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

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Abstract

Lectins or agglutinins are the 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 defense 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 amplified by PCR and cloned into the pET 30 b (+) vector with a C terminal His6 tag to get the over-expressed ASARI lectin. The His6 was used for the purification of Ni-NTA column chromatography. The over-expressed recombinant ASARI protein in *E. coli* /BL21 was an inactive inclusion body. The inclusion body contains lots of host cell proteins and cell components. To accomplish the active and native form of the protein from the inclusion body, it needs to be washed with different buffers to reduce the host cell components. For active and refolded forms of protein, it should be solubilized under denaturing conditions by (8M) urea. Then the protein was immobilized by metal affinity chromatography (IMAC) and gradually refolded by using a linear gradient of urea from 8.0 M to 0.0 M which showed that the protein was properly refolded. The 19.3 kDa protein showed positive agglutination with rabbit erythrocytes at a concentration of 12.5µg mg/ml

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

1. INTRODUCTION

Lectins are a large group of Carbohydrate-binding proteins that are important in plant defense mechanisms because they bind specifically to terminal carbohydrate residues on glycoconjugates of the surface of target organisms including pests and pathogens. Lectin was described by Sharon and Lis (1989) as a protein that readily binds to sugar moieties and is involved in biological recognition. These proteins recognize themselves with glycoconjugates on the cell walls or membranes of insects and pathogens and cause respective physiological alterations. In plants, most lectures serve as a defense mechanism against insect herbivores and microorganism pathogens. *Peumans and Van Damme (1995)* observed that lectins play an essential role in plant defense through linkages with chitin or glycoproteins in the exoskeleton or gut of insects thus affecting the uptake of nutrients that enhance the insect's growth and development.
Lectin's selectivity to specific carbohydrate structures makes them effective bio-recognition elements. *Chrispeels and Raikhel (1991)* noted that lectins can bind selectively with specific glycoconjugates on the

surfaces of insect or gut microorganisms and cause aberration of key activity like enzymatic action or nutrient transport. For instance, lectins isolated from snowdrops (*Galanthus nivalis*) target an N–-acetyl glucosamine residue on epithelial cells of insect midgut causing toxicity (Zhu *et al.,* 1996). Lectins are also important in defense against pathogens and diseased organisms. Wang *et al.,* (2015) showed that Arabidopsis lectin receptor kinases are involved in controlling defense response to fungal pathogens including *Phytophthora infestans*. Another benefit of lectins is that the raw materials that are used in their manufacture can readily be renewed. War *et al.,* (2012) noted that by employing genetically engineered lectin-expressing plants, there is less effect of pest control on other organisms in the similar environmental niche by way of undesirable pesticide content. Garlic (*Allium sativum*) lectins are promising candidate molecules for protection against chewing (lepidopteran) as well as sap-sucking (homopteran) insect pests. The molecular mechanism of toxicity and interaction of lectins with midgut receptor proteins has been described in many reports. Lectins show its effect right from sensory receptors of mouth parts by disrupting the membrane integrity and food detection ability. Subsequently, enter into the gut lumen and interact with midgut glycosylated proteins like alkaline phosphatase (ALP), aminopeptidase-N (APN), cadherin-like proteins, polycalins, sucrase, symbionin, and others. These proteins play a critical role in the life cycle of insects directly or indirectly. Lectins interfere with the activity of these proteins and cause physiological disorders leading to the death of insects.

Lectins are further transported across the insect gut, accumulated in various body parts (like haemolymph and ovary), and interact with intracellular proteins like symbionin and cytochrome p450. Binding with cytochrome p450 (which involve in ecdysone synthesis) might interfere in the development of insects, which results in growth retardation and premature death.

The productivity of important crops *viz.* Rice, mustard, chickpea, cotton, etc. suffers from huge losses due to the attack of homopteran pests (Patil, et al., 2020). These sapsucking homopteran insect pests cause physiological damage and nutritional depletion of the plants through stylet probing and feeding, as well as transmitting viral diseases by acting as vectors (Foissac *et al.,* 2000; Rao *et al.,* 1998).

