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Article type : Original Article

Title: **Immunoproteomic analysis of fish ectoparasite, *Argulus siamensis* antigens**

Running title: **Immunoproteomics of *Argulus siamensis***

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Conflict of interest

The authors declare no conflicts of interest.

Funding information

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/PIM.12837](https://doi.org/10.1111/PIM.12837)

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The funding support was provided by the Indian Council of Agricultural Research, New Delhi, India under the project ‘Consortia Research Platform on Vaccines and Diagnostics’ (AS/2/7/2016-ASR-IV).

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Author contributions

JM and PKS designed the project and participated in the coordination and management during the study. PD and MRB performed the experiments and wrote the manuscript. RRKR and ARS did the MS data acquisition and analysis. All authors read and approved the final version of the manuscript.

Abstract

Aim: An immunoproteomic approach was followed to identify immunoreactive antigens of fish ectoparasite, *Argulus siamensis* with rohu (*Labeo rohita*) immune sera for screening of potential vaccine candidates.

Materials and results: The whole adult *Argulus* antigen was run in 2D electrophoresis with IEF in 7 cm IPG strips of pH 4-7 and SDS-PAGE with 12% acrylamide concentration. Two parallel gels were run; one was stained with silver stain, and the other was western blotted to nitrocellulose paper (NCP) and reacted with rohu anti-*A. siamensis* sera. Fourteen protein spots corresponding to the spots developed in NCP were picked from the silver-stained gel and subjected to mass spectrometry in MALDI-TOF/TOF. The MS/MS spectra were analyzed in MASCOT software with taxonomy ‘other metazoa’ and the proteins identified based on similarity with the proteins from heterologous species. The gene ontology analysis revealed a majority of proteins being involved in binding activity in

‘molecular function’ and belonging to metabolic processes in ‘biologic process’ categories. The possibility of these proteins as vaccine candidates against *A. siamensis* is discussed in the paper.

Conclusion: Three of the identified proteins namely, bromodomain-containing protein, anaphase-promoting complex subunit 5, and elongation factor-2 could possibly serve as vaccine candidates against argulosis in carps.

Keywords: *Argulus siamensis*, Immunoproteomics, *Labeo rohita*, Vaccine

1. INTRODUCTION:

Aquaculture production makes a considerable contribution to the economy of developing countries. The growth of aquaculture is constrained due to different infectious diseases caused by bacteria, viruses, and parasites. Among different parasitic diseases, argulosis, a disease caused by a crustacean ectoparasite of the genus *Argulus* causes heavy economic losses to aquaculture production. *Argulus* spp. are the cosmopolitan fish parasites commonly known as fish lice with about 129 species are distributed throughout the world.¹ Depending upon species, these parasites live on a wide range of freshwater or marine fish species as hosts for the most part of their life cycle and the intensity of infections are higher on cultured fish than in the natural environment.² These ectoparasites feed on blood as well as on mucus and epithelial cells by puncturing the host’s skin by the pre-oral stylet and injecting a cytolytic toxin.³ This results in primary lesions at the contact sites. Hemorrhagic spots

develop particularly on the fins and at the base of fins, the head, and non-respiratory surfaces of the gill cavity, which may lead to secondary infections.⁴ Fish loss usually occurs due to direct effects such as dermal ulceration, osmotic imbalance, physiological stress, and immunosuppression.⁵

In India, argulosis has been reported from all major freshwater aquaculture zones and *A. siamensis* is the most prevalent species.⁶ It is controlled mainly by applications of several pesticides.⁷ However, these chemicals fail to provide complete protection against these parasites and, also possess direct deleterious effects on aquatic organisms and indirect effects on human health. Thus, for control of these parasites, there is an urgent need to look for safe alternative methods such as vaccination. However, unlike endoparasites, the development of vaccines against ectoparasites is difficult, since these parasites normally do not induce any effective adaptive immune response in the host. An indirect approach was utilized by Willadsen et al⁸ to develop a vaccine against cattle tick, *Rhipicephalus (Boophilus) microplus*. They found a protective protein Bm86, a concealed antigen of tick gut, and used it for immunization. The immunized cattle developed antibodies against the protein that entered the tick gut through blood-feeding leading to a reduction in engorgement weight of the ticks and a drop in egg-laying and ultimately death of the parasites; thus conferring protection. This approach has since been tried for vaccine development against several other ectoparasites of terrestrial animals as well as fish.⁹⁻¹⁰ However, barring a few isolated reports,¹¹⁻¹² no concrete studies have been undertaken on vaccine development against *Argulus* parasites.

