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Comparative proteomic profiling of silkworm hemolymph during *Nosema bombycis* infection

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Abstract
Silkworm pebrine, caused by *Nosema bombycis*, it causes great losses in sericulture all over the world. Research on host response in silkworm following *N. bombycis* infection has given interesting results, especially pertaining to the innate immune response to the pathogens. In this present study is to investigate the silkworm heamolymph proteome profile changes after the inoculation of *N. bombycis*. Silkworms were explorated with *N. bombycis*, and analyzed the differentially expressed proteins in both infected and uninfected hemolymph. Inoculated the pathogen spores ($1x10^8$) through *per oral* to the 5^{th} instar 1^{st} day of silkworm larvae and hemolymph were collected different time intervals after inoculation as well as control. Conducted Nano-drop spectrophotometry, electrophoresis and different staining methods for the detection of expression of protein profiling. The electrophoresis SDS-PAGE methods was used to identify differentiation of protein expression and analyzed the targeted band by using MALDI-TOF-MS and MASCOT tools for the characterization of the protein. After the inoculation hemolymph protein concentrations were up-regulated and down-regulated. The up-regulated 25 kDa protein band was targeted in hemolymph after 5^{th} dpi, based on its expression level at different intervals. Targeted protein was analyzed through the MALDI-TOF-MS and got the amino acid sequence. MASCOT analysis data have concluded that the targeted protein sequence matched 38% of homology with Neprilysin-3, a Metalloendoprotease protein found in *Drosophila melanogaster***.** Results will be valuable to further study of insect innate immunity and to aid in a comprehensive understanding of the silkworm immune mechanism after inoculation of the pathogens.

Keywords: Silkworm, *N. bombycis*, proteomics, electrophoresis, MALDI-TOF

Introduction
Insects have been particularly successful animals in evolution. Current estimates are that 90% of all known species within the animal kingdom belong to this insect class. In the insects hemolymph is in an open system that circulates among all organs, and functions in nutrient and hormone transport, injury, and immunity. In addition, hemolymph has a key role in innate immunity response that is triggered when bacteria or fungi enter the insect body. Insects plasma proteins, including those involved in immune responses, are synthesized primarily by the fat body, with some contribution from haemocytes and other tissues. Pathogens and parasites can alter this regulatory network. Hemolymph of many insects contains antibacterial proteins, lysozyme, an enzyme that degrades bacterial cell walls. All types of antibacterial, antifungal and antiviral proteins are identified in to the *Drosophila melanogaster* and silkworm *Bombyx mori* L hemolymph. Since the 1970s and earlier, the proteins of silkworm hemolymph have been studied to elucidate their role in silkworm development [1].

The silkworm *Bombyx mori* L., an important economic insect and one of the best models for studying insect immunity after the *Drosophila melanogaster*, possesses an efficient and sophisticated innate immune system against invasive microorganisms [2]. The final 5^{th} instar phase of *B. mori* is a critical period for its growth, development, metamorphosis and productivity. Days 4 and 5 of the fifth instar are the very stages for *B. mori* fast-growing. Proteomics results showed that silkworm hemolymph protein expressions varied with the insect's developmental processes. Li *et al.* [3], Hou *et al.* [4] and Li *et al.* [5] reported the protein profiling of the different developmental stages of silkworm hemolymph and identified the expressed protein characterization and some proteins play a vital role in the silkworm developmental stage. Cai *et al.* [6] were studied differential expression of hemolymph profile by using two-dimensional gel electrophoresis and MALDI TOF/TOF MS to the resistance of silkworm to nuclear polyhedrosis virus (BmNPV) infection.