Increasing productivity through crop protection is a vital issue in modern-day agriculture. Unfortunately, the productivity of several crops is affected worldwide by the attack of various insect pests. The ever-increasing demands on crop yields as a result of the alarming increase in the human population have led to intensive farming practices which, in turn, increase the severity of insect attacks (Pawar and Mali, 2022). The extensive use of chemical pesticides builds up resistance in insects to such compounds and adversely affects beneficial organisms, such as pollinators, nutrient cycles, and natural pest-controlling agents. Massive application of chemical pesticides exerts undesirable effects on both the environment and human health. Hence, deploying the desired resistance in cultivated crops through genetic engineering technology is preferred. In the last few decades, several genes from microbial and plant sources have been introduced into plants to confer resistance against damaging insect pests. In this context, *Bacillus thuringiensis* endotoxin (Bt) coding genes. These have been introduced in many plant species to control the attack of lepidopteran and coleopteran insects (Koziel *et al* 1993; Wunn *et al* 1996; Nayak *et al* 1997; Datta *et al* 1998; Kota *et al* 1999; Maqbool *et al* 2001). Sucking insect pests of the order Homoptera have not been reported so far to be controlled by Bt toxins. Their unique feeding strategy, nourishing on free amino acids and sugars directly from phloem tissue, makes them less dependent on digestive enzymes. However, protease inhibitor approaches have shown little effect in controlling these pests (Rahbe *et al* 2003). The insecticidal activity of carbohydrate-binding plant lectins against different insects belonging to the orders Coleoptera, Diptera, Lepidoptera, and Homoptera has been well studied (Gatehouse *et al* 1995; Schuler *et al* 1998; Carlini and Grossi-de-Sa 2002). Several carbohydrate-binding plant lectins, e.g. G*alanthus nivalis* agglutinin(GNA) (Gatehouse *et al* 1993, Nagadhara *et al* 2004), wheat germ lectin (WGA) (Kanrar *et al.,* 2002) and concanavalin A (Con A) (Gatehouse *et al* 1999), have been reported to have detrimental effects on homopteran pests. Recently, another mannose-binding 25-kDa homodimeric lectin from *Allium sativum* leaf (ASAL) has been shown to affect the survival of some sucking pests (Bandyopadhyay *et al.,* 2001; Roy *et al* 2002; Majumder *et al.,* 2004). The binding of ASAL to gut receptors is proposed to decrease the permeability of the membrane, thus affecting insects (Bandyopadhyay *et al* 2001).

For the production of insecticidal *Allium sativum* root lectin has two ways, one is to clone and expression in a yeast expression system and the second is to develop a transgenic plant expressing the lectin gene. But, these are time-consuming and expensive methods. However, the times have changed, and *E. coli*/BL21 has developed as the well-suited and successful host for the expression of heterologous recombinant proteins. The current paper is on the on-column refolding of overexpressed *Allium sativum* root lectin by Ni-NTA column chromatography and Purified protein was characterized using SDS-PAGE western blotting and Agglutination with rabbit erythrocytes.

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 Eurofins Genomics India Pvt. Ltd.Bangalore. Plasmid isolation used an 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 the manufacturer's protocol. Taq polymerase was purchased from Invitrogen. Restriction Endonucleases purchased from New England Biolabs (NEB). T4 DNA ligase was purchased from Promega. The DNA sequencing was performed using Eurofins 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. IPTG (Isopropyl –β-D-thiogalactoside), 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 blood was collected from Raj Biotech, Shirawal.

