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Molecular Cloning, Expression and On-Column Refolding Of Recombinant Modified *Allium Sativum* Root Lectin in *E. coli/BL21*

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Abstract

Lectins or agglutinins are sugar-binding proteins that bind reversibly to specific mono- or oligo-saccharides. They are widely distributed in plants, animals, and microbes. The physiological role of lectins in plant growth and development, plant defence against pathogens, and insect pests. Plant lectins have a severe effect on the growth and development of insects. In this study, The gene of *Allium sativum* root lectin (ASARI) was adopted from the National Centre for Biotechnology Information (Gene bank accession number AAB64238.1). The ASARI gene was modified (mASARI) by truncating 84bp at the 5' end and 126bp at the 3' end of the gene to enhance the binding activity to the target protein. The modified lectin was amplified by PCR and cloned into the pET 30 b (+) vector with C terminal His6 tag to get the over expressed modified mASARI lectin. The His6 is used for the purification of Ni-NTA column chromatography. But, the over expressed recombinant modified mASARI protein in *E. coli* is an inactive inclusion body. The inclusion body contains lots of host cell proteins and cell components. For receiving the active and native form of the protein the inclusion body has to be washed to reduce the host cell components. To get an active and refolded form of protein it should be solubilized under denaturing condition (8M) urea. Then the protein is immobilized by metal affinity chromatography (IMAC) and gradually refolded by using a linear gradient of urea from 8.0 M to 0.0 M showing that at least part of the protein was properly refolded. The 13.6 kDa protein showed positive agglutination with rabbit erythrocytes at a concentration of 6.25µg/ml

KEYWORDS: Recombinant ASARI Lectin, mASARI, PCR (Polymerase Chain Reaction), PET 30b (+), E. coli, SDS PAGE, refolding Western blotting, Ni-NTA column Chromatography, agglutination etc.

1. INTRODUCTION

The rising demand for environmentally sound practices in agriculture has led to rising interest in bio-pesticides based on natural products (*Pawar & Mali*, 2022). Of those molecules, plant lectins have been brought to the foreground owing to their highly effective insecticidal effects and the possibility of selecting pest species by carbohydrate-binding domains. Lectins, a diverse family of non-catalytic carbohydrate-binding proteins, have recently attracted much interest as multifunctional plant proteins that are involved in a variety of fundamental biological processes, including cell surface recognition and biotic stress responses. These proteins are defined according to their reversible, highly selective binding to mono- or oligosaccharides, which qualifies them for applications in agricultural biotechnology and therapeutic sciences *(Van Damme, 2021).* From these, the lectins

from *Allium sativum* (garlic) have become vital players in pest management; taking on *Hemiptera* pests which include aphids and whitefly, which are global threats to food security in agriculture *(Fitches et al., 2008)*. From a molecular point of view their insecticidal action can be linked to binding with glycosylated proteins present in insect midgut that causes inhibitory effects against the normal physiological processes that leads to developmental disabilities and death. These lectins therefore brighten prospects in natural pest control and management rather than the widely used chemical insecticides today.

Irrespective of the progress of agricultural biotechnology, recombinant protein production is crucial in the process (*Patil* et al., 2020). *Escherichia coli* is a well-used bacterial host, the reasons being it is an easy to handle, inexpensive system with high growth rates for recombinant protein production. Nevertheless, obtaining proper conformation of the functional activity of proteins expressed in *E. coli* requires elaborate methods of protein solubilization and refolding when they are present in the form of inclusion bodies *(Singhvi & Panda, 2022)*. The challenges are mitigated in the present study using the expression and refolding of a modified *Allium sativum* root lectin (mASARI) in *E. coli*. The construct used in the study comprised a nascent ASARI gene that was truncated in a manner that would optimise its binding activity, which was then cloned into the pET30b (+) vector carrying a histidine tail at the C-terminus for convenience in the purification process employing Ni-NTA affinity chromatography. The essence of the procedure is an on-column refolding that eliminates excessive loss of the native protein conformation since it entails a controlled removal of urea concentrations.