Microsporidia are obligate intracellular parasites classified in the Kingdom Fungi. Approximately 187 genera and more than 1300 species of Microsporidia have been described in the animal kingdom [7], among which more than half infect economically important arthropods, such as silkworm and honey bees. Apart from that pebrine is one among those caused by a microsporia species *N. bombycis* an obligate intracellular and transovarially transmitted parasite. *N. bombycis* attacks all insect tissues and developmental stages, and the signs and symptoms are observed in all life stages from egg to adult. Larvae infected by Pebrine exhibit a loss of appetite, have a reduced growth rate, vary in size, and display incomplete molts. *N. bombycis* affects maximum insects kingdom. Because of spore wall coated chitin protein it can survive in any ware. After infection it can multiply in the insect midgut. Within six days it completes the life cycle and multiplies in the tissue. Some micro and macro bioelements are present in the hemolymph. Zhang *et al*. [8] identified various microsporidian species is achieved using polyacrylamide gel electrophoresis. The spore polypeptides of *N. bombycis* and *Vairimorpha necatrix* were separated by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) [9]. PAGE electrophoresis provided unique reproducible electrophoretic profiles which were not influenced by the host species. The host proteins were not detected in the polypeptide profiles. Mixed spore samples of *V. necatrix* and *N. bombycis* could be separated in the presence of the profiles of the individual species. A total of 31 polypeptide bands were detected on electrophoretic profiles of spore mixtures, whereas individual profiles of *V necatrix* and *N. bombycis* had 24 and 26 polypeptide bands, respectively [9, 10]. Guoqing *et al*. [11] were summarized different microsporidian infection to the invertebrate host responses.

Studies detailing the pathological effects of *N. bombycis* on silkworm have focused mainly on consequences to mortality. Antunez *et al*. [12] and Chaimanee *et al*. [13] reported the effects of infection on expression of immune-related peptides after infection of the *N.apis.* Mayack C, Naug D [14, 15] reported in honey bees after infection of the *N.apis* at changes in nutritional or energetic states. However, our understanding of underlying physiological effects contributing to the disease process is incomplete. We used different proteomic techniques to measure relative changes in hemolymph after inoculation of the *N. bombycis*. Our objective was to determine whether infection with *N. bombycis* disrupts fundamental physiological processes for the silkworm cocoon formation.

2. Materials and Methods

2.1 Rearing & Inoculation of pathogens

Healthy silkworm (CSR2 breed) was reared under the standard rearing methods. 5th instar 1st day Silkworms were inoculated per oral at 1x10^{8} concentrations of spores with *N. bombycis* smeared on mulberry leaves. Healthy larvae also maintained simultaneously.

2.2 Sample collection

Hemolymph was collected from day 3 after inoculation till spinning (7th day) post infection by cutting the caudal horn of the silkworms. The collected samples were immediately stored at –80 °C for further use.

2.3 Protein estimation

Protein quantification method was carried out by the advanced Nano-drop Spectrophotometric analysis.

2.4 Sample preparation for gel electrophoresis

Sample preparation and separation for one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared. The protein estimated hemolymph samples (1 µl) were collected in a new sterile eppendorf tube and added autoclaved Milli-Q water (9 µl). Added equal volume of Laemmli buffer containing Tris-HCl (pH 6.8), SDS, 2-ME, Glycerol. Cyclomix the solution and boiled at 100 °C for 5 min.

2.5 Electrophoresis

12% gels were used for the detection of protein profile. Performed the electrophoresis using Bio-rad mini dual cell unit at 150 V, 30 mAh for 60 min or till run the gel to the bottom.

2.6 Staining methods

After the electrophoresis the extracted gels were performed the three different staining methods (Coomassie Briliant Blue, Silver Nitrate and Periodic Acid-Schiff's) for the detection of quantified proteins profiling. Periodic Acid-Schiff's method was used for the detection of glycoproteins in silkworm hemolymph.

2.7 Sample preparation for Maldi-TOF-MS

Targeted protein band was separated from SDS-PAGE gel, 5th day post infection hemolymph protein 25 kDa band was selected for the analysis. Protein bands were extruded from the gel and bands were stored in 7% Acetic acid and subjected to MALDI-TOF-MS analysis in Molecular Biophysics Laboratory, Indian Institute of Science, Bangalore.

2.8 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 Jensen *et al*. [16]. The tryptic peptides were extracted with 70% acetonitrile and 3% formic acid. After 10 min sonication, peptides were desalted using Zip Tip C18 microtips and eluted in 4 µl 50% acetonitrile. Tryptic peptides were analyzed on a MALDI-TOF-MS using Ultraflex TOF/TOF (Bruker Daltonics, Germany).