2.2. Isolation of full-length ASARI sequence from synthesized construct and preparation of expression construct

The full length lectin coding gene sequence was synthesized from GeneScript, USA. ASARI gene was amplified from synthesized construct using gene-specific primers (Table 1; **ASARIF**- 5'ATA TAT CAT ATG GGT CGT ACT ACT TCA TC 3' and **ASARIR**- 5'TAT ATA CTC GAG TCA AGC AGC ACC GC 3') with 5'Nde I and 3' Xho I restriction sites were added in the coding sequence of ASARI by using PCR (Polymerase chain reaction) based on the published data (Gene bank accession number AAB64238.1) The ASARI gene was amplified using Taq polymerase (Invitrogen), PCR machine (Eppendorf Thermal cycler, USA). The amplified PCR product 546bp of ASARI was further gel-purified using the Gel Extraction kit (QIAquick gel extraction kit, Germany). The gel-purified ASARI was ligated into the pGEMT easy vector by the T-A cloning method. The ligated mixture was transformed into *E. Coli* DH5 alpha and spread on an LB agar plate containing 100µg/ml) ampicillin. The plasmid was isolated from transformed colonies and characterized by sequencing.
For cloning of garlic root lectin sequence into the expression vector pET 30b (+) the plasmid was isolated from *E. coli* DH5 containing ASARI gene in pET 30b (+) vector were digested with *NdeI* and *XhoI*. The linearized pET 30b (+) vector and 546 bp ASARI insert from an isolated plasmid were further gel-purified using the Gel Extraction kit (QIAquick gel extraction kit, Germany). The gel-purified ASARI was ligated into the linearized pET30b (+) vector (Invitrogen, USA). The ligated mixture was transformed into *E. Coli* BL21/DE3 and transformed cells were spread on the LB agar plate containing kanamycin (30µg/ml). The transformed clones were characterized by restriction digestion and sequencing.

2.3 Expression of recombinant ASARI in *E. coli* BL21/DE3

Selected antibiotic resistance colonies were used 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) the highest protein expressing colony was used for protein purification and making glycerol stocks.

3. Recombinant ASARI protein production

The highest protein expressing glycerol stock of ASARI 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 500ml TB was inoculated with 10% overnight grown ASARI 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 ASARI Inclusion bodies

The overnight induced cell of ASARI in *E. coli* BL21/DE3 was centrifuged at 5000 rpm for 10 min at 4°C and removed the media and other cell components. Took 400 mg of inclusion bodies of ASARI in *E. coli* BL21/DE3 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 IBs were sonicated at 100 Amp for 5min (5 Seconds ON, 5 Seconds OFF) the sonicated IBs were centrifuged at 10000 rpm for 10 min at 4 ˚C. The pallet was washed with 40ml of wash buffer (0.5 M NaCl, 2% Triton X-100) then washed with 40 ml 0.5 M NaCl and finally IBs were 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) with 8M urea.

3.2 On-column refolding of soluble ASARI 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 mM NaCl, 80mM Imidazole, 6M Urea pH 8). The column was rinsed with a 5 column volume of wash buffer with decreasing amount 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 volume of wash buffer containing no urea. The refolded protein was eluted with 6 volumes of elution buffer (50mM Tris, 500mM NaCl, 300mM Imidazole. Eluted protein was analysed by SDS-PAGE and hemagglutination with rabbit erythrocytes.

3.1. Production of anti-ASARI and antibodies

Recombinant ASARI protein was used to raise polyclonal rabbit anti-ASARI antibodies. (Chromes Biotechnology Pvt. Ltd. Bangalore). Polyclonal anti-ASARI antibodies were raised using the standard provided protocol and used for the western blotting.

3.2 ASARI Haemagglutination assay

The Assay was carried out in a round (U) bottom clear microtiter plate. 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 room temperature.

3.3 SDS PAGE and Western blotting of ASARI

On-column refolded and purified protein samples of ASARI were collected and separated by SDS-PAGE. For western blotting the purified and on column refolded ASARI was separated by SDS-PAGE and transferred on PVDF membrane by using power blotter and detected by primary anti-ASARI antibody and secondary goat anti-rabbit antibody were diluted 1:2000.

4. RESULTS AND DISCUSSION

4.1 Preparation of ASARI 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) (pASARIF- 5'ATA TAT CAT ATG GGT CGT ACT ACT TCA TC 3' and pASARIR- 5'TAT ATA CTC GAG TCA AGC AGC ACC GC 3') Single-band was obtained approximately 546 bp. The fragment was purified, cloned, and characterized by restriction digestion and DNA sequencing.

