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Expression Studies of *BmNPV* Antiviral Proteins Serine Protease and Lipase in Mulberry Plant

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Sericulture is a significant agricultural cottage enterprise. The silkworm Bombyx mori L. is used in the commercial manufacture of silk. Silkworms are prone to fungal, bacterial, viral, and protozoan infections. *Bombyx mori* nuclear polyhedrosis virus (*BmNPV*) causes grasserie, a viral illness in silkworm that is a severe economic loss to the sericulture business. The current study focuses on the transformation of antiviral genes, serine protease, and lipase into mulberry leaves by agro infiltration. Antiviral proteins from BmNPV were cloned into the plant expression vector pBI121. The plant expression vector pBI121 was effectively transferred into Agro-bacterium cells for mulberry agroinfiltration investigations. PCR amplification and SDS-PAGE analysis validated the gene integration and production of antiviral proteins, which were discovered to be 885 bp of lipase gene with protein size of 29 Kda and 855 bp of serine protease gene with protein size of 24 Kda. Lipase and serine protease antiviral protein genes were cloned into the bacterial expression vector pET32a for bioassay experiments.

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Keywords: Bombyx mori; transformation; lipase; serine protease; antiviral protein; agroinfiltration; mulberry; silkworm.

1. INTRODUCTION

Sericulture is an one of the important ancient agarobased industry. The silkworm *Bombyx mori* L. is an economically significant lepidopteran insect that has been commercially employed to produce silk, and silkworm is used as a biological model system for investigating agricultural lepidopteron insect pests. It also contributes to our society's economic progress by providing jobs for rural residents. It is the only plant that can transform leaf protein into silk protein. Global silk output in 2016 was expected to be 2,02,072.83 MT, with China and India contributing 1,70,000 MT (84.13%) and 28,523 MT (14.11%), respectively (ISC, 2016).

Silkworms are susceptible to a variety of illnesses caused by fungi, bacteria, viruses, and protozoa. The main diseases of the silkworm Bombyx mori L. are flacherie, which is caused by several viruses and bacteria, grasserie, which is caused by the Bombyx mori nucleopolyhedrosis virus (*BmNPV)*, muscardine, which is caused by several fungal species, and pebrine disease, which is caused by the protozoan *Nosema bombycis*. The occurrence of silkworm illness is known to cause significant crop loss in sericulture. Disease causes less than 10% of crop loss in advanced countries like Japan, Italy, and China, but up to 30% in underdeveloped countries like India and Southeast Asia. It is believed that grasserie's contribute up to 15% of the loss in India.

Among these diseases, viral disease is the most damaging to the sericulture sector, frequently resulting in substantial economic losses [1]. Nuclear Polyhedrosis is a viral illness that is commonly known as grasserie, jaundice, milky sickness, and hanging disease [2]. Grasserie (BmNPV) disease causes more than 15% yield loss and 25-58% disease incidence [3]. *BmNPV* belongs to the Baculoviridae family. According to Gomi *et al*. [4] the *NPV* virus possesses a circular double-stranded DNA genome. Baculoviruses have developed to influence host cell defence mechanisms and to proliferate efficiently in the nucleus of infected cells [5].

Insects, like vertebrates, have a self-defense system against invading microorganisms. However, insects appear to lack any adaptive immune responses that function similarly to the well-documented antibody or histocompatibility adaptive immune responses found in invertebrates. Silkworms, like other insects, have an effective innate immune system that can recognise an invading organism as a foreign item and activate antiviral peptides. Apoptosis, also known as programmed cell death, is a phenomenon evolved by some invertebrates lacking humoral immunity to serve as an antiviral defence mechanism [6].

Disease management can be accomplished through a variety of methods. Several preventative management strategies for grasserie disease of silkworm have emerged. The use of disinfectants such as formalin and sodium hypochlorite, phenolic compounds, asiphor and bleaching powder, and sanitech for the control of *BmNPV* illness in silkworms is well established. All of these issues have forced the development of novel chemical disinfectants, and other tactics such as breeding of resistant silkworm breeds, boosting antiviral activity against NPV utilising transgenic approaches are emerging areas for disease management [7]. The genetic analysis tests were carried out in order to screen the resistant and vulnerable silkworm strains against viral infections. Various antiviral genes encoding proteins, such as serine proteases, lipases, red fluorescent proteins (RFP), gloverrin 4, gloverin 3, lebocin, serpin 5, arylphorin, promoting protein, cathespin B, and actin 3, are expressed against viruses during infection [8].

