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Identification and Molecular Characterization of Inducible Immune Protein in *Bombyx mori* L

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Abstract

Introduction: In insect immunity, antibacterial proteins are an important part of the immune system. These proteins are mostly produced by epithelial cells and released through hemolymph. Antibacterial proteins in insects belong to the five major families of cecropins, defensins, attacin-like proteins, proline-rich peptides, and lysozymes. Considering the importance of these proteins in fighting infections, the aim of this study was to evaluate and identify these proteins in silkworms (*Bombyx mori*).

Materials and Methods: In the present work, profiling of the proteins present in the hemolymph of control silkworms versus, those infected with bacteria was performed by SDS-PAGE, 2D gel electrophoresis, and image analysis. We also used MALDI-TOF and MS/MS to investigate novel and uncharacterized immune protein. For this aim, the silkworm hemolymph after inoculation and infection with *Staphylococcus aureus* bacteria was analyzed using SDS-PAGE, 2-dimensional gel electrophoresis, MALDI-TOF and MS/MS to identify the immune proteins. The Swiss-Prot and NCBI databases were used for protein identification.

Results: A novel protein with a molecular weight of 31.9 KDa was discovered on the fourth day of exposure to bacteria. The expressed protein showed effective activity against *S. aureus*, which was infected silkworms. In the MALDI-TOF/MS result and protein identification analysis, 90 numbers of mass values were searched, the mass values matched 16, the total sequence coverage was 46%, and their score was 57. According to the analyses, the expressed protein belongs to the hemolysin secretion protein (HlyD).

Conclusions: The results led to the identification of a new protein with antimicrobial properties in silkworm, although more information is needed.

Keywords: Silkworm, Antibacterial Proteins, Immunization, Electrophoresis, MALDI-TOF, MS-MS

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Introduction

In the last five decades of research on insects, immunology has developed significantly. In general, the immune response involves cellular and humoral components. In insects this is different from vertebrates; which have very effective immune systems composed of cellular and humoral components. The humoral immune system involves the synthesis and release of several antibacterial proteins. In insects, the fat bodies are like the liver in mammalian systems; they are the site of synthesis for peptides and proteins involved in killing pathogens.[1]

In the past 25 years, 1500 antimicrobial peptides (AMPs) have been isolated from a wide variety of plants, invertebrates, amphibians, and mammals, as well as from bacteria and fungi.[1,2] Cecropin is the first identified and isolated antibacterial protein in Hylophora cecropia (Gaint silkmoth). Antibacterial proteins are classified as cecropins, defensins, attacin-like proteins, proline-rich peptides, and lysozymes; these five major groups were isolated and identified in insects. More than 150 antibacterial proteins have been purified from the hemolymph of insects. Immune peptides belong to a large set of immune effectors with specificities for special classes of microbes that are expressed in insects in response to pathogen infections.[4,5] They have similar characteristics, such as low molecular weight (below 5 kDa), a positive net charge at physiological pH, and for most of them, amphiphilic a-helices, hairpin-like β-sheets or mixed structures.[6]

Bombyx mori is the only lepidopteran insect with a whole genome sequence, as well as a proteomic database, available.[2] Furthermore, it is the first lepidopteran insect to have documented an almost complete genome sequence.[7] With 18,510 predicted genes, about 400 mutant lines, and a relatively large body size, *B. mori* serves as a good model for the genetic and biochemical study of insect immune

responses.[5] Tanaka et al., reported AMPs genes upregulated in the larval fat body by the injection of bacteria and peptidoglycans.[8] Silkworms expressed AMPs after immunization with bacterial cell wall protein components lipopoly-saccharides,[9] peptidoglycan, and antibacterial proteins isolated from silkworms include Attacin,[10] Cecropin,[11] Enbocin, Glovirin,[12] Hemocyanin,[13] Lebocin,[14] Moricin,[15] Lysozyme,[16] and Nuecin.[17] In the silkworm, Bombyx mori, cecropins are classified into three subtypes, A, B, and D. Antibacterial proteins, Bm cecropin B, Bm attacin, and lebocin, are simultaneously regulated by bacterial cell wall components.