The sequence of 546 bp product was identical to the published mature sequence of the garlic root lectin ASARI with 100% similarity at the nucleotide and amino acid level. The expression constructs for ASARI which omitted the predicted signal peptide-encoding regions and stop codons were amplified by PCR primers. The final expression construct (pETASARI) contained the lectin sequence in frame with sequence from the vector, which encoded an N-terminal T7 promoter followed by initiation codon of ASARI and C-terminal his6 tags (Fig. 1).

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

Sequence of confirmed clone of pETASARI was transformed into the chemically competent *E. coli* BL21/DE3 cells. The cells were prepared using material and procedure provided into the PET Manual (Invitrogen, USA). Transformed colonies were screened by colony PCR using gene-specific primers. Expression of ASARI was

checked by SDS-PAGE and purification was done by washing of inclusion bodies with different buffers followed by on-column refolding by decreasing amount of urea i.e. 6, 4, 2, 1, 0 0.5, 0.25,0.125 and 0 M from wash and elution buffer. The purified and refolded ASARI was checked by SDS-PAGE and western blotting. For western blotting rabbit anti-ASARI as a primary and goat anti-rabbit hrp labelled as a secondary antibodies were used for the detection of ASARI. Purification of ASARI was done by a Ni-NTA column chromatography was successful in recovering recombinant garlic lectins from inclusion bodies at a purity of >95% (estimated by stained gels and comparison to standards; Fig. 3).
Analysis of purified protein from recombinant *E. coli* expressing ASARI by Western blotting gave monomers and dimers of refolded forms of ASARI which were shown immunoreactive with anti-ASARI antibodies (Fig. 4) these data suggest that the expressed and purified protein is intact and correctly refolded.

4.3 Functional Activity of ASARI by Agglutination Assay

The recombinant ASARI agglutinated rabbit erythrocytes down to a dilution of approx. 0.012 mg/ml, showing that the recombinant protein was functionally active. Positive control of native standard GNA lectin agglutinated to approx. 0.19 mg/ml and negative control (bovine serum albumin) showed no agglutination at any concentration, including the highest tested (1mg/ml).

DISCUSSION

Escherichia coli is one of the most commonly used prokaryotes 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 they expresses 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 ASARI 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 cause the degradation of recombinant protein. To overcome such problems we have been focused on the on-column refolding of the ASARI protein by washing inclusion bodies with different buffers and solubilised with urea-containing buffer. The solubilised ASARI 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 ASARI gene was amplified using gene-specific primers and the amplified PCR product was cloned into the primary vector pGEMT easy vector. The digested ASARI and linearized pET 30 b (+) expression vector were ligated. The expression construct pETASARI 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 ASARI protein using *E. coli*/BL21/DE3.

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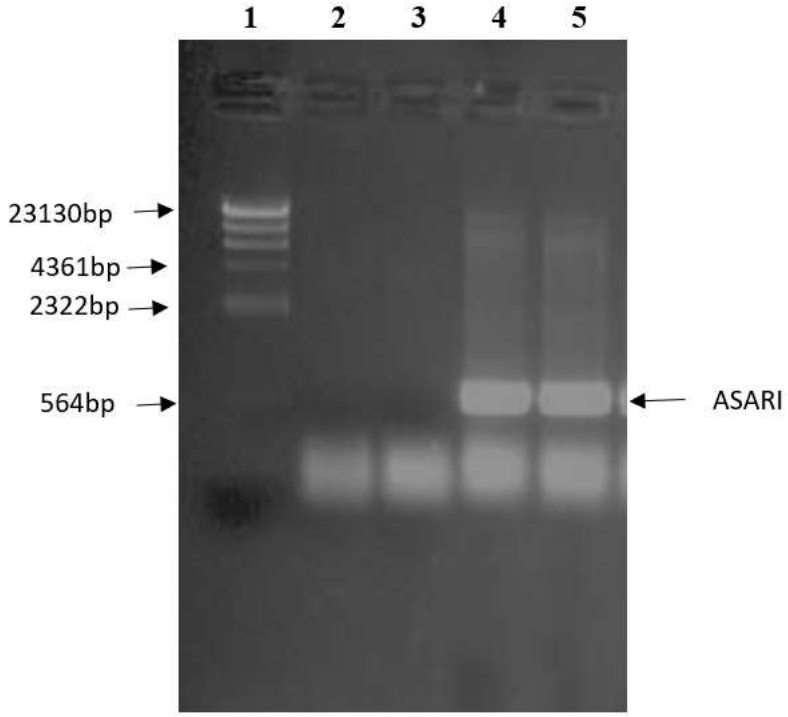