Even with enhanced breeds and scientific rearing technologies, the search for new solutions for silkworm disease control is essential. As a result, creating resistant strains using traditional or transgenic methods is one way to controlling silkworm illness. Traditional breeding approaches have drawbacks, such as improving disease resistance at the price of economically significant features [5]. Transgenic technology, such as antiviral protein overexpression and RNAi technology, which are two recognised gene regulatory mechanisms, may help to overcome the limitations of traditional breeding approaches. The problem still has to be handled, which includes improving anti-*BmNPV* features and discovering whether a single primary silkworm gene is responsible for *BmNPV* resistance (Jiang and Xia, 2014). Silkworm Bombyx mori L feeds only on mulberry plant which is sole food source

and disease transmission occurs through mulberry leaves in the mode of aerosol. Immunization of silkworm through mulberry leaves against *BmNPV* have not been reported earlier. The transient expression of antiviral proteins *Bm* lipase and *Bm* serine protease have been done in mulberry plants to check the efficacy of the transiently expressed antiviral proteins against *BmNPV* infection.

2. MATERIALS AND METHODS

2.1 Cloning and Molecular Screening of Antiviral Protein Genes Serine Protease and Lipase into Plant Expression Vector

The antiviral protein encoding genes serine protease and lipase present in the pGEMT cloning vector were amplified and cloned into the plant expression vector pBI121 using standard cloning procedures of restriction digestion and ligation A single colony from the each of cloned pGMET-Lp and pGMET-Sp plate was inoculated and grown overnight in Luria Bertani (LB) broth (HiMedia) at 37°C (Natesh J *et al*., 2017).

2.2 Development of Recombinant BmNPV-Lipase and BmNPV-Serine Protease Construct for Transformation Studies

Cloning of the amplified pGEMT-Lp and pGEMT-Sp was done in pBI121 expression vector using InsT/A cloning kit. The amplified pGEMT-Lp and pGEMT-Sp loaded in the gel and specific amplicons of ~855bp and ~885bp were excised using a sharp sterile scalpel on a low-intensity UV transilluminator and collected in sterile preweighed 2.0 mL microcentrifuge tubes. The product was extracted and purified using gel extraction kit (#K0691).

2.3 Preparation of Competent Cells

The competent E. coli (DH5) cells were produced according to the procedure. Sambrook and Russell (2001) referenced it with minimal changes. In 5 mL of Luria-Bertani broth (LB), an isolated colony from an E. coli (DH5) plate was injected. then incubated overnight at 37°C at 200 rpm. The culture was diluted the next day to 0.5 mL culture was added to 50 mL Luria broth at a ratio of 1:100. It was It was incubated for 2-3 hours until it reached an OD of 0.3-0.4 at 600 nm. The culture was then after 30 minutes on ice, 25 mL of culture was poured into two centrifuge tubes with a volume of 50 mL.

2.4 Transformation of Antiviral Protein Genes into Mulberry Leaves Using Agroinfiltration Technique for Transient Expression

Agrobacterium tumefaciens strain LBA4404 was incubated for 48 hours in 10mL LB Broth supplemented with appropriate antibiotics (Kanamycin and Rifampicin), and then 100L of the 48hour culture was inoculated (1%) into 100mL LB Broth with antibiotics. The culture was grown overnight at 28°C to log phase (OD 600), centrifuged, and suspended in induction buffer (10 mM MES, 10 mM MgCl2, 50 mM Acetosyringone) to a final OD 600 of 1.5 before centrifugation. The induced culture was then centrifuged and washed twice with wash buffer (10 mM MES, 10 mM $MgCl_2$). The cells were extracted by centrifugation and then dissolved in an infiltration buffer (10 mM MES, 10 mM MgCl2, 50 mM Acetosyringone, and 0.0001% tween-20) to an OD 600 of 0.3 of 1000 L of the bacterial suspension was infiltrated into the intercellular gaps of nearly completely grown cells. In a single Mulberry leaf, up to five invading patches separated by veins might be placed. After agro infiltration, the treated plants were kept in the greenhouse for 3-4 days before being tested for antiviral protein (Lipase and Serine protease) expression in the infiltrated regions using PCR amplification and SDS-PAGE.