The major requirement for any vaccine development program is to identify proper protective antigens of the pathogen. In conventional approaches, only one or a limited number of proteins can be analyzed as potential vaccine candidates at a time. The emergence of proteomics technologies, however, is aiding the vaccine development process by analyzing a large number of proteins simultaneously to find suitable protective antigens. Immunoproteomics, a branch of proteomics analyzes the entire antigenic components of a pathogen through immunological and proteomic studies and helps in identifying suitable immunogenic proteins as potential vaccine candidates.¹³ It particularly offers an advantage over other proteomic technologies in the sense that it involves practical evaluation of immune reactivity than theoretical analysis of genes and proteins based on bioinformatics. Among the number of immunoproteomic techniques, a combination of 2D

electrophoresis with western blotting is one of the most popular techniques for the identification of immunoreactive proteins.¹⁴ Several researchers have undertaken immunoproteomic studies to identify vaccine candidates against a number of microbial diseases.¹⁵⁻¹⁶

In the present paper immunoproteomic approach was followed with an objective to identify the immunoreactive antigens of *A. siamensis* parasite. Rohu (*Labeo rohita*), the most important carp species in Indian aquaculture and the most susceptible species to *Argulus* infestation¹⁷ was selected as the host to raise antiserum against *A. siamensis* whole antigens. The antiserum was used to detect immunoreactive protein spots in western blot from a 2D gel. The proteins were identified by mass spectrometry and the potentials of these proteins as probable vaccine candidates against this parasite are discussed.

2. MATERIALS AND METHODS:

2.1. Maintenance of *Argulus* parasite

Live adult *Argulus* parasites from infected fish were collected from the ponds of the CIFA farm and were maintained in 500 l FRP tanks in the Institute wet laboratory along with the host fish (rohu, *L. rohita*). Fish were fed with a commercial pellet feed *ad libitum*. Water quality parameters viz., pH, temperature, and dissolved oxygen were checked at regular intervals and maintained at optimum. Regular water exchanges were carried out taking care not to lose the parasites from the tanks.

The identification of the collected parasites was carried out by PCR with species-specific primers for *A. siamensis* available in the laboratory⁶ and used in our subsequent experiments.

2.2. Preparation of rohu immune serum against *Argulus* antigens

Rohu immune serum against *Argulus* antigens was prepared as per Das et al¹². In short, *Argulus* parasites were homogenized, centrifuged and the supernatant was used as the whole antigen for immunization of rohu (50-100 g size). Fifty numbers of rohu were collected from ICAR-CIFA pond with no prior record of *Argulus* infestation and maintained for 7 days in wet laboratory before the experiment. The fishes were apparently healthy with no attached *Argulus* parasites or any visible

skin lesions indicating possible infection. The antigen was emulsified with Freund's complete adjuvant and the fishes were intraperitoneally injected with the emulsion at a dose of 50 µg of protein/fish. The fishes were booster injected two times at 2 weeks interval with the same dose of antigen emulsified with Freund's incomplete adjuvant. Two weeks after the last booster the fishes were bled, serum separated, pooled, and preserved at -20 °C. The sera samples collected from unhandled fishes served as control. The reactivity of the serum sample was tested with dot blot before proceeding for 2D gel electrophoresis and western blotting.