2.9 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 SwissProt database for peptide and protein identifications.

3. Results and Discussion

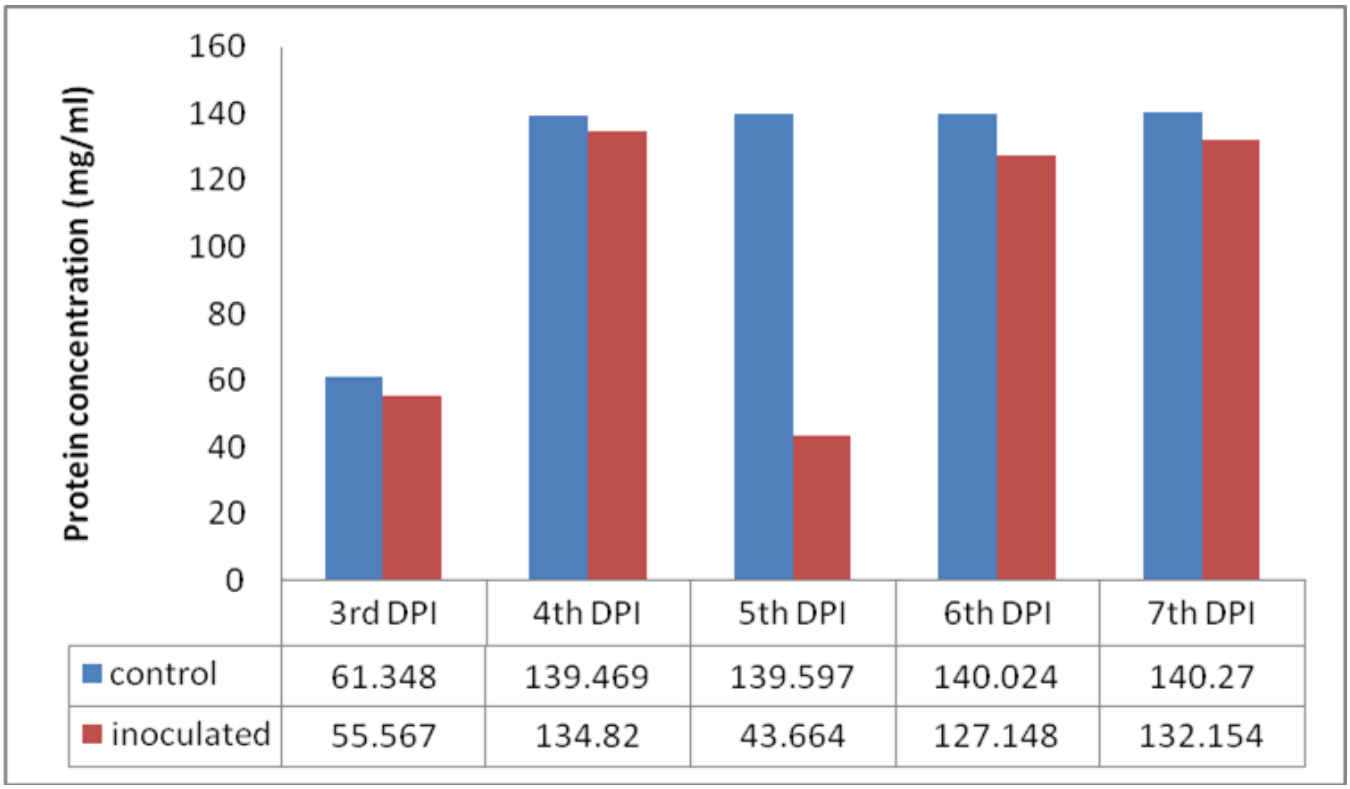
Silkworm hemolymph collected from the 3rd DPI of fifth instar to up to spinning and analyzed by Nano-drop spectrophotometric (Fig. 1) and SDS-PAGE (Fig.2). Nano-drop spectrophotometric analysis resulted in significant expression of total proteins in the infected samples compared with control. 61.348 mg/ml of protein concentration was found in the control hemolymph, where as in infected hemolymph samples the protein concentration were up-regulated (134.82, 127.14 & 132.15 mg/ml) on 4th, 6th, and 7th day respectively, but were down-regulated (55.56 & 43.66 mg/ml) on 3rd and 5th day respectively.