3. RESULTS AND DISCUSSION

3.1 Cloning and Molecular Screening of Antiviral Protein Genes Lipases and Serine Protease into Plant Expression Vector

Plasmid DNA from pGEMT lipase and pGMET lerine protease was extracted, and Bm Lp and Bm Sp genes were amplified using Taq DNA polymerase enzyme and gene-specific primers. The amplicon sizes of Bm Lp and Bm Sp were validated by 1% using agarose gel electrophoresis. Using Xba1 and Sac1, restriction digestion was performed on the plasmid DNA for the lipase, serine protease, and vector pBI121. Double restriction digestion of the reaction mixture using restriction enzyme and gene-specific primers revealed that the digested size was 885 bp of Bm Lp, 855 bp of Bm Sp, and 13.4 kb of pBI121 vector backbone that was

eluted and confirmed by one percent Agar-gel electrophoresis (Fig 1).

3.2 Cloning of Bm lipase and Bm Serine Protease Genes into pBI121 Plant Expression Vector

The components of ligation mix were added to 0.5 mL micro centrifuge tubes and ligated by using T4 DNA ligase and incubated at 4°C overnight for ligation. Blue and white colonies were observed after incubation at 37˚C overnight. Among them approx30 per cent colonies were in white color and remaining colonies were in blue color. When the PCR product was separated by an agarose gel, it revealed the existence of gene amplicons with lengths of 885 and 855 base pairs, respectively, for the pBI121 Bm Lp and Bm Sp. According to Natesh (2017), the pGEMT cloning vector contains the 885 bp Bm lipase gene and the 855 bp serine protease gene. The XbaI and SacI enzymes were used to digest the pBI121 Bm Lp and pBI121 Bm Sp clones. On agarose gel electrophoresis, the digested products were separated (Fig. 2 and 3). The SacI and XbaI restriction sites at the 5' and 3' ends of the Lp and Sp genes, respectively, make it easier to remove the Lp and Sp gene insert from the recombinant vector. The plant expression vector pBI121 includes XbaI and SacI restriction sites close to the -glucoronidase (GUS) gene's 3' terminal end and the 35S promoter of the cauliflower mosaic virus, respectively. These sites aid in ligation. The kanamycin resistance gene (11689- 12485 bp) of size 795 bp, which encodes aminoglycoside phosphotransferase and imparts kanamycin resistance, is carried by the plant expression vector pBI121. The cells that were transformed using pBI121 developed resistance and proliferated on Luria Bertani broth medium with kanamycin added.

3.3 Transformation of Antiviral Protein Genes into Mulberry Leaves Using Agro Infiltration Technique for Transient Expression

Agro-infiltration was carried out by injecting mulberry seedlings with the Agrobacterium-mediated transient transformation assay in mulberry (*Morus alba* L.) using a syringe. Agrobacterium-mediated transient expression assays were published by Lee and Yang [9] for in vivo investigation of constitutive or inducible gene expression in Arabidopsis plants. He claimed that transient expression tests could be carried out in 3 days without the need for expensive equipment or laborious processes by simply injecting agrobacterium cells containing the relevant gene constructs into Arabidopsis leaves.