2.3. Detection of anti-*Argulus* antibodies in immunized rohu serum by dot blot

Dot blot was carried out on the nitrocellulose membrane to detect the anti-*Argulus* antibodies in immunized rohu serum. Two µl of *Argulus* homogenate sample containing 3 µg of protein concentration was placed onto two separate nitrocellulose membranes each. The samples were allowed to dry on the membranes, blocked with 5% skim milk (prepared in TBS) for 2 h, and developed as per Das et al¹⁸. In brief, the membranes were incubated sequentially with rohu serum (*Argulus* immunized serum or control serum in 1:500 dilution), guinea pig anti-rohu IgM serum (1:1000) (available in the laboratory), and goat anti-rabbit ALP conjugate (1:1000) (Genei, India) for 1 h each. The dilutions of all reagents were carried out in TBS. After incubation with each reagent, washing of the blot was carried out with TBST (TBS with 0.1% tween 20) 3 times at 5 min intervals. Finally, the membranes were developed with the substrate, BCIP/NBT (MP Biomedicals, OH, USA) for the development of color.

2.4. 2D gel electrophoresis and western blotting of *Argulus* antigens

2.4.i. Preparation of protein samples from *Argulus* parasites

Live *Argulus* parasites were collected in an Eppendorf tube and washed thoroughly with Millipore water by centrifugation. Approximately 50 numbers of parasites were homogenized in 0.5 ml of Millipore water along with 10 µl of protease inhibitor cocktail (Promega, WI, USA) with the help of Super FastPrep-1 homogenizer (MP Biomedicals, OH, USA) using lysing matrix C at a speed setting of 25 (4000 cycles per min) for 10 s. The homogenate was centrifuged at 10,000 rpm for 30 min. The supernatant collected was used for protein precipitation by adding ice-cold acetone and

trichloroacetic acid (TCA) in the proportion of 1:8:1 (sample: acetone: TCA) for 2 h at -20 °C. The sample was centrifuged for 30 min at 10,000 rpm and the pellet was collected. The pellet was washed with ice-cold acetone and was dissolved in 150 µl of rehydration buffer containing urea (7 M), thiourea (2 M), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (2%), dithiothreitol (DTT) (50 mM), pharmalyte (0.002%) and bromophenol blue (0.001%). The protein was quantified by Bradford method¹⁹ using bovine serum albumin (BSA) as standard.

2.4.ii. Isoelectrofocussing (IEF)

IEF was carried out in Ettan™ IPGphor 3 (GE Healthcare, UK) system using two 7 cm IPG strips of pH 4-7. The protein content in the sample prepared above was adjusted to 100 µg/125 µl and the strips were rehydrated with the sample. The IEF was run for 10,000 Vh by specifying the number and pH of strips in IPGphor 3 Control Software.

2.4.iii. SDS-PAGE

The second dimension SDS-PAGE was carried out to separate the *Argulus* proteins according to their molecular weight. After running IEF, the strips were incubated with equilibration buffer-I (6 M urea, 2% SDS, 20% glycerol, 0.375 M tris HCl pH-8.8, and 130 mM DTT) and equilibration buffer-II (6 M urea, 2% SDS, 20% glycerol, 0.375 M tris HCl pH-8.8 and 135 mM iodoacetamide) for 10 minutes each. The strips were subsequently loaded onto 12% acrylamide gels and sealed with 1% agarose. The gels were run in miniVE electrophoresis unit (GE Healthcare, UK) at 150 V till the bromophenol blue dye reached the bottom of the gel. After the run, one gel was stained with mass spectrometry compatible silver staining protocol following Chevallet et al²⁰. The other gel was used for western blotting.

2.4.iv. Western blotting of 2D gel

One 2D gel was blotted to nitrocellulose paper (NCP) following Towbin et al²¹. The transfer was carried out in miniVE blot module (GE Healthcare, UK) in transfer buffer (25 mM tris base, 192 mM glycine, and 20% methanol) for 2 h at 25 V.

After the transfer, the NCP was immediately put in 5% skim milk and left overnight at 4 °C for blocking. The NCP was subsequently immunostained as per the protocol mentioned for the dot blot above. The paper after development was washed with Millipore water and dried prior to photography. The experiment was repeated once to confirm the immunoreactive spots. Blots were also similarly developed with control rohu sera as well as non-sera (only buffer).

