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In-silico and *in-vitro* expression of *Vigna aconitifolia* lectin for insecticidal activity

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ABSTRACT

Lectin proteins are ubiquitously present in all life forms with various biological functions. Current study covers *in silico* analysis of *Vigna aconitifolia* lectin (Valectin) protein sequences, aiming identification of functional conserved domains and to predict its tertiary structures with different ligands. This study was carried out at ICAR-NIPB and division of entomology at ICAR-IARI, Pusa,New Delhi during 2015-18. Molecular Docking study of Valectin against alanylaminopeptidase N (APN) receptor of Acyrthosiphon pisum (pea aphid) revealed that ASP159, SER161, GLU164, SER181, ASN178, VAL177, SER35, VAL94, ASN92, LYS141, ARG85, GLU138 and TYR139 residues of Valectin are critical for the interaction with insect receptor. The efficacy of Valectin protein was confirmed using wet lab experiments including molecular cloning, *in-vitro* expression and aphid bioassay. Western blot analysis using Anti-His antibody conjugate confirmed the presence of recombinant Valectin protein and characterized as ~30 kDa in size. The insecticidal potential of this protein was explored and bioassay results showed mortality of 59.25%, 66.66%, and 70.36% at concentration of 1, 10 and 20 µg/ml upon 96 h incubation against *Lipaphis erysimi*. Present studies by using various bio-computational tools will augment our understanding about the Valectin structure and could be used as a potential candidate gene for generating transgenic of crop plants for increased aphid resistance.

Key words: Alanylaminopeptidase N, Aphid bioassay, Heterologous expression, Lectin, Molecular modeling

Lectin proteins are originally carbohydrate binding proteins that can bind different simple or complex carbohydrates in specific manner. Legume lectins are considered as largest and best characterized plant lectins family, present mainly in the seeds of legume plants. Moth bean (*Vigna aconitifolia*) is an important legume crop with excellent reserve of digestible protein. Lectin from common bean, pea and soybean showed adverse effect on various insects of the order Lepidoptera and coleopteran. They have proved equally effective against the sap sucking insects like aphids, leaf and plant hoppers, against whom till date no Bt toxins has been proved effective (Rao *et al.* 1998; Gatehouse *et al.* 1999). *In planta* bioassay of *Aphis gossypii* showed 69% of population reduction in T1 transgenic plants with *Sclerotium rolfsii* lectin. Whereas, 100% insect mortality was observed in *Spodoptera litura* larvae within 96 h on same plants (Vanti *et al.* 2018).

The anti-parallel beta sheets are characteristics features of lectins secondary structure and have tightly-bound calcium and manganese ions. They are devoid of alpha helices. All legume lectins share similar 3D structure. Generally, the 3D structure of plant lectin is characterized by presence of β-sheets that are connected by α-turns, β-turns and bends (Vazquez-Moreno *et al.* 2000; Sharon *et al.* 2002). The monomer structures display a jellyroll motif structure of 25–30 kDa containing a carbohydrate recognition domain (CRD) and metal binding domain for divalent cations (Ca^{2+} and Mn^{2+}). However, although presence of strong similarities in primary, secondary and tertiary structures of legume lectins, the quaternary structures shows substantial disparities that lead to changes in the monomer–monomer interactions and the presence/absence of protein modifications, such as glycosylation (Ambrosi *et al.* 2005).

The present study was carried out to study the physicochemical properties, secondary structure attributes and 3D model of *Va*lectin protein. The insect receptor binding potential with *Va*lectin protein was evaluated through docking analysis and further efficacy of the protein was confirmed by aphid bioassay.

MATERIALS AND METHODS

Cloning and heterologous expression of Valectin Gene in E. coli

The *Valectingene* **(**Accession No. JN561787.1) was amplified using gene specific primers with the addition of CACC in the forward primer (Table 1). The PCR was

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Table 1 Primers used for amplification of complete coding sequences