Protein identification is an important step to identifying the function and roles of proteins in the cell. Improvements in 2D gel electrophoresis, mass spectrometry, and bioinformatics provide new tools for characterizing proteins involved in physiological processes, such as the immune response of the silkworm insect model *Bombyx mori*. To analyse AMPs with higher molecular masses, a strategy based on 2D gel electrophoresis was developed. Hou et al., used SDS-PAGE/2DE, tryptic digestion of a protein band or spot can be chemically derivatized and sequenced by MALDI-TOF, MS/MS[18] to analyse silkworm hemolymph peptides.

In the present work, profiling of the proteins present in the hemolymph of control silkworms (*Bombyx mori*) versus, those infected with bacteria was performed by SDS-PAGE, 2D gel electrophoresis, and image analysis. We also used MALDI-TOF and MS/MS to investigate a novel and uncharacterized immune protein. Through this differential analysis, more than 30 out of 160 spots were up- or down-regulated by at least fivefold after bacterial immunization.

Materials and Methods

Isolation of Bacterial sp.

The collected silkworm cadavers showing bacterial infections were taken, and their surface was sterilized with 0.1% of mercuric chloride solution and washed twice with Milli-Q water. The pathogenic bacteria were isolated from the infected silkworm body fluid on a nutrient agar medium. The isolated colonies were selected and identified using standard methods, and confirmed strains were stored at 4 °C for future use.

Insects

Bombyx mori (NB7) layings were procured from the Central Sericulture Germplasm Research Centre (CSGRC), Hosur, Tamilnadu. The bivoltine race NB7 was used in the present study as bivoltine silkworms have low resistance against pathogen infection. Bacteria were isolated from silkworms infected with bacterial diseases in Dharwad and surrounding villages. *Bombyx mori* (NB7) larvae were reared at room temperature (27 + 2 °C and 75+5% relative humidity) on fresh S36 variety mulberry leaves and maintained as per the standard rearing method.

Immunization

Septicemia The pathogen (*Staphylococcus aureus*) was grown in nutrient broth (NB) and log-phase *S. aureus* was isolated. Bacteria were diluted in 1 ml of saline solution. The fifth-instar, 2-day-old larvae were injected with 1 X 105/20 µl of the bacterial saline suspension using a Hamilton syringe. A total of 150 larvae were used in this study (100 immunized larvae and 50 control larvae were both injected with insect physiological saline under the same conditions).

Sample Collection

Hemolymph samples can be collected from the immunized as well as control silkworms at different intervals (0, 24, 48, 72, and 96 h) after the vaccination by making small incisions on the silkworm surface using a sterile scalpel blade. The hemolymph can then be aspirated into an eppendorf tube containing a few crystals of phenylthiourea (PTU) added to stop the melanization reaction. The collected samples were centrifuged at 10,000 rpm for 10 min (4 °C). The hemocytes and tissue debris were discarded, and the supernatant was stored at -20 °C until use.

Protein Determination

The amount of protein measured by spectrometry using the Bradford[19] method of a calibration curve was prepared with different concentrations of BSA (bovine serum albumin) as standards. Foline-Ciocalteu reagent was used as a colour reactant, and concentration was calculated in response to absorbance at 595 nm in a spectrophotometer.

SDS-PAGE

Hemolymph protein was denatured by mixing with sample buffer (containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris HCl pH 6.8) and heating at 100 °C for 5 min. 15% of the polyacrylamide gel was used. The electrophoresis was performed using a Bio-rad protean tetra cell at 150 V, 30 mA, and 1.30 min. After electrophoresis, gel was stained with Coomassie brilliant blue R-250.

First Dimension Electrophoresis (IEF)

The immunized, as well as those who controlled hemolymph flow, performed IEF according to our standardized protocol.[20] Briefly, in the first dimension, we are using the Bio-rad Protean II chamber. Glass capillary tubes were loaded with IEF gel solution, and after polymerization, 20 l of GOS (Gel Overlaying Solution) were loaded on the two gels, and the remaining space was filled with 20 mM NaOH (upper tank buffer/cathode electrode buffer). Fill the lower tank with anode electrode buffer (7.8 M orthophosphoric acid). The upper tank and lower tank buffer were degassing under high pressure. The IEF has performed a pre-run with a constant voltage of 200 V for 15 min, 300 V for 30 min, and 400 V for

30 min. After the pre-r, the estimated protein sample was loaded with a 1 ml tuberculin syringe (loaded in each tube was 100 µg of protein). Load 20 µl of GOS and fill the upper tank buffer. Connect to the power supply and begin a 12-hour constant voltage of 600 V and a 1-hour constant voltage of 700 V at room temperature.