This work goes further on the insecticidal potential of ASARI by improving the recombinant production to enhance practical usage. That 84bp of the 5' end and 126 bp of the 3' end of the gene was excluded was made deliberately to promote the protein's bioactivity principally in terms of its agglutination properties. Thus, the current study established the functional integrity of the purified and refolded mASARI by demonstrating the hemagglutination activity of the recombinant protein at micromolar concentrations of the rabbit erythrocytes. This is particularly important since lectins require an exact tertiary structure to express the right specificity and binding capability. The successful recovery of mASARI from inclusion bodies not only supports the optimised refolding process but also trusts the on-column refolding method to solve the solubility problems arising from the *E. coli* expression system *(Yamaguchi & Miyazaki, 2014)*.

There are a number of insect pests including the whiteflies *(Bemisia tabaci)* that continue to pose a significant threat to world agriculture hence the need to develop new strategies in the management of these pests. The mode of action of mASARI involves its associations with glycosylated gut proteins such as aminopeptidase-N and alkaline phosphatase which are important nutrient uptake and cell signaling molecules in insects. This interaction inhibits the smooth functioning of the digestive system and results in toxicity which in turn affects the growth and development of insects besides affecting their reproductive system. It also opens up the possibility of using mASARI in transgenic plants as well as adopting it as a bioinsecticide, in response to the issues of environmental toxicity and pest resistance to common chemical pesticides *(Kumar et al., 2021)*. In particular, the application of the transgenic approach to produce crops with the inserted gene mASARI would allow increasing the crops' resistance to pests and decrease their dependence on chemical pesticides.

However, the overall production of functionally active recombinant lectins has always been a challenge owing to solubility problems and post-translational modification. The present study adds to the elimination of these challenges by showing that 8M urea followed by on-column refolding works as a strong solubilization protocol for inclusion bodies. This process recovers the biological activity of mASARI without the need for refolding buffers or refolding assistance thereby reducing downstream complexity. Molecular characterization of the refolded lectin protein by SDS-PAGE and western blotting supported the structural features of the protein and this, in turn, enabled subsequent functional studies and practical applications to be drawn from the enzyme.

Furthermore, the capability of mASARI to form erythrocyte agglutination of rabbits at 6.25µg/mL reveals it functional biomolecule for agriculture and health sciences. This is consistent with the insecticidal effects of mASARI identified in this report together with high specificity for glycan structures of insect guts meaning that there will be a more focused action on the target with fewer side effects. This duality is not an exception for lectins like mASARI, where they also serve as molecular probes in glycobiology due to their CSDs that enable the investigation of glycan interactions across different biological systems *(Mattox & Bailey-Kellogg, 2021)*.

The utilization of mASARI in improving agriculture pest control methods is further supported by recombinant *E. coli* system of production for efficient production of large quantities of protein. The strategic truncation made in this study for increasing the binding activity of mASARI is representative of any rational protein engineering exercise; specific changes to the structure could be used to improve function without significant loss of stability. Hence, this approach implemented along with recent accomplishments in the expression

systems and the purification technologies creates a path to produce the bioactive lectins at a better efficiency eliminating the difficulties for its application in agriculture and other uses.
Under these circumstances, this study steps forward in the biotechnological exploitation of *Allium sativum* lectins for pest control. Combing the recombinant expression and refolding difficulties in *E. coli*, it sets up a valid and effective process for creating functional mASARI. Therefore, the findings of this work have a universal application on the establishment of new bio-insecticides and the practical control of insect pests in support of food security and agricultural production impacted by bio and abiotic stresses.

