Original Research Article

Expression study of Bmlipase in silkworm B*ombyx mori* midgut against BmNPV

ABSTRACT

Silkworms are susceptible to various pathogens, including bacteria, fungi, viruses, and protozoa. The present study focused on the viral disease known as nucleopolyhedrosis virus, which causes severe infections in silkworms. Silkworms possess several antiviral proteins that play a crucial role in preventing the spread of infection. One protein, which is found in the digestive juice produced in the silkworm's midgut, was focused, and it is lipase. This protein is responsible for controlling the virus infection. To investigate the role of lipase expression in silkworm for virus control, its concentration after post-infection was measured. This was quantified and analysed using SDS-PAGE. The band of interest, with a molecular weight of 29 kDa, was further analysed through MALDI-TOF/MS, and it showed 70% homology to the lipase enzyme.

Keywords: NPV, Proteins, SDS-PAGE, MALDI-TOF/MS.

1. INTRODUCTION

Silkworm is an insect belonging to the Bombycidae family. The silkworm is the second organism that is used as a model organism in genetics and also in the field of insect research as an important economic insect [1]. The silkworm is an important model in various research areas, including human disease, screening of antimicrobial agents, environmental safety monitoring, and antitumor studies [2,3]. Additionally, there are rich genetic resources associated with silkworms. The silkworm and other insects are infected by bacteria, fungus, and viruses, which also cause severe diseases. As such, the silkworm is also affected by viruses and bacteria that cause severe diseases in them. The most deadly disease is nucleopolyhedrosis virus (NPV), which affects all the internal organs of the silkworm and causes grasserie. Due to its effects, the yield of the silk decreases drastically. In India, >30% of silk cocoon crop losses are attributed to BmNPV infection [4].

The insect midgut plays a major role in resistance to various parasites. It is an accepted axiom that the natural portal of entry for pathogenic insect viruses is via the digestive tract, specifically the midgut region. The generalised alimentary tract of the insect is composed of three distinct

areas: the foregut, the midgut, and the hindgut. The foregut and hindgut are of ectodermal origin and are lined with cuticles, thus forming a significant barrier to infection. The gut juice, or digestive juice, produced in the midgut contains many proteins that fight against bacteria, fungi, viruses, and protozoan pathogens [5,6]. Digestive juice is secreted by cylindrical and cup cells in the midgut and has an alkaline pH between 9.2 and 9.8 and a highly alkaline pH between 10.5 and 11.0 in the central midgut region [7]. The gut juice of the silkworm, *Bombyx mori*, contains a substance that can inactivate nucleopolyhedrovirus (NPV), which is the antiviral protein [8]. The midgut lumen is a vulnerable portal of entry for pathogens, including viruses such as *Bombyx mori* nucleopolyhedrovirus (BmNPV) that infect midgut cells before spreading to other tissues. Many antiviral proteins, such as red fluorescent protein, Bmlipase-1 (*Bombyx mori* lipase), and BmSP-2 (*Bombyx mori* serine protease), are induced and are important for defence against infection [9]. The antimicrobial proteins that are produced by silkworms can be used to treat many diseases in mammals and insects. Lipase is one of the most important enzymes in the insect's midgut. It will play a major role in the immune system, especially in controlling viral infections [10].

The present study is focused on the expression of Bmlipase in silkworms post-infection with the virus. BmNPV has inoculated silkworms and isolated the proteins from the midgut. The expression level of Bmlipase in both controls and BmNPV-infected silkworms and found significant results.

2. MATERIALS AND MATHODS

2.1 BmNPV purification

The BmNPV pellet was suspended in 1 ml of distilled water, vortexing briefly for 3 to 5 minutes for better mixing. The above purification method was followed and later it was inoculated to the silkworm larvae for further procedure.

2.2 Inoculation of BmNPV

Larvae (100) were separated into each tray for the infection process, and the mulberry leaves were cut into a uniform disc shape for equal distribution of the BmNPV. 500µl of the purified BmNPV sample was smeared onto the entire disc-shaped leaf and partially dried the leaf. The prepared leaves were fed to the larvae.

2.3 Sample collection

After infection of the fifth-instar silkworm larvae were collected and subjected to starvation for 18 hours. After starvation, each larva was centrifuged for six minutes at around 3,000 rpm. Immediately after centrifugation, the larvae were treated with chloroform vapor. The larvae vomit, and it is collected in the Falcon tube. The above procedure was followed until the fifth day of infection when the sample was collected to perform further procedures.