Second-Dimensional Electrophoresis

Extrude the IEF gel from the capillary tube by using an extruded needle with Laemmli buffer (0.125 mM Tris [pH 6.8], 20% glycerol, 10% mercaptoethanol, 10% SDS, and 0.004% bromophenol blue). The extruded gel was immediately stored in lamelli buffer at -80 °C. The IEF gels were kept in lamellae buffer for 30 min because, in the second dimension, the protein resolution is increased. For the second-dimension SDS-PAGE, we used the PROTEAN II XL system (Bio-Rad). Overlaid immobilized IEF gel on top of 12.5% of polyacrylamide gel and sealed with 0.1% of low-melting agarose solution. Gels were run for 2.15 h at 150 V, 30 mA on an ESP1000 (GE Health care) until the dye front had run off the edge of the gel. The gels were fixed in the fixative solution. The separated proteins were stained with silver nitrate according to the Amersham protocol, with an additional developing step to reduce background staining. The stained gels were stored in a 7% acetic acid solution.

Protein Purification

Hemolymph was fractionated by salting out with the ammonium sulfate precipitation method and subjected to 80% ammonium sulfate. The precipitates were incubated for 4 h. After incubation, the sample was centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was discarded. The pellet was dissolved in 0.5 M ammonium acetate (pH 7) buffer, and the solution was transferred to the dialysis membrane (cut-off range: 12 KDa). Sealed, the dialysis pack was kept in a 0.3 M ammonium acetate (pH 7) buffer overnight. Sephadex G-75 beads were used. Wash the column with 0.3 M ammonium acetate buffer. The column was first washed with 0.3 M ammonium acetate to remove the non-adsorbed proteins. The dialysate was loaded onto a column and eluted stepwise with 0.5 M ammonium acetate buffer. The flow rate was 2 ml per 10 min. The collected fraction readings were monitored at 220 nm in a UV-Spectrophotometer. The collected fraction was lyophilized to dry and store at -20 °C.

Antibacterial Activity

Antibacterial activity against *Staphylococcus aureus* was analyzed by an inhibition zone assay. LA (Luria agar) was used. A 10 µl aliquot of bacterial dilution was then added to 10 ml of fresh media and poured over the petri dish. After the agar solidified, two wells were prepared for the growth inhibition zone. 20 µl of purified protein and pure hemolymph were added and incubated at 37 °C for 12 h. Antibacterial activity was indicated by the formation of a clear zone on the surface of the agar. The determination of the clear zone without bacterial growth was recorded.

Trypsin Digestion, Mass Spectrometry Analysis, and Identification of Proteins

Spots of interest were excised from the gels within 6 h after staining and digested using trypsin according to the standard protocol. 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 ZipTip C18 microtips (Millipore, Bedform, MA, USA) and eluted in 4 µl 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 (337 nm) at 50 Hz was used for taking an average of 100-150 laser shots. The resulting spectrum was analyzed using Bruker Daltonics Flex Analysis Software.

Protein Identification

Swiss-Prot and NCBI-nr databases were used for protein identification through the search engine Matrix Science-Mascot (http://www.matrixscience.com) and MS Fit in the Protein Prospector v.4.27 (http://prospector.ucsf.edu/protein-prospector).

Immune Gene Annotation

The MS spectral signature was used for a search of related CDs in the silkworm database for its annotation (http://silkworm.swu.edu.cn/silkdb/doc, http://www.silkdb.org/silksoft/blast2-simple.html).

Results

Characterization of Organisms

The isolated bacterial strains were identified using biochemical testing kits. At the NA plate, the colony was colourless. The colony was used for biochemical tests. Through simple staining tests, rod- and spherical-shaped bacteria were observed. In negative staining, colourless spherical-shaped bacteria were identified. Gram staining revealed blue-colored rods and spherical-shaped gram-positive bacteria. On acid-fast staining, blue-colored, spherical- shaped bacteria were identified. In bacterial endospore staining, red-coloured vegetative cells and green-colored endospores were observed. We found *Bacillus subtilis* and *Staphylococcus aureus*.