Quantified hemolymph samples subjected to SDS-PAGE analysis for protein profiling. After electrophoresis, different

staining methods were used for staining, such as CBB, Silver nitrate and PAS methods. We found total 35 protein bands in control hemolymph and found some differentiate protein profiling in the different time intervals of infected hemolymph by using CBB staining (Fig. 2a). Identified 45 protein bands by using a silver nitrate staining method (Fig. 2b). 20 proteins identified by PAS method for detection of glycoproteins (Fig. 2c). Total expressed proteins also listed in the Fig. 3.

The SDS-PAGE map showed that hemolymph proteins, particularly the proteins around 15 kDa, 25 kDa, 30 kDa and 80 kDa, underwent dramatic changes. Detected 80 kDa protein expression level gradually increased from the 3rd day of fifth instar, reaching a maximum at the spinning stage. Whereas some proteins were up-regulated like 25 kDa, 30 kDa proteins, these two proteins belongs to the lepidopteron low molecular weight proteins. Still not identified these Lepidopteron low molecular weight proteins functions. Fat bodies are secreted this proteins. Proteins of approximately 30 kDa appeared in the hemolymph at the 4th day of fifth instar, increased gradually as the silkworm grew, and became a major component of hemolymph in fifth instar and expression patterns, these proteins predicted to be storage proteins and 30 K proteins of silkworm hemolymph. They expressed in the fat body and secreted to the hemolymph, and constituted the major part of plasma proteins. 30 kDa protein expression levels increased after inoculation of the *N. bombycis*. In addition to the low molecular weight lipoprotein 30 kDa protein, small proteins also showed remarkable variation in the silkworm hemolymph maps. It identified only in the 8th day hemolymph of pupa [17]. These two proteins identified as 32 kDa apolipoproteins. Diapauses bio-block protein having 15 kDa protein expression levels also increased after inoculation, which is involved in the silkworm diapauses eggs and transient ATPase activity [18]. 27 kDa Ribosomal proteins S7E protein was expressed in the inoculated silkworms; it belongs to processing of pseudogenesis activity [18, 19]. On 4th and 5th DPI, one of the important hypothetical protein (77 kDa) expressions levels was very high it may be pathogenesis. Immunoglobulin super family receptor (20 kDa) protein was found to be gradually increasing after inoculation of the pathogen, because of induced immune response of the host against the pathogen [18].

Based on the observations made from different staining methods for SDS-PAGE gel at different time intervals, a 25 kDa molecular weight protein was selected and extruded for MALDI-TOF-MS analysis. m/z values obtained from the MALDI-TOF-MS (Fig. 4) results were subjected for MASCOT search analysis to search the protein sequence in Swiss-Prot database and characterize the identified protein. MASCOT analysis of the MALDI-TOF-MS data has concluded that the targeted protein sequence matched 38% of homology with Neprilysin-3, a Metalloendoprotease protein found *in Drosophila melanogaster*. Neprilysin-3 is a neurohormonal peptides secreted by the central nervous system brain, ventral ganglion and midgut. By using NCBI iCn3D (web-based 3D viewer) browser site, we designed the interested protein 3D structure (Fig. 5).



	3rd DPI	4th DPI	5th DPI	6th DPI	7th DPI
control	61.348	139.469	139.597	140.024	140.27
inoculated	55.567	134.82	43.664	127.148	132.154