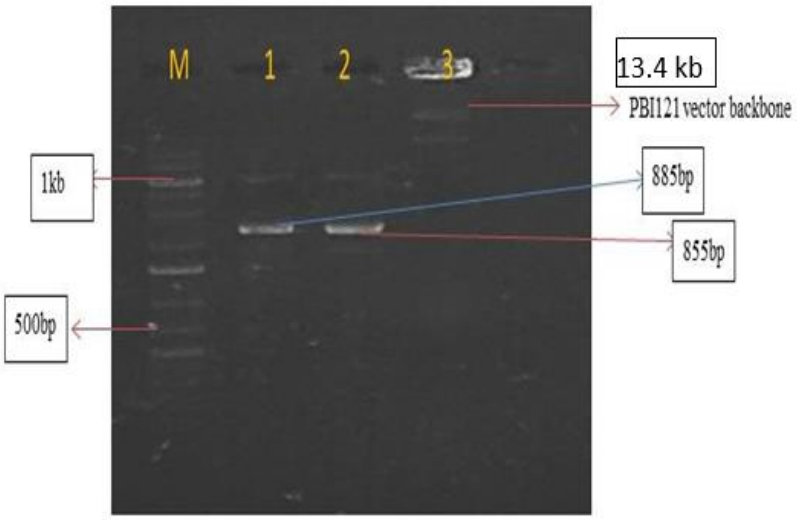

Fig. 1. Agarose gel electrophoresis showing confirmation of restriction digestion of lipase and serine protease genes and pBI121 vector

M: DNA ladder (1kb),
Lane1: Restriction digestion of Lipase with Xba1 and sac1, Lane2: Restriction digestion of serine proteases with Xba1 and sac1
Lane3: Restriction digestion of pBI1211 with Xba1 and sac1

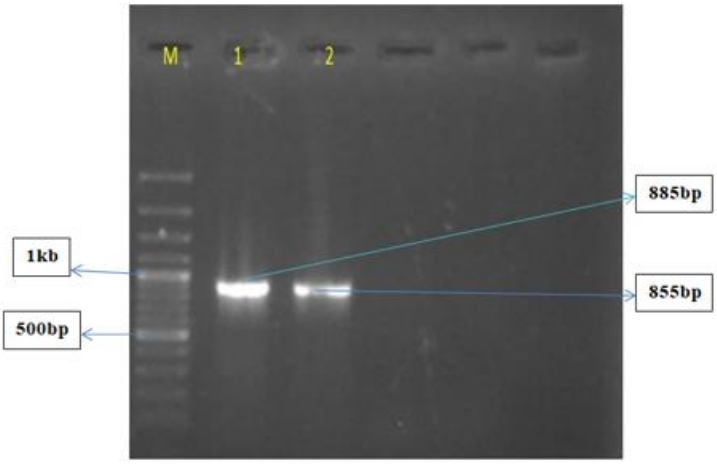

Fig. 2. Agarose gel showing restriction digestion of pBI121-lipase and pBI121- serine protease from transformed cells

M: DNA ladder (1kb)
Lane1 : Restriction digestion of pBI121-lipase constructs with Xba1 and Sac1
Lane2 : Restriction digestion of pBI121-serine protease constructs with Xba1 and Sac1

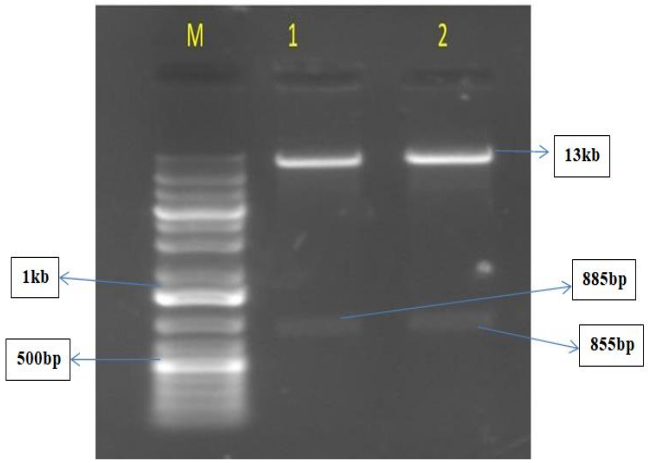

Fig. 3. Agarose gel electrophoresis showing PCR amplification for Transformedcolonies

M:1 kb DNA ladder
Lane1 : PCR amplification for lipase gene
Lane2 : PCR amplification for serine protease gene and Sac1

3.4 Molecular Screening of Gene Integration and Expression of Antiviral Protein Genes in Mulberry Leaves

Bleach agarose gel electrophoresis (1%, w/v) was used to validate the total RNA obtained from agro-infiltrated mulberry leaves. The Prime script 1st strand c-DNA synthesis kit was then used to convert total RNA into c-DNA.Using gene-specific primers, the converted c-DNA was amplified for lipase and serine protease. An amplicon size of 885 bp for Bm Lp and 855 bp for Bm Sp was validated by 1% agarose gel electrophoresis. The 28.974 Kda Bm lipase protein and the 24.271 Kda of serine protease protein, which were extracted from the digestive juice of the mulberry silkworm Bombyx mori (Fig. 4), were similarly described by Ponnuvel et al. [1] and Nakazawa et al. [10]. From the hemolymph of the fifth instar larvae, two distinct haemolymph proteins (P-I and P-II) of the Japanese silkworm *A. yamamai* were isolated. According to gel filtration and SDS-PAGE results,