The Molecular Weight of the Novel Protein

The novel protein molecular weight was measured using SDS-PAGE with a standard medium-range molecular marker (14-97 KDa). The newly expressed single-band protein's molecular

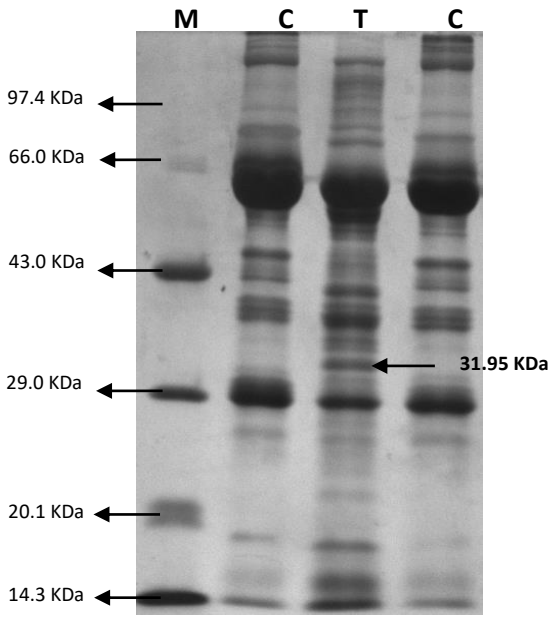

Figure 1. Protein Identification through SDS-PAGE Analysis in both Control and Immunized Hemolymph. **M**: Protein Molecular Marker; **C**: Control; **T**: Immunized hemolymph.

weight was 31.95 kDa. The protein photograph and molecular weight were determined by using the Vilbert Lourmet Gel Doc system and its molecular weight analysis. We analyzed the gel by using E-Box software for the expressed protein molecular weight and expression level.

2DE-Analysis

In the present study, 2DE was employed to compare the control and immunized silkworm hemolymph. All the steps from sample preparation to 2DE were carried out simultaneously, and the operation conditions were also the same. The protein spot distribution pattern was nearly the same in the two gels. One of the gels (Figure 2a) was designated as the reference gel (control) and was matched with the other immunized gel (Figure 2b). The results showed that the spot-matching rate of the two gels was very high and that most of the main protein's expression volume in the two gels was almost the same. In the immunized sample, some new proteins were expressed.

1-DE Immune Protein Search against Drosophila Database

A coomassie-stained protein band of 31.9 kDa excised from the 1-DE gel was subjected to MALDI-TOF, MS/MS for identification and confirmation. The resulting MALDI-TOF fingerprints were analyzed using a protein prospector search engine, and the hits obtained are presented in Figures 3 and 4. Eventually, a 31.9 kDa protein expressed in *B. mori* larvae is identified for the first time in the range of its molecular weight.

Identification by MALDI-TOF and MS/MS

Another protein identification method is based on peptide sequencing. The expressed immune protein band was analyzed