Fig1: PCR amplified ASARI

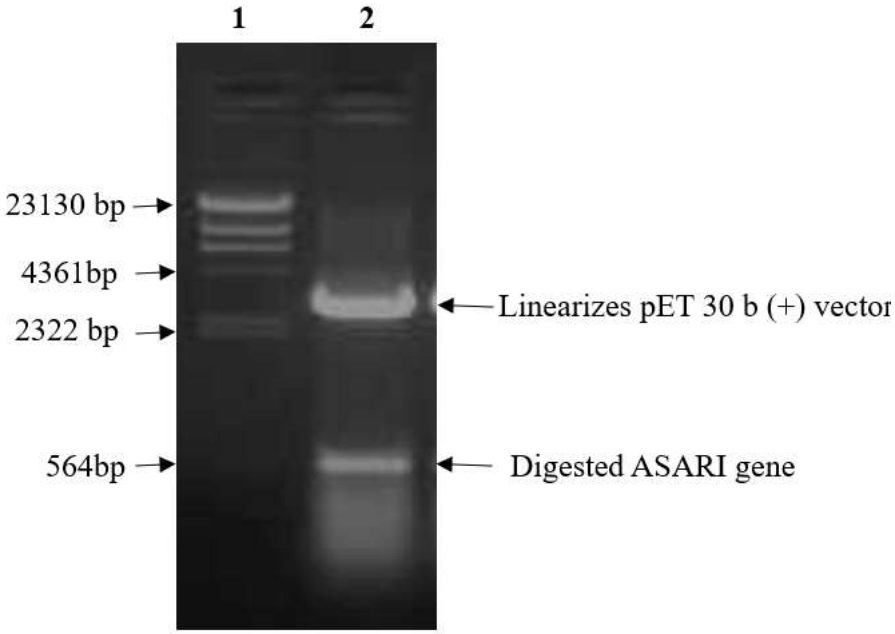

Fig 2: ASARI in pET 30 b (+) digested with *NdeI* and *XhoI*

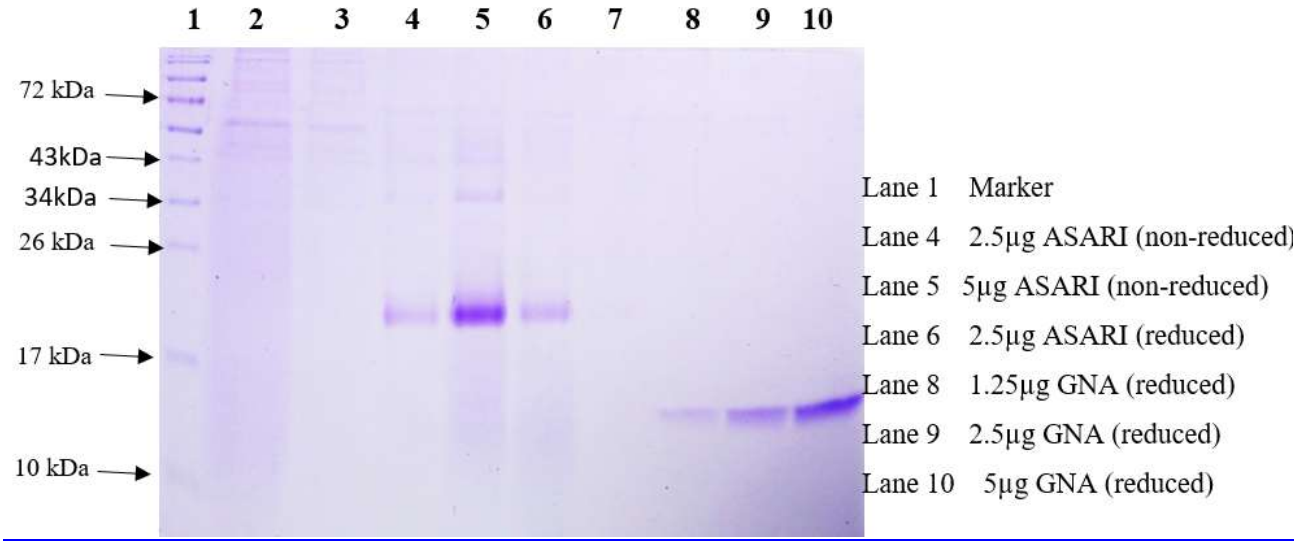

Fig. 3: SDS-PAGE of on-column purified ASARI from *E. coli*/BL21