2.5. Identification of immunoreactive proteins by mass spectrometry

The immunostained spots in the NCP were compared with the spots in the silver-stained 2D gel. Fourteen corresponding spots were picked from the gel manually and used for mass spectrometric identification using the protocol described earlier²² with minor modifications. The gel pieces were destained (1:1 proportion of 60 mM potassium ferricyanide and 20 mM sodium thiosulphate), washed (alternative washing with 25 mM ammonium bicarbonate and, equal proportion of 25 mM ammonium bicarbonate and 100% acetonitrile) and dehydrated (100% acetonitrile). The dried gel pieces were digested with trypsin (Promega, WI, USA) digestion solution (12.5 ng/ml in 25 mM ammonium bicarbonate) overnight at 37 °C and the solution was extracted. Further extraction was carried out with trifluoroacetic acid at three sequential concentrations of 0.1%, 0.5%, and 5% in 50% acetonitrile solution. The extracts were pooled and dried in a speedvac concentrator (DNA plus vacuum centrifuge, Heto, Germany). All 14 spots were processed for MALDI-TOF/TOF mass spectrometry. The peptide samples were reconstituted with 5 µl of the solution containing 50% acetonitrile and 0.1% trifluoroacetic acid. Then 1.5 µl of the reconstituted sample was mixed with 1.5 µl of matrix α -cyano-4-hydroxycinnamic acid (CHCA, 5 mg/ml). One µl of the sample mixture was spotted on the MALDI plate in triplicate and the plate was loaded in the MALDI-TOF/TOF (AB Sciex 5800 system, USA) for MS/MS data acquisition. The peak lists of each protein generated by MALDI were analyzed by online MASCOT MS/MS Ions search for identification of proteins. It was performed with the database NCBIprot (AA), taxonomy as 'other metazoa', enzyme as trypsin, and allowed up to one missed cleavage. The modification followed were carbamidomethyl as fixed modification and oxidation as variable modification. It possessed peptide tolerance to ± 100 ppm and MS/MS tolerance to ± 0.8 dalton. It had a peptide charge of 1+ and followed monoisotopic. In the MASCOT analysis, the protein with the highest MASCOT score was taken as the identified protein.

2.6. Gene ontology (GO) analysis

The gene ontology (GO) annotations of the identified proteins based on the BLAST results were made using Blast2GO version 5.2.5.²³ The resultant proteins were functionally categorized using the Web Gene Ontology Annotation Plot (WEGO 2.0, <http://wego.genomics.org.cn>).²⁴

3. RESULTS:

Anti-*A. siamensis* serum prepared in rohu showed reactivity with *Argulus* antigens in dot blot, whereas the control serum was negative {Figure 1(i)}. In 2D gel of *Argulus* proteins run with IEF in narrow range IPG strip of 4-7 pH followed by SDS-PAGE, the resolution of the spots were distinct {Figure 1(iiA)}. Western blot from 2D gel developed with rohu anti-*A. siamensis* serum depicted only a limited number of immunoreactive spots {Figure 1(iiB)}. The spots were found to be reproducible in repeat blot and there were no specific spots visible in blots treated with control serum and no-serum (results not shown).

Fourteen clear spots corresponding to the immunoreactive spots were picked from the 2D gel and subjected to mass spectrometric identification. All the 14 proteins matched with proteins of heterologous species (Table 1). Except for two of these proteins from organisms that belong to phylum Mollusca (*Octopus bimaculoides*) and Nematoda (*Caenorhabditis latens*); all others matched with phylum Arthropoda to which *A. siamensis* too belongs. Besides, four out of fourteen were hypothetical proteins.

To understand the functions of the identified proteins, the protein sequences were annotated using GO terms obtained from the NCBI nr databases through BlastP search in Blast2GO. Out of 14 proteins, four proteins did not show any GO annotation in the database. The remaining 10 proteins were functionally categorized into cellular component, molecular function and biological process according to GO hierarchy using WEGO {Figure 1(iii)}. These proteins were mainly classified as binding proteins by GO molecular function category and were primarily involved in metabolic processes in the biological process category.