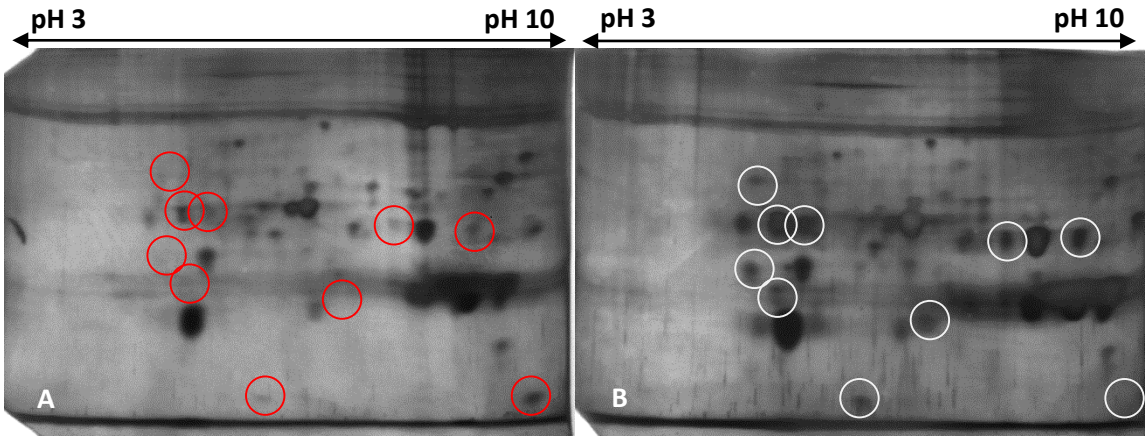

Figure 2. Hemolymph 2-DE Analysis. Proteins were separated according to their pI values on pH gradient 3-10 range, and then separated by 12% SDS-PAGE. The white boxes indicated the protein up regulation and down regulation in both control as well as immunized hemolymph.

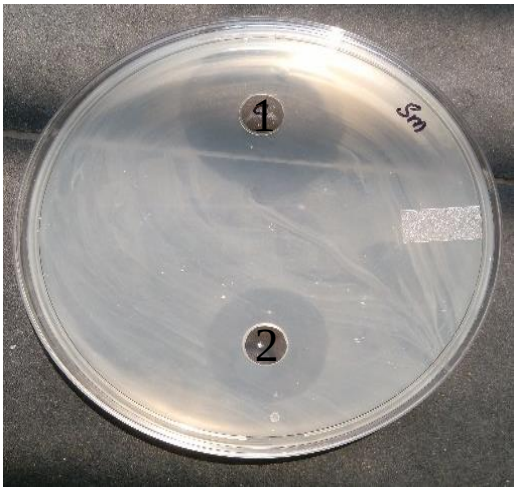

Figure 3: Growth Inhibition Assay against the *Staphylococcus aureus* of Both Purified Protein and Crued Hemolymph. **1**: Purified new protein; **2**: Crued hemolymph.