2. MATERIALS AND METHODS

2.1. Materials and Molecular techniques.

Allium sativum root lectin (ASARI) gene was synthesized from GeneSript, USA and used as a source of gene for PCR (Polymerase Chain Reaction) amplification. The pET 30 b (+) vector, *E. coli* DH5α, and BL21/DE3 (Invitrogen). Oligonucleotide primers were synthesized from Eurofines genomics India Pvt. Ltd.Bangalore. Plasmid isolation used alkali lysis protocol from Sambrook and Russell. Agarose gel electrophoresed amplified and linearized DNA fragments were purified by using a Gel Extraction kit (QIAquick gel extraction kit, Germany). According to manufacturer's protocol. Taq polymerase was purchased from Invitrogen. Restriction Endonucleases purchased from New England Biolab (NEB). T4 DNA ligase was purchased from Promega. The DNA sequencing was performed using Eurofines genomics India Pvt. Ltd. Bangalore. Luria-Bertani broth, Yeast extract, Peptone, and Glycerol were purchased from HiMedia Laboratory Private Limited, India. Petri dishes were purchased from Tarsons Products Private Limited, India. Ampicillin, tetracycline and Kanamycin were purchased from Sigma. All buffers and growth media were prepared in autoclaved Milli-Q water. Ni-NTA his bind resin purchased from Thermo Fisher Scientific. Bromophenol blue, acrylamide, ammonium persulfate, bisacrylamide, beta-mercaptoethanol, sodium dodecyl sulfate, a sodium salt of ethylenediaminetetraacetic acid (EDTA), N, N, N', N'-Tetramethylethylenediamine (TEMED), ammonium bicarbonate, Trizma®Base were also purchased from HiMedia Laboratory Private Limited, India. The rabbit erythrocytes were Raj Biotech, Shirawal.

2.2. Isolation of modified mASARI sequence from synthesized construct and preparation of expression construct

The synthesized *Allium sativum* root lectin (ASARI) gene was used for the designing of primers. In the forward primer added 5'Nde I and the reverse primer added 3' Xho I. The coding gene sequence of mASARI was amplified from the synthesized construct using gene-specific primers (pmASARIF- 5' ATATATCATATGAGGAACCTACTGACGAAC3' and pmASARIR- 5' TATATACTCGAGTCTGTAGGTACCAGTAG3') by PCR (Polymerase chain reaction) based on the published data (Gene bank accession number AAB64238.1) The modified mASAIL gene was amplified using Taq polymerase (Invitrogen), PCR machine (Eppendorf Thermal cycler, USA). The amplified PCR product of mASARI 339bp was further gel-purified using the Gel Extraction kit (QIAquick gel extraction kit, Germany). The gel purified mASARI was ligated into the pGEMT easy vector by the T-A cloning method. The ligated mixture was transformed into *E.Coli* DH5 alpha. The transformed clones were characterized by sequencing.
For cloning of modified garlic root lectin sequence into the expression vector pET 30b (+), the pGEMT easy vector with mASARI was digested with *NdeI* and *XhoI* and loaded into the agarose gel electrophoresis. After electrophoresis, the linearized pET 30b (+) vector and mASARI were gel purified using a gel extraction kit. Gel purified modified mASARI were ligated into the pET30b (+) vector (Invitrogen, USA). The ligated mixture was transformed into *E.Coli* BL21/DE3 and transformed clones were spread on the LB agar plate containing kanamycin (30µg/ml). The transformed clones were characterized by sequencing.

2.3 Expression of recombinant modified mASARI in *E. coli* Bll21/DE3

Selected antibiotic resistance colonies were selected for expression of recombinant protein by growing them in small scale i.e. 5ml LB media with kanamycin 30µg/ml. The culture was grown up to O.D. 0.6 at 600nm with 180 rpm at 37°C. The culture was induced with 1mM IPTG and grown for further 5 hrs with 180 rpm at 37°C. The induced cells were used for the analysis of SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using anti-mASARI antibodies. The highest protein expressing colony was used for protein purification and making glycerol stocks.

3. Recombinant protein production of mASARI

The highest protein expressing glycerol stock of modified mASARI in *E. coli* BL21/DE3 was inoculated in to 50 ml of terrific broth (TB) with kanamycin 30 µg/ml. The cells were grown overnight at 37˚C, 180 rpm. Next day, The TB was inoculated with 10% overnight grown modified mASARI in *E. coli* BL21/DE3 with kanamycin 30 µg/ml. the cells were grown up to O.D. at 600 nm is ~ 0.6. The culture was induced by adding 1mM IPTG (Isopropyl –β-D- thiogalactoside) and grown overnight up to stationary phase at 37˚C, 180 rpm.

