinal symbiotic microbes may directly or indirectly act on host pathogens. The direct ways in which intestinal microbes act on pathogens involve interfering in the infection process, inhibiting cellular growth, and destroying the pathogens by enzymes, antimicrobial peptides, or secondary metabolites, etc., which mainly present as antagonistic effects. Indiragandhi et al. (2007) reported that the symbiotic Pseudomonas sp. PRGB06 in the intestine of the diamondback moth (Plutella xylostella L.) inhibited the spore germination and mycelia growth of entomopathogenic fungi such as Beauveria, Bisporedium, and Penicillium. Lactobacillus acidophilus, which was found in the gut of greater wax moth (Galleria mellonella L.) protected host from Candida albicans infection by inhibiting the biofilm formation (Vilela et al. 2015). The indirect ways mainly involve in stimulating host immunity. The colonization of symbiotic intestinal bacteria may indirectly inhibit the infection and proliferation of pathogenic microbes by stimulating host basic immune effect (Cirimotich et al. 2011). Comparing with bacterial and fungal pathogens, the interactions between virus and intestinal microbes remained largely unknown. According to the published reports, intestinal microbes might either promote or inhibit viral infection. In one hand, intestinal microbes may be essential for viral infection. For example, the symbiotic bacterium Serratia odorifera in the intestine of Aedes aegypti assisted dengue virus infecting mosquitoes (Apte-deshpande et al. 2012). Rat intestinal microbes provided lipopolysaccharide (LPS) which was essential for poliovirus infection (Karst 2016). On the other hand, intestinal microbes may protect host from viral infection. For example, a research has shown that salmon intestinal bacterium Lactobacillus sakei MN1 enhanced host immunity to prevent hematopoietic necrosis virus infection (Nácher-Vázquez et al. 2015). In some cases, symbiotic microbes may either inhibit or promote viral infection. According to the published reports, the nonintestinal symbiotic Wolbachia might benefit for host survival during viral infection (Hedges et al. 2008, Pan et al. 2012), or increase the mortality caused by viral pathogens (Graham et al. 2012). The microbes–virus interactions generally depend on microbial, viral, even host species. In fact, microbes might directly act against viral pathogens. By preincubating with intestinal probiotics,
vesicular stomatitis virus showed retarded infectivity (decreased to 40\%) to IPEC-J2 cells in vitro (Botić et al. 2007). In the rat model, the infant intestinal probiotic *Bifidobacterium longum* preincubated with human rotavirus showed antiviral activity both in vitro and in vivo (Muñoz et al. 2011). In this study, we used the similar ‘in vitro preincubation strategy’ to explore whether the bacterial lipase was the potential antiviral substance. BmNPV showed decreased infectivity by preincubating with the recombinant *B. pumilus* lipase, which suggested the direct interaction between this antiviral enzyme and the virus. We assumed that the recombinant BpLipase might directly destroy the viral envelope of BmNPV, which then affected the infectivity. Despite lacking some of the detailed mechanisms, our research clearly proved that intestinal microbes directly acted against viral pathogens by secreting bioactive substances.

The domestic silkworm is a famous captive rearing insect with great economic values. From ancient to modern society, the sericulture industry is one of the most important pillars of economy in some areas. However, the viral disease caused by BmNPV brings great threat to modern sericulture industry (Rahman and Gopinathan 2004). The major strategies of BmNPV damage control are disinfecting by using environment-friendly disinfectants, and breeding new silkworm strains with specific antiviral pattern by traditional hybridization (Chen et al. 1991) or transgenic technology (Jiang et al. 2014). To date, several antiviral silkworm lines have been successfully generated; however, none have been applied in sericulture due to some limitations, such as the poor quality of important economic characteristics or susceptible to other pathogens (Jiang et al. 2014). Our results suggested that the intestinal microbes of silkworm might also involve in the host defense against the BmNPV. Using microbes to control BmNPV disease may have specific advantages, e.g., the microbes could be developed as probiotic supplements, which was not only very easy to use, but also avoid the limitations of the specific silkworm strains. Future research will continuously focus on screening valuable intestinal microbes with significant antiviral activity, exploring the detailed antiviral mechanism, as well as developing bio-control additives. Based on the results, we presumed that some bacteria in silkworm intestine might reduce the infectivity of BmNPV by producing antiviral lipase. This study also suggested a potential application of host intestinal microbes in controlling viral diseases.

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