P-I has an apparent molecular weight of 31000 and 35000 daltons, respectively. According to gel filtration and SDS-PAGE results, P-II has a molecular weight of 22000 and 25000 Daltons, respectively [11]. By using the techniques of gel filtration, ion exchange, and hydroxyapatite column chromatography, Uchida et al. [12] purified a protein that may precipitate nuclear polyhedrosis virus (NPV) in vitro. This protein was recovered from the digestive juice of silkworm larvae (Bombyx mori). The centrifugal and electrophoretic tests on SDS-polyacrylamide gels revealed that the isolated material was a homogeneous simple protein. The purified protein has a molecular weight between 27,000 and 8,000 Da and a sedimentation coefficient of 2.61 S. This protein also activated the NPV of *B. mori* in vitro, which is similar to how serum proteins neutralise antibodies in the body.

3.5 Cloning of Lipase and Serine Protease into a Bacterial pET32a Expression Vector

Both XbaI and SacI were used to break down the pET32a vector (Fig. 5). The digested products were separated in an agarose gel, and the backbone of the release vector was then extracted and purified. The T4 DNA ligase was used to ligate the ligation mix's components in 0.5 mL micro centrifuge tubes, which were then incubated at 4°C overnight. After an overnight incubation at 37° C, blue and white colonies were seen. Using the Thermo Scientific Instaclone PCR cloning kit, the ligated product was converted into E. coli -DH5 cells. After an overnight incubation at 37 °C, altered colonies were seen. Based on blue-white screening, transformed colonies were chosen, and the presence of the gene in white colonies was verified. The colony from the transformed *E. coli* DH5 cell was cultivated, and plasmid restriction digestion using Xba1 and Sac1 and a fragment of 885 bp and 855 bp was used to validate the presence of the Bm Lp and Bm SP genes in the pET32a vector plasmid. SDS-PAGE was used to evaluate the expression of antiviral protein in the pET32a vector. The electrophoretic protein SDS-polyacrylamide gel sizes for lipase, which is 29 kDa, and serine protease, which is 24 kDa (Fig. 6 and Fig. 7). In a similar manner, 28.974 Kda Bm Lipase protein and 24.271 Kda of serine protease protein that were recovered from the digestive juice of mulberry silkworm Bombyx mori were described by Ponnuvel et al. [1] and Nakazawa et al. [10].