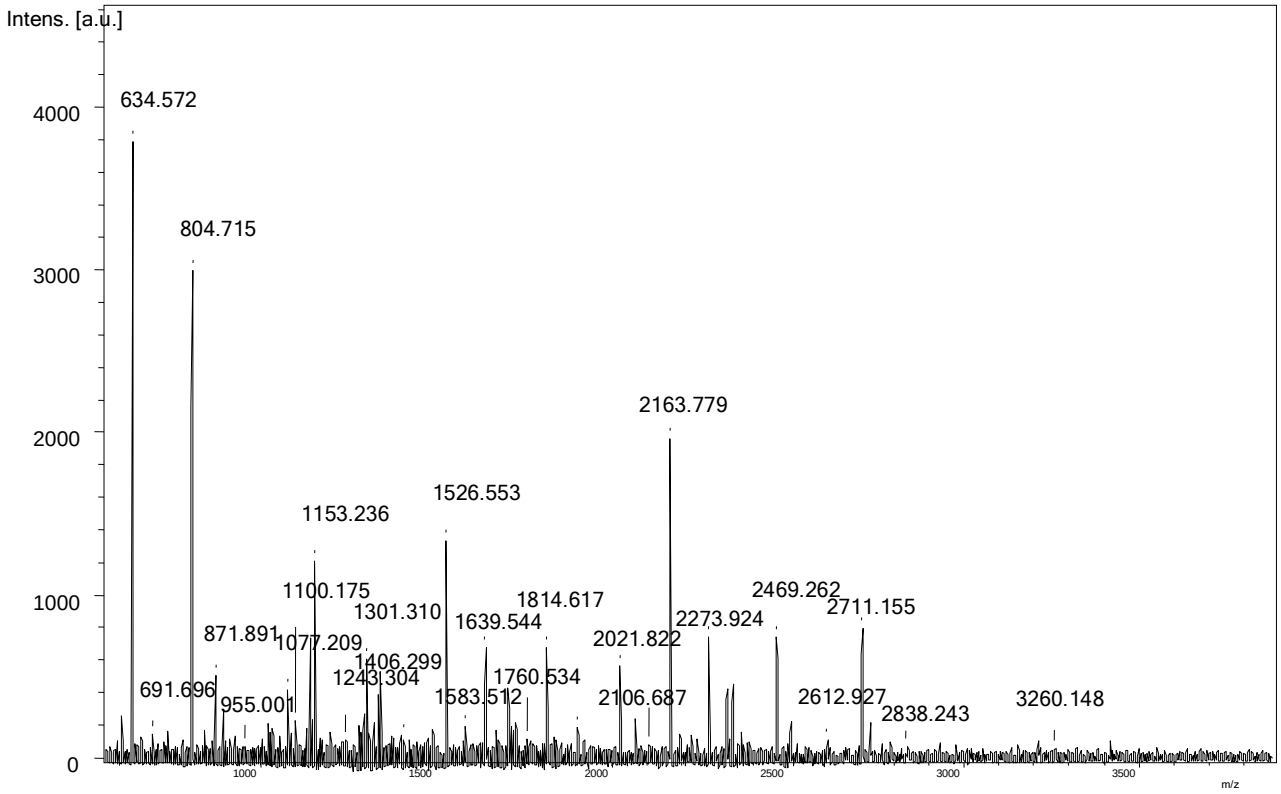

Figure 4. Identification of Newly Expressed Protein by MALDI-TOF/MS Spectrum.

through MALDI-TOF. The expressed protein belongs to the HlyD family protein. The results were shown in Figures 3 and 4. In these 90 numbers of mass values searched, mass values matched 16, total sequence coverage 46% and their score was 57.

Antibacterial Activity

The antibacterial activity of the hemolymph as well as the purified protein, were studied in the zone of inhibition of growth assay against *Staphylococcus aureus*. The results of the antibacterial activity are presented in Figure 3.

Discussion

As a result, our findings emphasize the significance of immune function in the maintenance of larval phenotypes and provide a detailed investigation into the molecular mechanism underlying the immunological properties of the tropical silkworm, *B. mori*. Immune proteins play an important role in biochemical processes in the biological system, particularly the immune system. Most studies on insect innate immunity have focused on bacterial and fungal infections.[21,22] Early immunological studies conducted at the beginning of the 20th century focused on the role of the morphotic elements of the

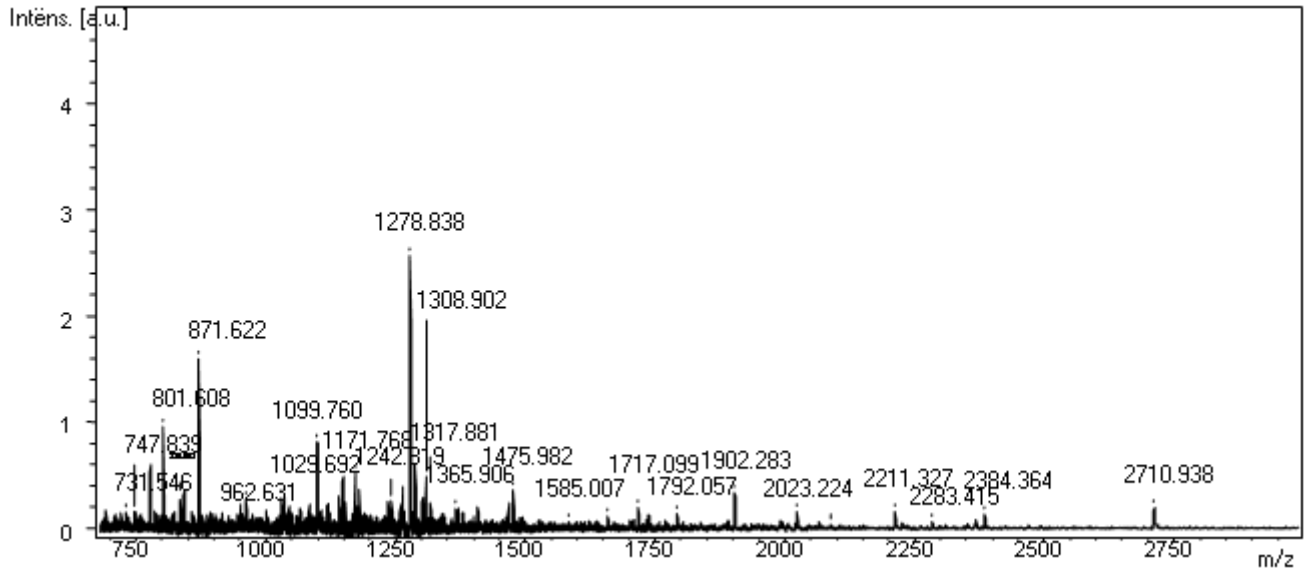

Figure 5. Identification of Newly Expressed Protein by MS/MS Analysis Spectrum.