Fig 1: Control and infected silkworm hemolymph protein concentration

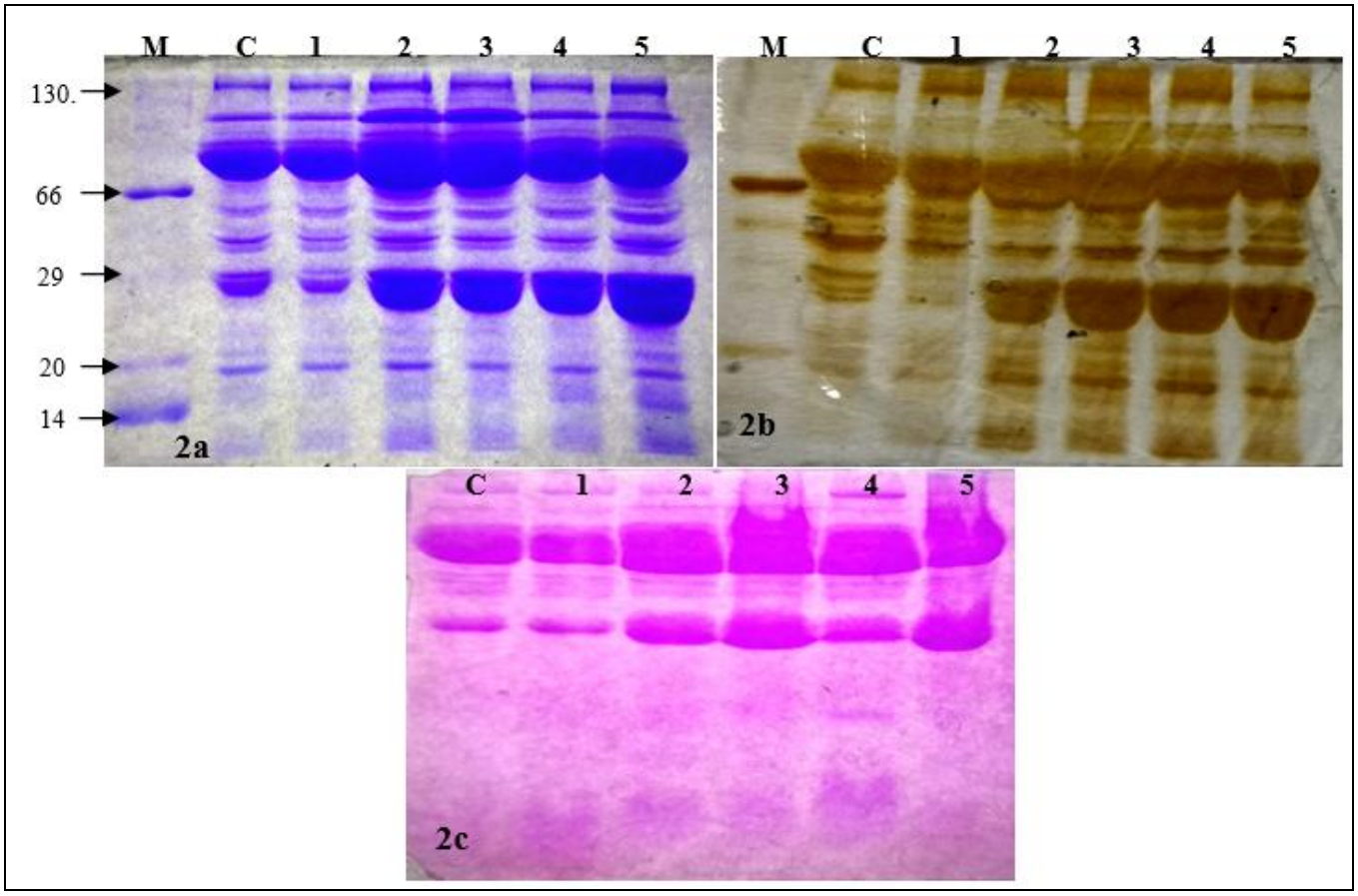


Fig 2: Proteome profiling of control as well as *N. bombycis* infected silkworm hemolymph by using 2a:CBB staining, 2b:silver nitrate and 2c:PAS staining M: Marker, C: control heamolymph, lane1:3-DPI, 2: 4-DPI, 3: 5-DPI, 4: 6-DPI, 5: 7-DPI