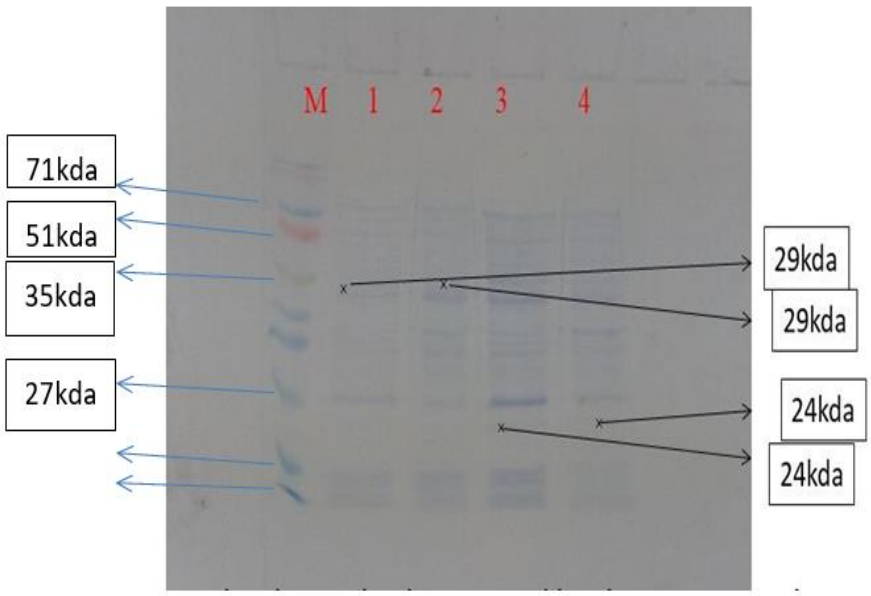

Fig. 4. SDS PAGE (10%) gel showing expression of lipase and serine protease proteins
M : Protein Marker (puregene)
Lane 1 : Lipase expression In V-1
Lane 2 : Lipase expression In S-36
Lane 3 : Serine protease expression In V-1
Lane 4 : Serine protease expression In S-36

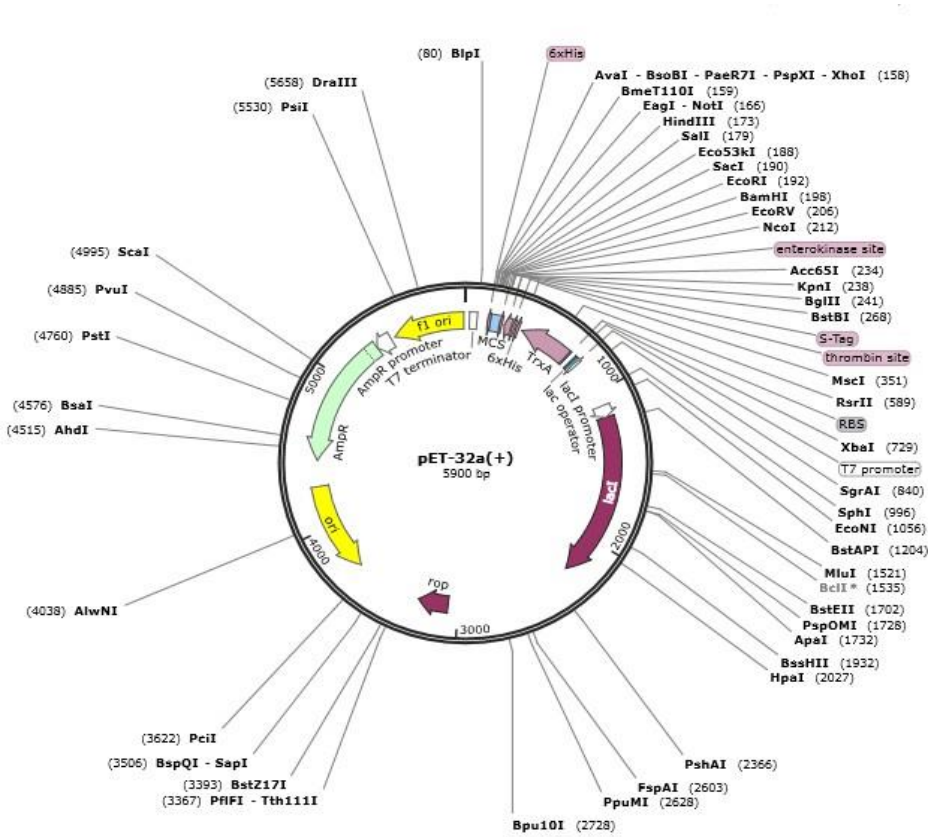

Fig. 5. Genome map of vector pET32a (Snapgene viewer)