Gene	Primer	Amplicon size (bp)
Valectin	FP: CACCATATGGCTTCTCTTCAAACCCAAATG RP: AAGCTTATGCATCTGCAGCTTGCTTAGAACTC	828

carried out with *Phusion DNA polymerase* enzyme by denaturing the template at 98ºC for 30 sec followed by 35 cycles of amplification (30 sec at 98ºC, 1 min at 60ºC and 30 sec at 72°C) and by extension at 72°C for 10 min. The amplified PCR product cloned into pENTR-D-TOPO and generates entry clone by using single shot TOPO10 cell, then expression clone with pET300/301 DESTC-terminal fusion vector (Invitrogen).Positive recombinant clones were sequenced and BL21 (DE3) pLysS competent cells were transformed with recombinant expression clone for protein expression studies (Sambrook and Russell, 2001). The induced soluble recombinant protein suspension was centrifuged for 15 min at 6000 rpm at RT. The clear supernatant and remaining pellet were analyzed on 12% SDS-PAGE to verify the location of the expressed recombinant protein. Colony Blot of the transformed colonies was done by placing the colonies from LA plate containing kanamycin (50µg/ml) and IPTG (1 mM) to nylon 6,6 transfer membrane as per user manual.

Protein purification and western blotting of Valectin protein

In order to purify the protein, BL-21 (DE3) pLysSstrain of *E. coli* harbouring the cloned gene was grown at 37 ºC until the $O.D_{600}$ reached 0.6. Purification was accomplished using QIAexpress® Ni-NTA Fast Start Kit (Qiagen, USA). The recombinant protein was detected through western blotting using Anti-His Antibody (Invitrogen, USA) as per manufacturer's protocol.

Aphid bioassay for insecticidal efficacy

Aphid bioassay was performed in division of entomology, Indian Agricultural Research Institute, New Delhi, India. The assay was carried out against second instar nymphs of *Lipaphis erysimi* by feeding them on artificial diet incorporated with purified proteins of *Va*lectin. Nymphs of *L. erysimi* used for bioassay were maintained in the laboratory on artificial diet at constant temperature of 26 ± 2 ºC with 80% relative humidity. Liquid diet of 200µl supplemented with different concentrations range (1, 10, 20 µg/ml each) of purified protein was used for bioassays. These experiments were carried out at division of entomology at ICAR-IARI, Pusa, New Delhi during 2015-18.

IN SILICO CHARACTERIZATION OF *VALECTIN* PROTEIN

Sequence retrieval and analysis

The full length amino acid sequence of *Va*lectin (accession #JN561787.1) protein was retrieved from NCBI database. The functional domains of lectin were determined using the InterPro tool available on the EBI web page (www.ebi.ac.uk/interpro/). Probability of protein disorder was determined by the PrDOS (Protein disorder prediction server) tool (http://prdos.hgc.jp). The subcellular location and molecular functions of protein were predicted by using CELLO2GO (http://cello.life.nctu.edu.tw/cello2go/) web server. To analyze the conserved motifs among protein sequences related to legume lectin, MEME (http://meme-suite.org) programme was used. All the *in silico* studies of Valectin were performed at ICAR-NIPB, Pusa, New Delhi during 2015-18.

Homology-based modelling, molecular docking and active site mapping

The secondary structure and solvent accessibility of *Va*lectin protein was determined by the RaptorX protein structure server (Kallberg *et al.* 2012). The 3D structure of the target protein *Va*lectin and APN *Acyrthosiphon pisum* membrane alanyl Aminopeptidase N (APN; DQ440823) was generated with SWISS Model (https://swissmodel.expasy.org/) through homology based modelling. The authenticity of the predicted models was further validated by employing RAMPAGE tool (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). In order to accomplish the docking studies using *Acyrthosiphon pisum*APN, which was found to be a probable ligand for *Va*lectin, ClusPro Docking server (http://cluspro.bu.edu/) was used and results were viewed through Discovery Studio 4.1. PDBSumtool (http://www.ebi.ac.uk/pdbsum) was used to further identify the amino acid residues binding with the sugar moieties present on the lectin binding.

RESULTS AND DISCUSSION

Cloning and heterologous expression of Valectin Gene in E.coli

The *Va*lectin gene of 828 bp were PCR amplified and cloned in pET301/CT-DESTC-terminal fusion vector using Gateway cloning system to generate the expression clone BL21 DE3 pLysS cells transformed with this for its optimum expression. The colony PCR was done to confirm the presence of the *Va*lectin gene. *E.coli* BL21 (DE3)pLysS cells harbouring Valectin gene downstream to T7 promoter, when induced with 1mM IPTG, exhibited ~30 kDa induced protein bands maximally after 4 h of incubation at 37ºC.

Protein purification, western blotting and aphid bioassay of Valectin

Western blot analysis using Anti-His antibody conjugate confirmed the presence of recombinant *Va*lectin expression of ~30 kDaprotein both in the crude and purified samples.