3.1 Washing of Inclusion bodies of mASARI

Overnight induced cell of modified mASARI in *E. coli* BL21/DE3 was centrifuged at 5000 rpm for 10 min at 4°C removed media and other cell components. Took 400 mg of inclusion bodies of modified mASARI lysed with adding 20 ml of lysis buffer (50 mM Tris-Cl , 50 mM EDTA, 15% sucrose, pH 8 5mg/ml of lysozyme) and incubated for 1hr at 37˚C,180 rpm. The incubated IB'S were sonicated at 100 Amp for 5min (5 Second ON, 5 Second OFF) the sonicated IB'S were centrifuged at 10,000 rpm for 10 min at 4 ˚C. The pallet was washed with 40 ml of wash buffer (0.5 M NaCl, 2% Triton X-100) then washed with 40 ml 0.5 M NaCl and finally washed with 40 ml Distilled water. IB'S were solubilized in Tris-Cl buffer pH 8 (50mM Tris, 500mM NaCl, 5mM Imidazole, 6M Urea pH 8).

3.2 On column refolding of insoluble modified mASARI by Ni-NTA column chromatography

The column was packed with NI-NTA and equilibrated with an equilibration buffer (50mM Tris, 500mM NaCl, 6M Urea pH 8). The solubilized IBs were loaded onto the pre-equilibrated column. After loading, the column was washed with wash buffer (50mM Tris, 500 NaCl, 80mM Imidazole, 6M Urea pH 8). The column was rinsed with 5 column volume of wash buffer with decreasing amounts of urea i.e. 6, 4, 2, 1, 0 0.5, 0.25 and 0.125 M. Finally, the column was rinsed with 10 column volumes of wash buffer containing no urea. The refolded protein was eluted with 10 volumes of elution buffer (50mM Tris, 500 NaCl, 300 Imidazole. Eluted protein was analysed by SDS-PAGE and hemagglutination with rabbit erythrocytes.

3.1. Production of anti-mASARI antibodies

Purified recombinant modified mASARI was used to raise polyclonal rabbit anti-mASARI antibodies. (Chromes Biotechnology Pvt. Ltd. Bangalore). Polyclonal anti-mASARI antibodies were raised using the standard provided protocol. Antisera was collected from immunized rabbits and used for antibody purification. The polyclonal antibodies were purified by protein A affinity chromatography followed by ion-exchange chromatography. Purified antibodies were used for western blotting.

3.2 Haemagglutination assay of mASARI

The Assay was carried out in a round (U) bottom clear microtiter plate. The standard GNA (*Galanthus nivalis*) was purchased from Sigma Aldrich (USA) and used as a positive control. The rabbit erythrocytes were collected and washed with 1ml 1X PBS. In 96 well microtiter plates, added 50µl PBS to each well, then added 50 µl of serially diluted lectin (starting from 5µg to 2ng per well) was added to each well. Finally, added 50ul of 2% erythrocyte suspension to each well. The agglutination reaction was monitored visually after incubation for 2 hrs at RT.

3.3 SDS PAGE and Western blotting of mASARI

On the column refolded and purified protein samples were collected and separated by SDS-PAGE. The separated proteins were transferred on the PVDF membrane and detected by Anti-rabbit antibody. For western blotting primary anti-modified mASARI and detection anti-rabbit antibody were diluted 1:2000.

4. RESULTS AND DISCUSSION

4.1 Preparation of modified mASARI expression construct into pET 30 b (+) vector

The ASARI mature coding sequence was amplified from synthesized plasmid DNA using PCR primers designed from the published sequence for the garlic root lectin ASARI (Gene bank accession number AAB64238.1). Single-band was obtained at approximately 339bp (Fig. 1) the fragment was purified, cloned, and characterized by restriction digestion and DNA sequencing.

The sequence of the 339p product was identical to the published mature sequence of the garlic modified root lectin mASARI with 100% similarity at the nucleotide and amino acid levels. The expression constructs for mASARI which omitted the predicted signal peptide-encoding regions and stop codons were amplified by PCR primers. The final expression construct (pETmASARI) contained the lectin sequence in frame with

sequence from the vector, which encoded an N-terminal T7 promoter followed by initiation codon and C-terminal his6 tags (Fig. 2).

4.2 Production and purification of recombinant modified garlic root lectin expressed in pET 30 b (+) vector.

Sequence confirmed clone of pETmASARI was transformed into the chemically competent ***E. coli*** BL21/DE3 cells were prepared using material and procedure provided into the PET Manual (Invitrogen, USA). Transformed clones were screened by colony PCR using gene-specific primers. Expression of mASARI was checked by SDS-PAGE and purification was done by washing of inclusion body followed by on column refolding by decreasing the amount of urea i.e. 6, 4, 2, 1, 0 0.5, 0.25,0.125 and 0 M from wash buffer. The purified and refolded modified mASARI was checked by western blotting. Anti-histidine HRP conjugated antibody was used for the detection of modified mASARI. Purification by Ni-NTA column chromatography was successful in recovering recombinant garlic lectins from culture supernatants at a purity of >98% (estimated by stained gels and comparison to standards; Fig. 3).