2.4 Protein Quantification

The digestive juice collected is checked for the protein concentration present in it by Nanodrop. The sample was subjected to protein quantification by using nano drops to determine the average concentration of proteins present as well as their purity.

2.5 Sample Purification

Ammonium sulphate is added to the digestive juice collected by taking 500 µl of the sample in an Eppendorf tube to get 40% saturation and incubating at 4 □ until precipitation appears. The sample is checked for precipitation, and as soon as precipitation appears, it is centrifuged at 5,000 rpm at 4 □ for 15 minutes. After centrifugation, the pellet form is saved, and the supernatant is discarded. The pellet collected was dissolved completely in the 40 mM phosphate buffer (pH 7.4). Store this above-processed sample at 4 □ for further processing.

2.6 Dialysis

The dialysis membrane is activated by treating it with 1 mM EDTA that was kept in a boiling water bath for 10 minutes. The membrane with the sample was placed in the phosphate buffer and dialyzed against it. The buffer was changed at intervals of every 2 hours. After dialysis, the sample was stored at 4 □.

2.7 Sample preparation

10 µl of the dialyzed sample was pipetted into an Eppendorf tube. In the same tube, 50 µl of sample buffer was added. The sample was made up to 100 µl by adding 40 µl of water. The above sample was kept in a boiling water bath for 10 minutes.

2.8 SDS-PAGE analysis

The experiment was carried out in accordance with our previous standardized protocol. Hemolymph protein was mixed with sample buffer (4% SDS, 20% glycerol, 10% 2-Mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris HCl, pH 6.8) and heated at 100 °C for 5 minutes. We used 15% of the polyacrylamide gel. A Bio-rad protean tetra cell was used for

electrophoresis at 150 V, 30 mA, and 1.30 min. The gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis [11].

2.9 Sample preparation for MALDI-TOF/MS

The targeted protein band was separated from the SDS-PAGE gel, and the 3rd day post-infection digestive juice protein 29 kDa band was selected for the analysis. Protein bands were extruded from the gel, stored in 7% acetic acid, and subjected to MALDI-TOF/MS analysis in the Molecular Biophysics Laboratory, Indian Institute of Science, Bangalore [11].

2.10 In-gel digestion and MS analysis

Spots of interest were excised from the gels within 6 h after staining and digested using trypsin according to a protocol described in Gadwala et al., (2023) [11]. The tryptic peptides were extracted with 70% acetonitrile (Merck, Darmstadt; Germany) and 3% formic acid (Prolabo, Paris, France). After 10 min of sonication, peptides were desalted using Zip Tip C18 microtips (Millipore, Bedform, MA, USA) and eluted in 4µl 50% acetonitrile. Tryptic peptides were analyzed on a MALDI-TOF-MS using Ultraflex TOF/TOF (Bruker Daltonics, Germany). A saturated matrix solution was prepared in 50% CAN with 0.1% TFA. After centrifugation, the supernatant was applied to the spots. A pulsed N2 laser-337nm at 50 Hz was used for taking an average of 100–150 laser shots.

2.11 Database search

The resulting files from MALDI-TOF/TOF MS detection were subjected to the MASCOT search engine (version 2.0; Matrix Science, London, UK) with GPS Explorer software (version 3.0; Applied Biosystems, Foster City, CA, USA) against the Swiss Prot database for peptide and protein identifications.

3. RESULTS AND DISCUSSION

In the present study, the protein quantification was estimated by nanodrop analysis was compared with control, and analyzed the digestive juice sample collected from the fifth instar larvae of the silkworm.

3.1 SDS-PAGE analysis

The control sample of digestive juice showed a low concentration of protein concentration compared to the NPV-infected digestive juice sample; the protein concentration increased day by day as the infection spread to separate the proteins. SDS-PAGE gel electrophoresis was performed. For the visibility of bands on gels, silver staining is performed where it detects

minute protein bands (>10mg). We observed different molecular-weight proteins, but our target was 29 kDa. After electrophoresis, we observed (Fig. 1) prominent results in protein expression. The expected lipase expression is higher from the 1st to the 3rd day of infection, and on the 4th and 5th days, the protein expression is low. According to previous reports, the antiviral protein expression is higher in the initial days, that is, from the 1st to the 4th days.

3.2 Identification by MALDI-TOF-MS

Based on the observation of the SDS-PAGE results, we extruded the 29 kDa protein based on the expression level of this protein in the different staining methods and subjected it to MALDI-TOF analysis. The expressed protein band was analyzed through MALDI-TOF. The protein matched the homologous protein lipase enzyme with a 70% homology (Fig. 2).