4. DISCUSSION:

An immunoproteomic analysis was performed in the present study to identify immunoreactive proteins of fish ectoparasite *A. siamensis* paving the way for their use as vaccine candidates. Identification of a specific protective antigen is central to any vaccine development process against microorganisms and of late, immunoproteomic studies are complementing the search for new potential antigens as vaccine candidates. Antiserum-based immunoproteomic studies have been carried out for identifying such potential antigens from several parasites including ectoparasites.²⁵⁻²⁶

Initially, the *A. siamensis* parasite species was identified by PCR and maintained in wet laboratory on rohu as a host for our experiments. We developed immune serum in rohu to probe *Argulus* antigens in western blot transferred from the 2D gel to identify the immunoreactive proteins. Rohu was used as the model fish, as it is cultured primarily and widely in the Indian freshwater aquaculture system and the most susceptible carp species to this parasite.¹⁷ Further, this parasite species also causes a huge loss in rohu carp.²⁷ Hence, any subsequent studies relating to vaccine development against this parasite can directly be applied to this fish species. The positive reactivity of immune serum with *A. siamensis* antigens was confirmed in dot blot before proceeding for immunoproteomic study. Our initial 2D gel electrophoresis was conducted in the IPG strip of pH 3-10 (results not shown), where separation of the protein spots was not distinct and most of the proteins were found within the pH range of 4-7. Hence, our subsequent experiments were performed in strips of 4-7 pH that improved the resolution of the spots. A similar observation has also been made by Wang et al²⁸ while analyzing excretory-secretory proteins of parasite *Trichinella spiralis* muscle larvae. Western blotting was carried out in the present study with the 2D gel of the same pH and length as the silver-stained gel to facilitate easy identification and extraction of the corresponding immunoreactive protein spots. Following analysis of the 2D gel and western blotting results, 14 immunoreactive protein spots were identified from *Argulus* antigens by mass spectrometry. Since there is no species-specific database available for this parasite, the analysis was carried out using NCBIprot database with taxonomy as 'other metazoa'. As can be seen from the results in Table 1, all the proteins identified were from heterologous organisms. GO analysis suggested that these protein spots were mainly involved in the metabolic processes and hence, blocking these proteins with antibodies through vaccination could help in controlling *Argulus* infestation in general.

Out of the 14 proteins identified from *Argulus* parasites, 4 were hypothetical proteins and the rest 10 were proteins possessing different functions. Spot 1 was found to be sporulation-specific protein 15-like isoform X3 of *Varroa jacobsoni* species. This protein is reported to be associated with spindle pole bodies and plays an important role in the initiation of spore membrane formation.²⁹ Another protein identified from the parasite is the mediator of RNA polymerase II transcription subunit 4, which is a component of the Mediator complex, a coactivator involved in the regulated transcription of RNA polymerase II-dependent genes.³⁰ RecQ-mediated genome instability protein 1 is a component of an evolutionarily conserved protein complex that is critical for the maintenance of genomic stability.³¹ Another protein is microtubule-associated protein RP/EB family member 1 (MAPRE1; also known as EB1) which is a highly conserved key member of the plus-end tracking protein (+TIP) group that localizes preferentially to the end of growing microtubules and modulate microtubule dynamicity. This protein is involved in the accurate chromosome segregation during mitosis and in the polarization of the microtubule cytoskeleton in migrating cells.³² The NADH dehydrogenase subunit 1 is a mitochondrial protein that plays a major role in the electron transport chain of the mitochondrial energy-generating pathway, oxidative phosphorylation.³³ The diphthamide biosynthesis protein 1 performs posttranslational modification of histidine on translation elongation factor-2 for the biosynthesis of diphthamide, a unique amino acid in eukaryotes.³⁴ This diphthamide modification promotes the progression of the translation process and maintains translational fidelity.³⁵ The helicase POLQ like protein is found in archaea to metazoan organisms. This DNA helicase helps DNA replication to overcome encounters with DNA damage and assists in repairing the damage.³⁶

The potential of all the above proteins as vaccine candidates have not been recorded against any parasite. The remaining three proteins that have been evaluated as vaccine candidates or drug targets are bromodomain-containing protein, anaphase-promoting complex subunit 5, and elongation factor-2.