Analysis of purified and refolded clones expressing mASRI by Western blotting gave multiple bands of monomer, dimer, trimer and tetramer of approx. 12.6,25.2, 37.8 and 50.4kDa, which were immune reactive with modified anti-ASARI antibodies (Fig.4) and anti-His antibodies (Data not shown) these data suggest that the expressed and purified protein is intact, refolded not cleavage by proteases. In GNA there is no band observed because antibodies are specific only for the mASARI. The SDS-PAGE analysis showed approx. 12.6, 25.2, 37.8 and 50.4kDa intact bands.

4.3 Functional activity of modified mASARI by Agglutination Assay

The recombinant modified mASARI agglutinated rabbit erythrocytes down to a dilution of approximately 0.012 mg/ml, showing that the recombinant protein was functionally active. Positive control of native standard GNA lectin agglutinated to approximately 0.05 mg/ml and negative control (bovine serum albumin) showed no agglutination at any concentration, including the highest tested 1mg/ml (Fig.5).

DISCUSSION

Escherichia coli is one of the most commonly used prokaryotic organisms for the production of commercial eukaryotic proteins like therapeutic proteins and plant lectins. Compared with the other expression systems like *Pichia pastoris,* Mammalian cells (CHO) and Insect cells. These host cells have disadvantages like limitations to over-express the recombinant protein resulting in them expressing a very small quantity of recombinant protein. Overcome this problem *E. coli* has several advantages, like growth on economical carbon source, rapid accumulation of biomass, high cell density fermentation, and simple to process scale-up and availability of many cloning and expression vectors with respective host strains. Considering these advantages, various attempts were made to produce mASARI lectin using *E. coli* resulting it the required to produce protein refolded by using the on-column refolding method. Thus attempted to produce full-length ASARI lectin with co-expressing protein which supported to production of soluble protein into the cytoplasm but the cytoplasm of *E. coli* is very thin however the expressed protein yields very little.

The expression of plant genes in *E. coli* systems requires refolding of over expressed protein. The prokaryotic organisms produce endotoxins which causes the degradation of recombinant protein. To overcome such problems we have been focused on the on-column refolding of the mASARI protein by washing inclusion bodies with different buffers and solubilised with urea-containing buffer. The solubilised mASARI was bound to the NI-NTA resin. Washed resin with decreasing concentrations of urea from i.e. 6, 4, 2, 1, 0 0.5, 0.25, 0.125 and 0 M. Finally on-refolded protein was eluted by elution buffer without urea.

The Full-length mASARI gene was amplified using gene-specific primers and the amplified PCR product was cloned into the primary vector pGEMT easy vector. The digested mASARI and linearized pET 30 b (+) expression vector were ligated. The expression construct pETmASARI was transformed into the *E. coli*/BL21/DE3. Isolated colonies were grown on a kanamycin-containing medium and screened for positive transformed colonies. *E. coli*/BL21/DE3 achieved higher levels of expression in the inclusion body. In this study, we represented successful cloning, expression, and activity of on-column refolded mASARI protein using *E. coli*/BL21/DE3.

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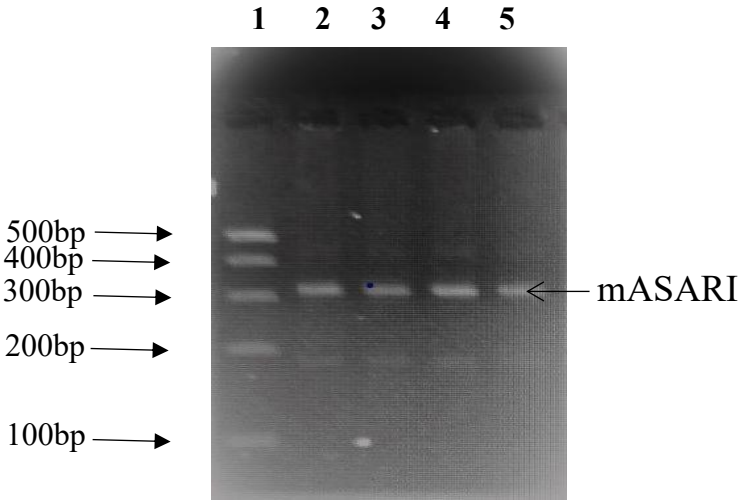