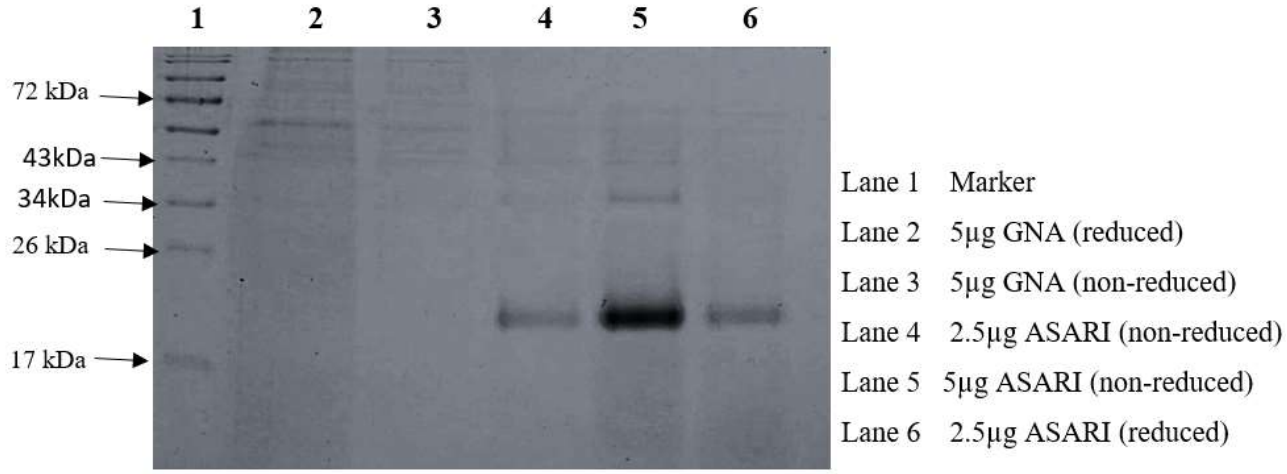

Fig. 5: Western blotting of on-column purified ASARI from *E. coli*/BL21

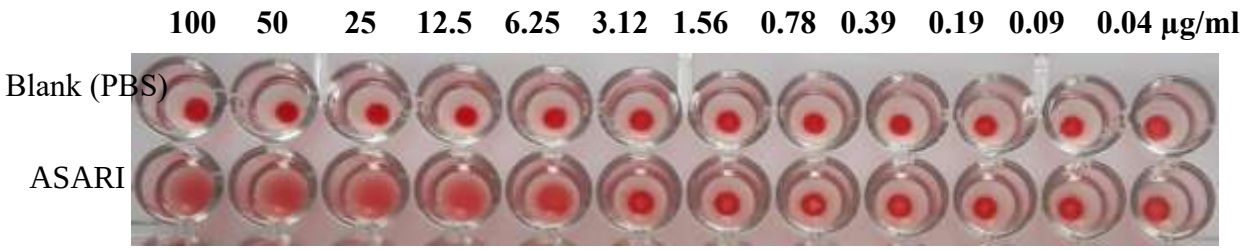

Fig. 5: Agglutination assay of ASARI lectin with rabbit erythrocytes.