The bromodomain-containing proteins possess a domain, bromodomain that binds to acetylated lysine residues particularly in histone proteins, and are involved in transcription regulation.³⁷ Deregulation of these proteins has been linked to several human diseases including neurological and inflammatory diseases as well as cancer and hence, bromodomains have been

targeted with small molecule inhibitors.³⁸ These proteins also have a potential role in parasite viability and invasion. Disruption of gene expression by interfering with lysine acetylation has been shown detrimental to the survival of protozoan parasites.³⁹ Bromodomain inhibitors have been proposed to act as novel anti-parasitic agents against many parasites such as *Plasmodium falciparum*⁴⁰, *Toxoplasma gondii*⁴¹, and *Trypanosoma cruzi*⁴². Considering the potential of these proteins as drug targets against parasites, the protein found in the present study may be evaluated as a possible vaccine candidate against *A. siamensis*.

Anaphase-promoting complex subunit 5 (APC5) is one of the subunits of the anaphase-promoting complex/cyclosome (APC/C) that controls the mitotic cell division cycle in eukaryotic cells. Recently, Guleria and Jaiswal⁴³ studied the malarial parasite, *P. falciparum* through transcriptomic studies and suggested the APC as a potential vaccine candidate based on its important role in the control of cell division cycle, extracellular location, and high antigenicity score. APC5 in particular is required for viability in organisms and that has conclusively been shown in *Drosophila melanogaster*.⁴⁴ Against *A. siamensis* hence, this protein might be effective as a vaccine candidate.

The eukaryotic elongation factor 2 (eEF2) is important for protein synthesis and cell survival.⁴⁵ This protein has been suggested as a potential drug target in several parasitic infestations, e.g. poultry coccidiosis⁴⁵ and malaria⁴⁶. Also, this protein has served as a vaccine candidate, particularly in leishmaniasis. Elongation factor-2 was identified in *Leishmania donovani* through proteomics as one of the soluble leishmanial proteins that induced a T helper type 1 (Th1) immune response.⁴⁷ Later on, its efficacy as a vaccine candidate has been shown in hamsters against *L. donovani*⁴⁸ and in mice against *L. infantum*⁴⁹ in challenge studies. Thus, this protein could serve as a vaccine candidate in *A. siamensis* infection in carps.

Research on vaccine development in freshwater fish lice belonging to the genus *Argulus* is very limited. Besides whole antigen extracts of *A. foliaceus*⁵⁰ and *A. siamensis*¹², ribosomal P0 peptide¹¹ has been tried as a vaccine candidate against *A. siamensis*. Compared to freshwater fish lice, however, sea lice belonging to genera *Lepeophtheirus* and *Caligus* have received more attention towards vaccine development over the years. The initial research on sea lice species has started with crude antigenic preparations from the parasites⁵¹. Later on, researchers proposed gut antigens⁵²,

antigens of reproductive system⁵³, and chitinases in molt processes⁵⁴ of sea lice as potential vaccine candidates. Further, a number of proteins have also been evaluated against sea lice with varying successes e.g. a novel gene (denoted as my32) from *C. rogercresseyi* that showed the highest identity with the *L. salmonis* gene akirin-2⁵⁵; putative Toll-like receptor 6 and, potassium chloride and amino acid transporter from midgut function and blood digestion of *L. salmonis*¹⁰, and a peptide from ribosomal P0 protein of *L. salmonis* fused with T cell epitopes⁵⁶. The new antigens reported here add to this repertoire of proteins already been suggested as vaccine candidates in fish lice/sea lice. However, further studies need to be carried out to determine the sites of expression of these proteins in *A. siamensis* by immunolocalization. Besides, gene silencing through RNAi technology also may be employed in evaluating these vaccine targets deciphering the effectiveness of these antigens prior to the proteins are tried as vaccine targets.