hemolymph. As a result, we focused on identifying immune proteins in *B. mori*. Based on the molecular weight in 1-DE and protein spots in 2-DE, the expressed immune protein was predicted. Notably, 2-DE separation of hemolymph proteins isolated after immunization of larvae revealed a discrete variation in the distribution of protein spots between control and immunized larvae of NB7. In the control hemolymph protein profile of the 2-DE, 185 isoforms were identified; in immunized hemolymph, 214 isoforms were identified. There are 29 newly expressed isoforms in total, with some isoforms having different molecular weights. The expressed protein was sequenced through MALDI-TOF and MS/MS. Before sequencing, the gel band was treated with trypsin digestion. The PMF analysis using a search engine matrix science mascot against Swiss-Prot and the NCBI-nr database uncovered different isoforms of immune-related proteins in *B. mori* for the first time. Different immune protein isoforms found in NB7 could undoubtedly play a role.

Pawel et al., discovered a strong, heat-stable antibacterial activity induction after immunizing insects with low doses of virulent pathogens.[23] Song et al., evaluated to understand the BmDefensin-like peptized effect of immune challenge and antimicrobial activity. Semi-quantitative RTPCR analysis revealed that bacterial injection induced BmDefensin-like peptied gene expression, with the highest level of expression 8 h after bacterial injection.[24] Our results provide novel immune proteins and their molecular characterization. The currently expressed protein has a large molecular weight of 31.4 kDa and is a highly anionic peptide. analyse and observe the antibacterial activity of purified as well as crude hemolymph. The purified fractions are very effective against bacteria, and crude hemolymph has some inhibition zone as well. Characterization of expressed immune proteins has incorporated 1-DE and 2-DE supported by MALDI-TOF and MS/MS in light of advances in proteomics research.

Hence, our approach and present findings differ, considering the hemolymph of silkworm larvae for immune study, from other proteomic research that is tissue-specific, including silk glands,[25] embryos,[26] colleterial glands,[27] midgut,[28] hemolymph,[29,30] Malpighian tubes,[31] and fat bodies.[32] After identifying immune proteins that were correlated with the drosophila genome database, the same spectral signature was used to search the *B. mori* genome database in NCBI-nr. We have extracted the amino acid sequence from the resultant NCBI BLAST search gi:220914948 and subjected it to multiple sequence alignments. The primary observation of Mascot search results was concerning 7.96, MW 37.7 kDa. The total number of amino acid residues is 354. We demonstrated that integrating proteomic assays, such as 2-DE coupled with MALDI-TOF, MS/MS, and bioinformatics tools and techniques, could help with AMP annotation. Since some proteins were identified using the EST database, which undoubtedly slowed progress towards a well-annotated lepidopteran genome, the current data derived from the proteomic approach may play a critical role in genes in the validated protein-coding genes in the integrated silkworm genome assembly. Thus, different AMPs that are expressed due to inducing bacteria in *B. mori* larvae may not find a match in the silkworm genome database, whereas MS can reliably demonstrate the presence of protein isoforms. The application of proteomic strategies in the present study for the first time facilitated our uncovering of AMPs annotation in the silkworm genome, which is a promising example for the generation of an accurate gene catalogue for *B. mori* and other lepidopterans.

Conclusion

The immune system in insects is simply by not having a complex immune system and immune organs, but the immune

system is extremely effective, as evidenced by insects' survival in almost all habitats. The use of two-dimensional electrophoretic techniques will help us understand more about the way insects have evolved themselves in nature through a simple but very effective mechanism. Generally, they are distinguished as being heat stable and having a low molecular weight with no adverse effects on eukaryotic cells. In general, they are characterized as heat-stable with no adverse effects on eukaryotic cells. These characteristics contribute to the potential use of these proteins in human and veterinary medicine and animal nutrition. Depending on their mode of action, insect AMPs may be applied as single peptides, as a complex of different AMPs, and as an active fraction of insect proteins in the nutrition of different livestock.

Authors' Contributions

All authors were concerned in the scientific assessment, analytic for the cases describe in this document. All authors read and accepted the concluding version.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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