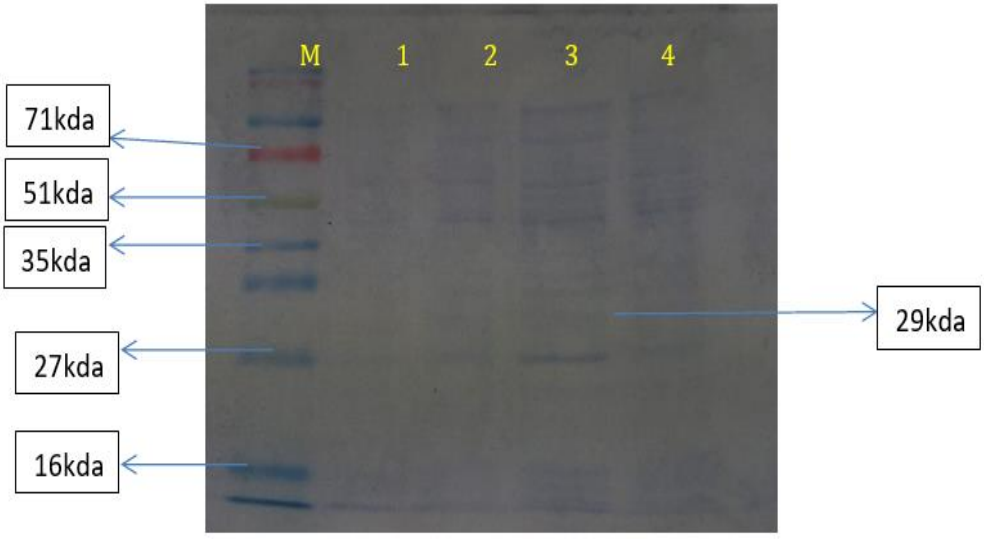

Fig. 6. SDS PAGE (10%) gel showing expression of recombinant lipase protein
M: Protein Marker (puregene)
Lane 1 : Un-induced lipase supernatant
Lane 2 : Un-induced lipase pellet
Lane 3 : Induced lipase supernatant
Lane 4 : Induced lipase pellet

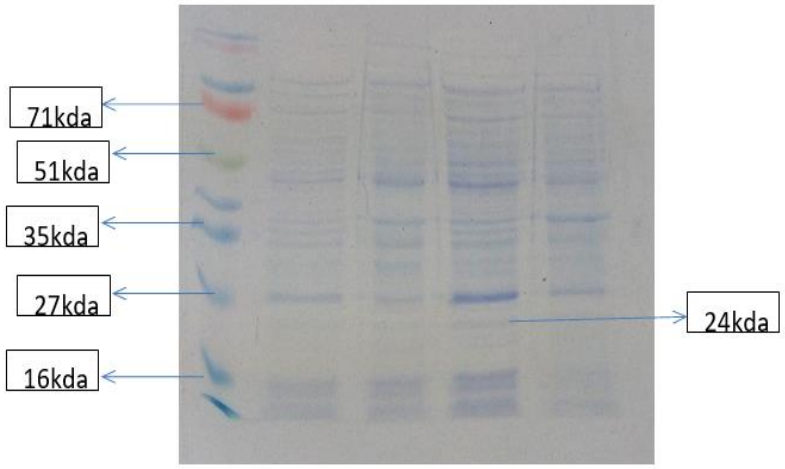

Fig. 7. SDS PAGE (10%) gel showing expression of recombinant serine proteaseprotein
M: Protein Marker (puregene)
Lane 1 : Un-induced serine protease supernatant
Lane 2 : Uninduced serine protease Pellet
Lane 3 : Induced serine protease supernatant
Lane 4 : Induced serine protease supernatant

4. CONCLUSION

The pGEMT cloning vector contains the antiviral genes Bombyx mori lipase and serine protease. The plant expression vector pBI121 was used to express BmNPV antiviral proteins. Amplified antiviral protein gene products from the pGEMT vector were then cloned into the vector after being restriction digested with Xba1 and Sac1. The blue-white colony screening method was used to check the pBI121 altered colonies. By using PCR amplification and restriction igestion, the transformed colonies for the Bombyx mori serine protease and Bombyx mori lipase genes were verified. The lipase gene's 885 bp and the serine protease gene's 855 bp were amplified and released, according to the results. For agro infiltration investigations in mulberry, the plant expression vector pBI121 was effectively transferred into Agro-bacterium cells. Successful invasion of mulberry leaves by Agrobacterium strain LBA4404 carrying the individual gene constructs pBI121 Lp and pBI121 Sp. PCR amplification and SDS-PAGE analysis validated the gene integration and production of antiviral proteins, which were discovered to be 885 bp of the lipase gene with a protein size of 29 Kda and 855 bp of the serine protease gene with a protein size of 24 Kda.

FUTURE LINE OF WORK

1. Development of stable transgenic mulberry plants with antiviral protein genes will enhance the antiviral mechanism against *Bm*NPV infection.
2. Development of artificial diet using transgenic mulberry for feeding early stage silkworm will help in immunizing silkworms against *Bm*NPV infection.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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