Fig. 1: Amplification of mASARI

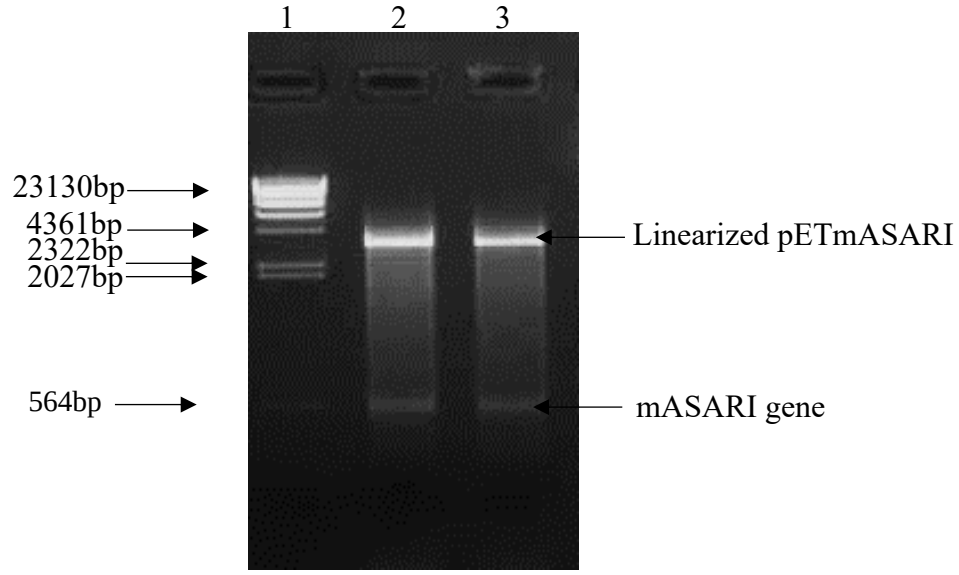

Fig. 2: pET30 b (+) vector with mASARI digested with *NdeI* and *XhoI*

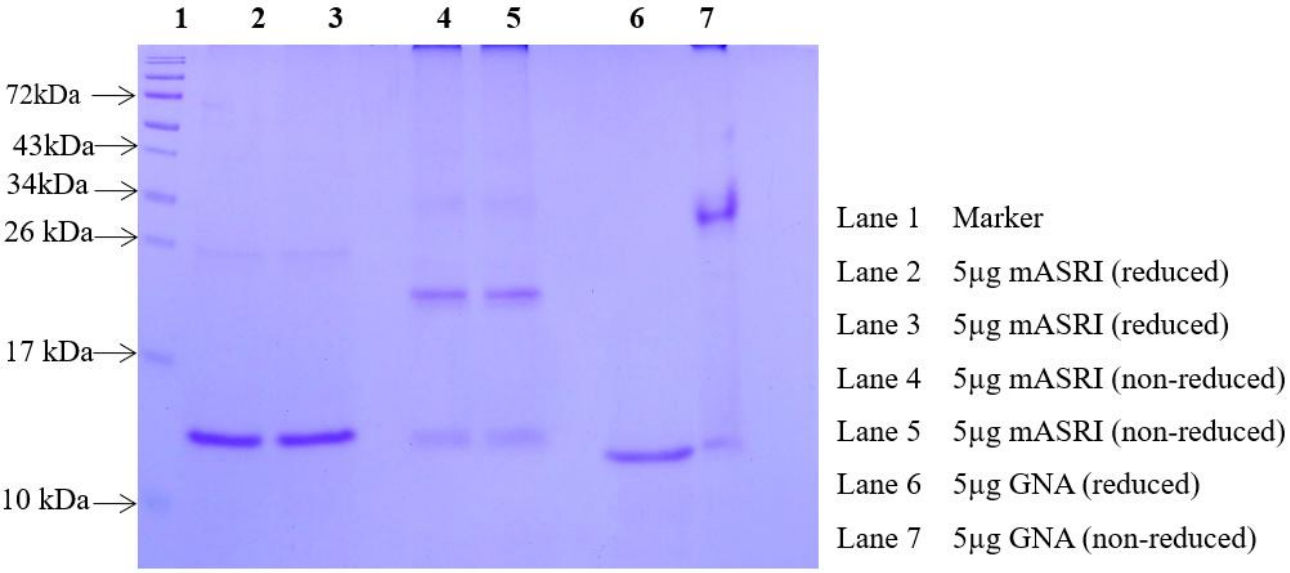

Fig. 3: SDS-PAGE of purified mASARI from *E. coli*/BL21