Aphid bioassay was carried out using the artificial diet containing the *Va*lectin protein to assess the insecticidal activity against second instar nymphs of *L. erysimi.* Aphid bioassay using *Va*lectin protein revealed that 1 µg/ml, 10 µg/ml and 20 µg/ml of the protein was sufficient to cause 59.25%, 66.66% and 70.36% mortality respectively, against *L. erysimi* within 96 h. Whereas, using the same concentration of GNA protein with the diet showed 33.33%, 55.5% and 62.95% mortality against *L. erysimi* within 96 h. Hence, the *Va*lectin protein proved to be more effective compared to GNA lectin.Wu *et al.* (2006) has reported the insecticidal toxicity of another agglutinin family gene (*Amaranthus caudatus*) on the cotton aphids and showed significant effect of ACA on the growth inhibition of cotton aphids. Another report with *Vigna radiata* lectin protein in artificial diet exhibited that 15µg/ml and 25µg/ml of the protein was sufficient to cause 50% to 100% mortality in *Lipaphis erysimi*, respectively within 6 days upon exposure (Singh *et al.* 2016).

IN-SILICO CHARACTERIZATION OF *VA*LECTIN PROTEIN

Sequence analysis and characterization

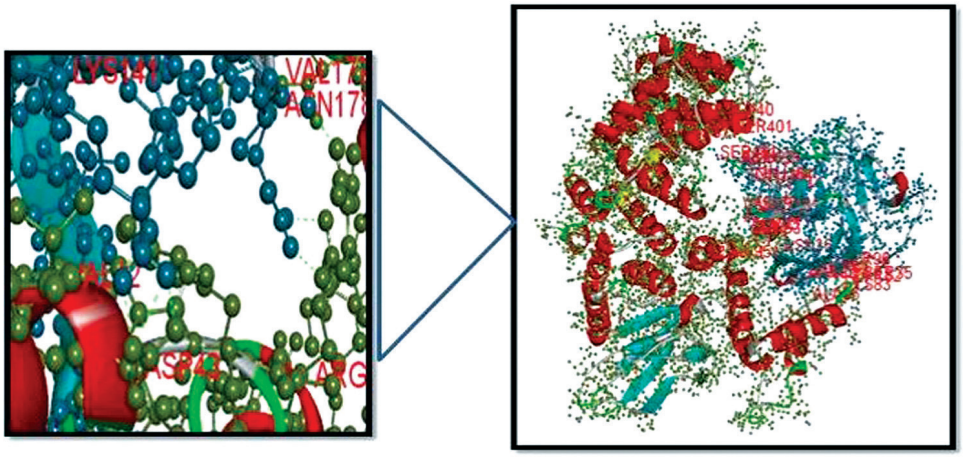
The subcellular localization of *Va*lectin protein is plasma membrane as predicted by CELLO2GO tool with a reliability score of 2.441. Recently Moraes *et al.* (2017) reported that most of the legume lectins are located in the extracellular medium or associated with the plasma-membrane and play important function in ion binding, kinase activity and enzyme regulator.The instability index for *Va*lectin was 30.24, classifying it as a stable protein which also justify with the result obtained from PrDOS tool. Two disordered regions were predicted in the protein sequence, of which the longest disordered region was found between Ser^{258} to Ala^{268} comprising 11 amino acid residues.

Structural analysis and homology-based modelling

The 2D structure generated with RaptorX predicted a total of 5% α helices, 44% β pleated sheets and 50% coil. Its 3-class secondary structure hovering over a residue will display the predicted distribution for that residue. It also revealed solvent accessibility of this protein as 27% residues are buried into structure, 35% residues are exposed and 37% were medium.Similar results were obtained from secondary structure of *Cajanus cajan* lectin (CCL) which showed of 7% α-helices, 42% β-pleated sheets and 50% coil with solvent accessibility of 27% residues were buried, 30% residues were exposed and 41% were medium (Prajapat *et al.* 2018). All these proteins contain about 40–50% β-sheet, 35–45 % β-turn and 0–10% α-helix, and thereby fall into a structurally distinct class of proteins. The β-sheet, α-helical and β-turn content predicted here agrees well with X-ray structure determination of *Con* A. The 3D model of *Va*lectin was generated by SWISS-MODEL using pea lectin (ID-2bqp.1) as template with identity of 81.47% and coverage was 87% .The 3D model of APN with sequence identity 30.59% and coverage 89% generated by same server using APN1 from *Anopheles gambiae* as a template (ID-4wz9.1.A). The SWISS modelling of APN from pea aphid also showed that Zn is most probable ligand for its activity. The generated 3D models were further validated by analysing Ramachandran plot using MOLPROBITY server.