3.3 Function

Lipase is one of the antiviral proteins found in the digestive juice of silkworms, with a molecular weight of 29 kDa (Fig. 3). In insects, lipase plays a crucial role in various biological processes, including survivability, reproductive capacity, oocyte maturation and development, and sex pheromone biosynthesis. Additionally, lipase exhibits strong antiviral activity against BmNPV, a virus that affects *B. mori* larvae. The immune response of insects against NPV infection can be divided into two phases. The primary defense occurs in the alimentary canal, primarily targeting the ODV (occluded viral form). The secondary defense takes place in other tissues such as the fat body, trachea, and hemocytes, and is aimed at combating the BV (budded viral form).

4. DISCUSSIONS

Insects possess innate immunity, which is characterized by cellular and humoral responses against invading pathogens. However, continuous rearing of silkworms has led to the loss of certain natural characteristics, including their resistance or tolerance to microbial infections [12]. As a result, silkworms have become susceptible to various pathogenic groups, such as fungi, bacteria, viruses, and protozoans. Nonetheless, silkworms have developed an effective host defence mechanism through their immunological and genetic resistance [13].

In the silkworm species *B. mori*, immunological responses are primarily carried out by circulating haemocytes, which provide innate immunity. Antimicrobial proteins serve as the arsenal that insects have evolved to combat pathogens [14]. The immune system of insects responds to NPV infection in two phases: the primary defence against ODV in the alimentary canal and the secondary defence against BV in other tissues such as the fat body, trachea, and haemocytes [15,16].

It has been reported that red fluorescence protein (RFP) can be obtained in vitro from midgut proteins and chloride a, a prosthetic group derived from chlorophyll-A [17]. However, it was determined that the anti-BmNPV action is attributed to Chlide a rather than the protein itself [18]. Various RFPs of different molecular sizes have been identified, including a 65 kDa 40 kDa of RFP [19], a 24 kDa serine protease, a 29 kDa lipase RFP, and more recently a 302 kDa lipocalin. Santhosh et al. (2011) partially isolated and obtained red fluorescent proteins (RFPs) from the gut fluid of multivoltine and bivoltine silkworm races in their fifth instar [20]. These RFPs were observed as multiple bands in electrophoretograms and chromatographic eluates. Bioassay experiments indicated that the fluorescent bands exhibited anti-NPV activity. Shen et al. (2022) identified and reported that Bmlip10584 was highly expressed in the epidermis, fat body, trachea, testis, and ovary of the silkworm [21,22]. Xialong et al. (2015) investigated changes in protein composition in the digestive juice of silkworms induced by BmNPV infection using shotgun proteomics and MS sequencing [23]. They identified a total of 75 proteins, 44 of which were unknown, in the digestive fluid of third-day, fifth-instar silkworm larvae. Additionally, a total of 106 proteins, 39 of which were unknown, were discovered in the digestive fluid of larvae exposed to BmNPV six hours prior. Following the BmNPV challenge, more secreted proteins with antiviral and digestive features were observed. Several proteins have been reported to interact with BmNPV, providing insights into the host-pathogen interaction and revealing potential functions of the digestive juice during persistent BmNPV infection.

Lipase is one of the antiviral proteins that are present in the digestive juice of the silkworm; the molecular weight of the protein is 29 kDa [24]. The lipase in insects is related to survivability, reproductive capacity, oocyte maturation and development, and sex pheromone biosynthesis [21] Shen et al., 2022. Lipase has strong antiviral activity against BmNPV from *B. mori* larvae. However, the antiviral innate immune response of silkworms has not been systematically studied, although specific antiviral molecules such as BmSTING, BmAtlastin-n, BmNOX, small heat shock protein [25] and Bmlipase-1 have been identified [26]. Guohui et al. (2017) identified Bm-SP142 transcripts that were significantly up-regulated at 24 hours post-infection (hpi) in BmBDV-resistant strains inoculated with BmBDV and BmNPV-resistant strains of NB inoculated with BmNPV [27].