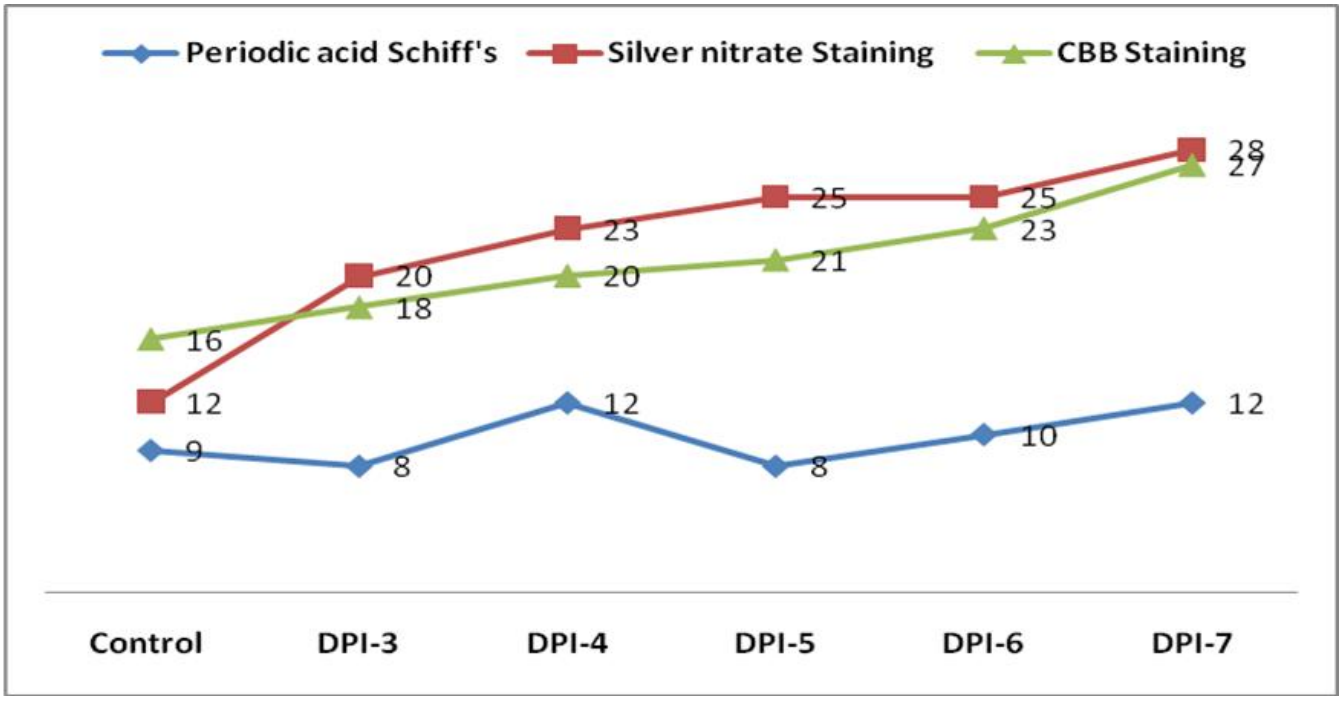


Fig 3: Number of Protein bands expression by using different staining

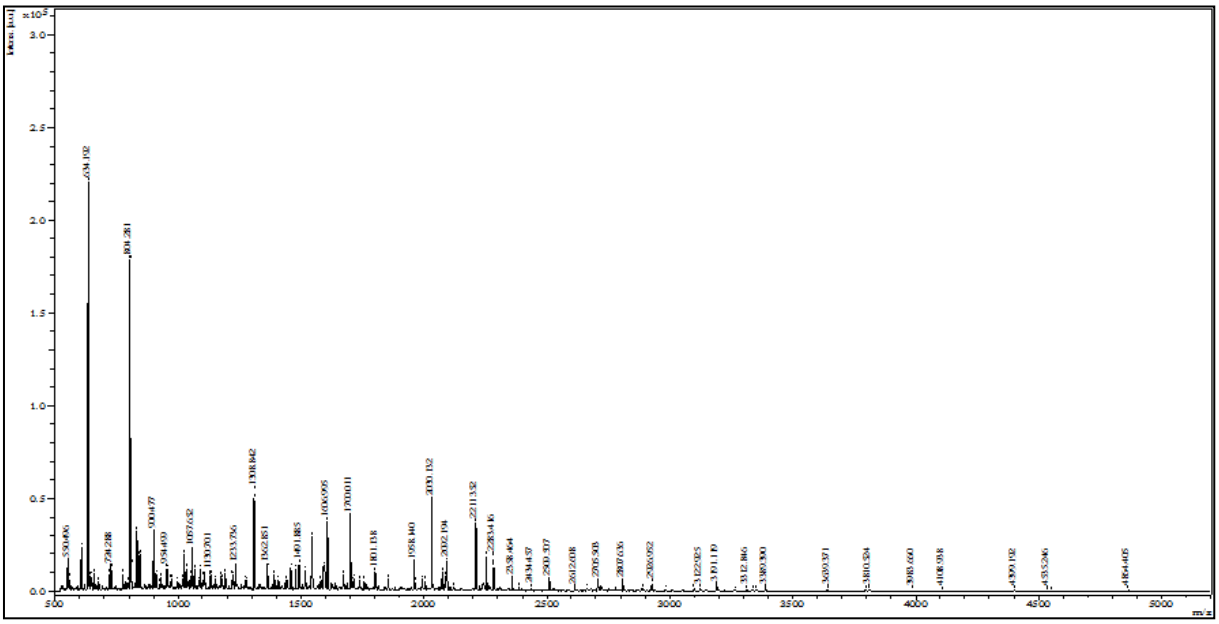


Fig 4: MALDI-TOF-MS spectra of the targeted 25 kDa protein band

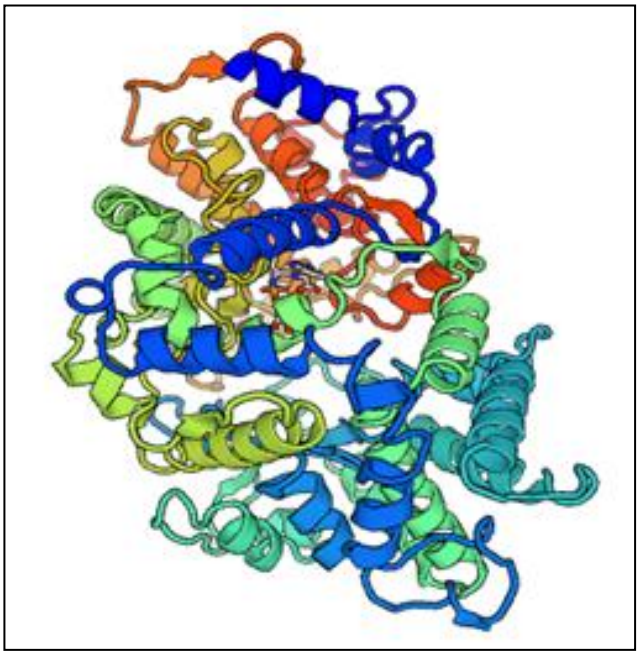


Fig 5: 3D Structure of the up-regulated protein Neprilysins like protein.

4. Conclusion

Proteomics research not only provides a method for investigating protein expression patterns, but also identifies lots of attractive candidates for further investigation. In this work, silkworm hemolymph proteins from different intervals of after inoculation of *N. bombycis* were analyzed by proteomic tools SDS-PAGE and MALDITOF/MS. Insects protect themselves against foreign invaders via both humoral and cellular response. Some proteins are involved in the immune response called immune proteins. Some proteins, including 30 K proteins, low molecular weight lipoproteins, proteins related to PCD, proteases, protease inhibitors, enzymes involved in metabolism, proteins related to immunity, and a few novel proteins were identified from silkworm hemolymph of different intervals of after infection of *N. bombycis* pathogen. In this study, we demonstrated that up-regulated protein which was expressed in the heamolymph after the inoculation of the pathogen. Taken it together, our results will be valuable to further study on insect innate immunity and to aid in a comprehensive understanding of silkworm immune mechanism after inoculation of the pathogens. As we identified a 25 kDa neprilysins like protein Up-regulation in *N. bombycis* inoculated silkworm hemolymph samples, we need to perform structural analysis as well as it's functional role in silkworm immunity.

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