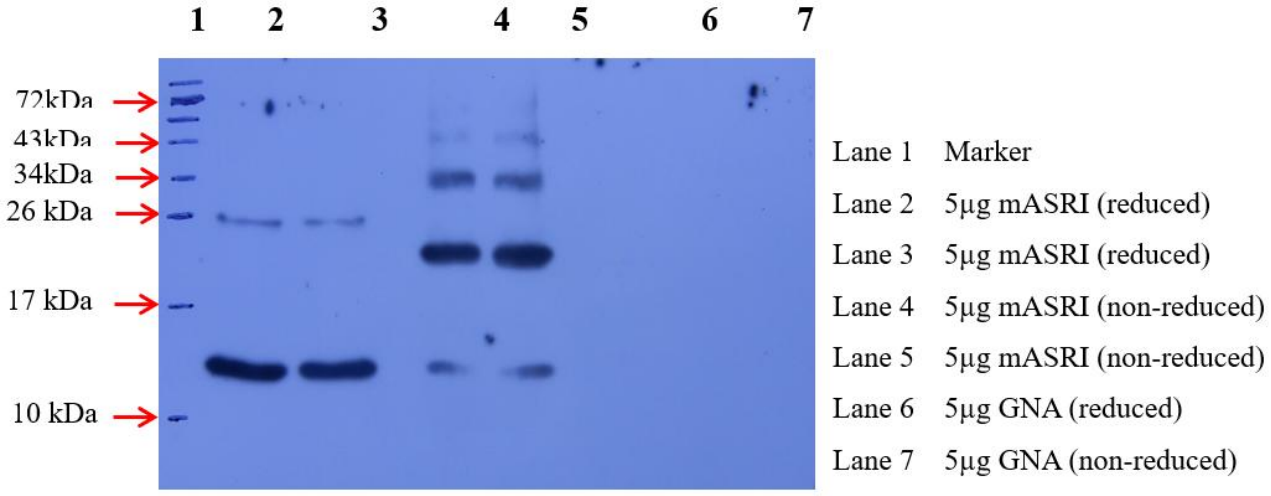

Fig.4: Western blotting of purified mASARI from *E. coli*/BL21

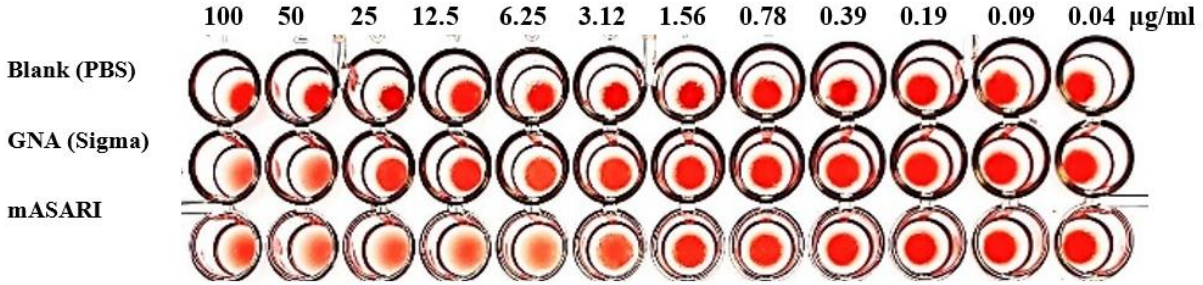

Fig. 5: Agglutination assay of lectin(s) with rabbit erythrocytes.