In conclusion, 2D electrophoresis and western blot were used in the present study to screen the immunoreactive antigens from *A. siamensis* proteins. Fourteen immunogenic spots recognized by rohu anti-*A. siamensis* sera were identified by MALDI MS/MS. Only three of these proteins like bromodomain-containing protein, anaphase-promoting complex subunit 5, and elongation factor-2 might be effective as vaccine antigens against argulosis in carps.

ACKNOWLEDGEMENTS

The authors sincerely thank the Director, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar for providing the necessary facilities for the study. The financial support from the Indian Council of Agricultural Research, New Delhi under ‘CRP on Vaccines and Diagnostics’ project is also duly acknowledged. The authors also acknowledge Central Proteomics Facility, Institute of Life Sciences, Bhubaneswar for Mass Spectrometric analysis.

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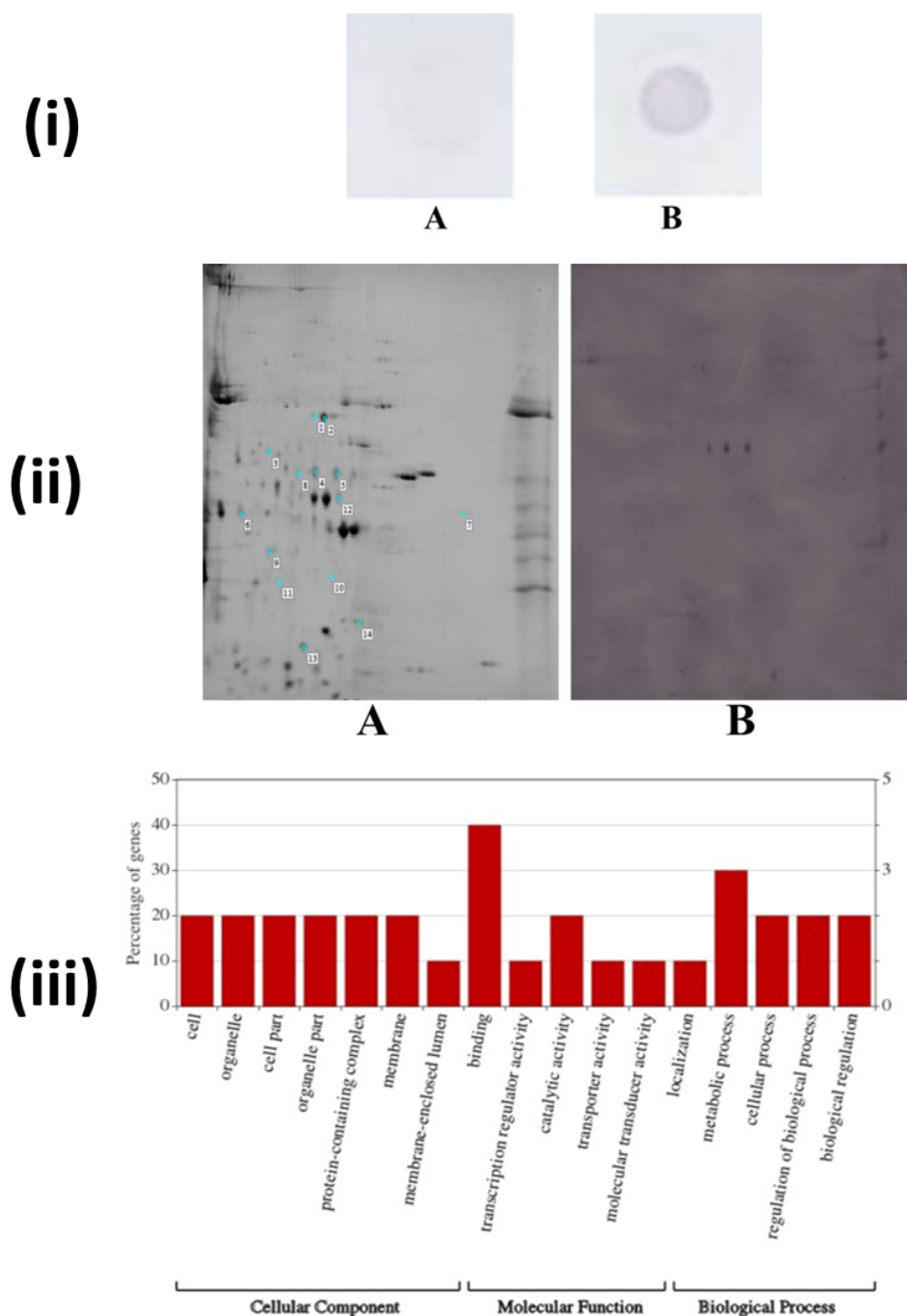
FIGURE LEGEND:

FIGURE 1 (i) Reactivity of rohu serum with *A. siamensis* whole antigens in dot blot developed with control serum (A) and anti-*A. siamensis* serum (B); (ii) Silver-stained 2D gel of *A. siamensis* whole antigens showing the immunoreactive protein spots (A) corresponding to the spots in 2D blot developed with rohu anti-*A. siamensis* serum (B); (iii) Gene ontology (GO) annotation of immunoreactive proteins of *A. siamensis* recognized by rohu immune sera; categorized into cellular component, molecular function, and biological process by WEGO according to their GO signatures.

Table 1. Immunoreactive proteins of *A. siamensis* identified by MALDI-TOF/TOF MS/MS and MASCOT analysis.

Sl. no.	Protein accession	Protein description	Organism	Mass (Da)	pI	Mascot Score	Peptide match	Sequence coverage
1	XP_022692316.1	sporulation-specific protein 15-like isoform X3	<i>Varroa jacobsoni</i>	274804	5.32	70	24	11%
2	XP_013193961.1	mediator of RNA polymerase II transcription subunit 4	<i>Amyelois transitella</i>	27632	5.92	55	7	42%
3	KXJ75014.1	hypothetical protein RP20_CCG012471	<i>Aedes albopictus</i>	186021	9.04	66	15	11%
4	KOF95159.1	hypothetical protein OCBIM_22039315mg	<i>Octopus bimaculoides</i>	9361	7.98	63	6	64%
5	XP_014226553.1	recQ-mediated genome instability protein 1-like	<i>Trichogramma pretiosum</i>	90160	5.27	69	12	24%
6	XP_019866806.1	microtubule-associated protein RP/EB family member 1 isoform X1	<i>Aethina tumida</i>	31072	5.47	62	8	43%
7	OZF87616.1	hypothetical protein FL83_22837, partial	<i>Caenorhabditis latens</i>	10095	7.96	57	5	57%
8	AAC28679.1	NADH dehydrogenase subunit 1, partial (mitochondrion)	<i>Lymantria obfuscata</i>	12562	9.26	43	5	35%
9	AUW84056.1	putative diphthamide biosynthesis	<i>Xylinada sp.</i> INB054	25874	8.28	54	6	36%

		protein 1, partial						
10	XP_012535676.1	bromodomain-containing protein DDB_G0278469-like	<i>Monomorium pharaonis</i>	12468	4.75	68	6	69%
11	KYN11023.1	anaphase-promoting complex subunit 5	<i>Trachymyrmex cornetzi</i>	85734	6.4	69	9	15%
12	OWR43841.1	hypothetical protein KGM_209173	<i>Danaus plexippus plexippus</i>	104068	6.66	75	9	13%
13	AAR01313.1	elongation factor-2, partial	<i>Rhinotus purpureus</i>	82191	6.32	63	12	22%
14	OWR45988.1	helicase POLQ like protein	<i>Danaus plexippus plexippus</i>	96925	8.02	60	12	17%



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