Since there is little knowledge of the digestive lipases of insects, the current study aimed to identify and characterise the enzyme in the midgut of *B. mori* after BmNPV inoculation. The antiviral activity of a protein encoded by the lipase gene of the intestine bacterium *B. pumilus* of silkworms was examined [28]. A relatively high concentration of the recombinant lipase

inhibited BmNPV infectivity in vitro, according to the antiviral test, which led to a drop in viral DNA quantity and viral occlusion bodies. Thus, the quantity of OB-forming cells can be used to directly measure the antiviral activity of the recombinant BmLipase against BmNPV. Zhang et al. (2020) have revealed that some proteins in *Bombyx mori* larvae's digestive juice show antiviral activity [29]. The response to BmNPV infection in the digestive juice of *B. mori* larvae was found to be mediated by BmLipase member H-A (BmLHA). Ponnuvel et al. (2003) purified the protein from the digestive juice of *B. mori* larvae and purified the enzyme [30]. The purified protein shows antiviral activity against BmNPV. A homology search of the deduced amino acid sequence of the protein cDNA revealed 56% homology with *Drosophila melanogaster* lipase. The results showed that the protein has lipase activity, suggesting that lipase is involved in immune mechanisms against viral infection in the alimentary canal.

5. CONCLUSION

The gut juice of silkworms contains numerous proteins and enzymes that play roles in digestion and primary defence mechanisms. Among the antiviral proteins identified are lipase, red fluorescence protein, serine protease, and others. The lipase protein, with a molecular weight of 29 kDa, exhibits antiviral activity against NPV infection, and its levels increase as the infection spreads. The lipase is isolated from the gut juice, purified, and separated using SDS-PAGE to obtain distinct protein bands. These bands are then stained using silver staining, particularly as lipase is a low molecular weight protein. The stained bands are compared against a protein marker scale and sequenced using MALDI-TOF/MS, which revealed a 70% homology with the lipase enzyme. To further understand how insect digestive lipases inhibit viral growth in the midgut, it is essential to explore their biochemical characteristics.

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Table 1: Protein concentration in gut juice

Control	1.009	
DPI 1	2.829	
DPI 2	4.906	

DPI 3	7.03	
DPI 4	7.29	
DPI 5	7.46	

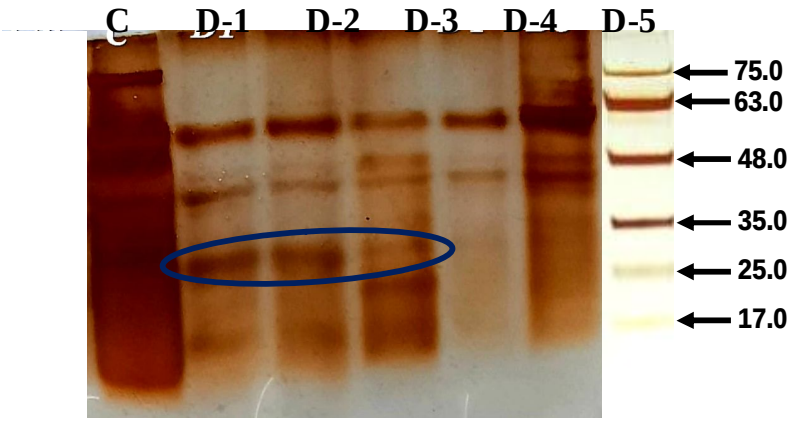

Fig 1: SDS-PAGE analysis of midgut juice with silver staining

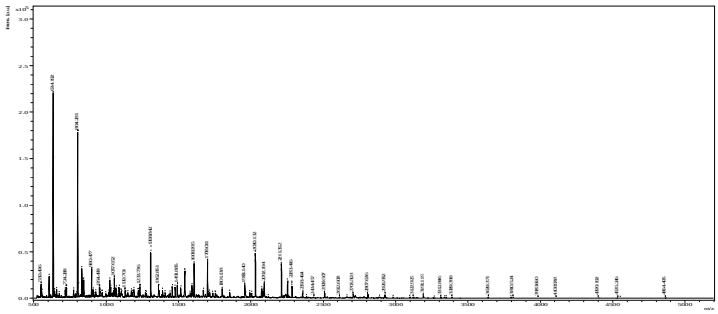

Fig 2: MALDI-TOF-MS results of

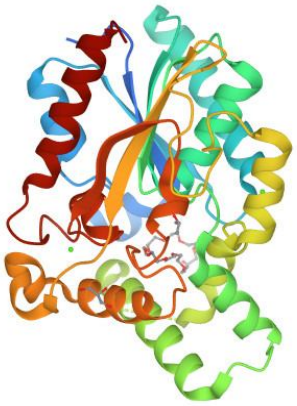

Fig. 3: 3-D structure of Lipase like proteins