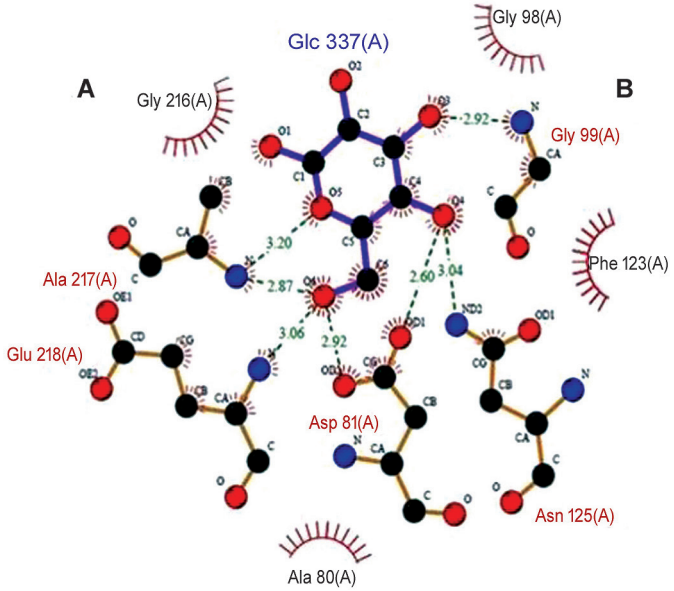
Molecular docking and active site mapping

Molecular docking through ClusPro revealed positions of interaction between *Va*lectin and APN receptor. The amino acid residues of *Va*lectinprotein ASP^{159}, SER^{161}, GLU^{164}, SER^{181}, ASN^{178}, VAL^{177}, SER^{35}, VAL^{94}, ASN^{92}, LYS^{141}, ARG^{85}, GLU^{138} and TYR^{139} interact with SER^{401}, ASP^{371}, ARG^{3}, GLU^{16}, ARG^{51},TYR^{82}, ALA^{85}, ASP^{43}, ASN^{36}, ARG^{440} and VAL^{42} residue of APN receptor through H-bond (Fig 1). These results were confirmed by docking studies of PPL from *Cajanus cajan* using same receptor showed that ASN, ASP, TRP, VAL and SER residues were involved in interaction with SER, ASP, GLY, VAL and SER residues of APN receptor from *Acyrthosiphon pisum* membrane.It's



MBL	Recptor (Aphid gut)
ASP159	SER401
SER161	ASP371
GLU164	ARG3
SER181	ARG3
ASN178	GLU16
VAL177	ARG51
SER35	TYR82
VAL94	TYR82
VAL94	LYS83
ASN92	ALA85
LYS141	ASP43
ARG85	ASN36
GLU138	ARG440
TYR139	VAL42

Fig 1 Interactions of Valectin protein with APN receptor of Acyrthosiphon pisum visualized by PRED_PPI interaction server.



VAL Protein		Ligand glucose		
Res. name	Res. No.	Res. name	Res. No.	Distance(Ao)
ASP	81	GLC	337	2.73
ASP	81	GLC	337	2.90
GLY	99	GLC	337	2.89
ASN	125	GLC	337	2.95
ALA	217	GLC	337	3.04
ALA	217	GUC	337	2.92
GLU	218	GLC	337	3.06

Fig 2 LIGPLOT of *Valectin* interactions involving ligand GLC.

indicated that most of amino acid involved in interaction during docking was conserved but their position in the chain may be different (Prajapat *et al.* 2018). Active site mapping for determining the residues involved in ligand binding, i.e. glucose for *Valectin* protein is done by LigPlot using PDBSum tool (Fig 2). Most plant lectins preferentially bind to complex oligosaccharides such as *N- and O-* linked glycans (Bhat *et al.* 2010; Smith *et al.* 2010; Upadhyay *et al.* 2011; Ghazarian *et al.* 2011; Inamdar *et al.* 2016). Homology-based modelling using SWISS Model also revealed that Ca^{2+} and $Mn^{2+}$are metal ligand for *Va*lectin, which is required for carbohydrate binding. The domain analysis revealed metal binding sites of *Va*lectin protein are GLU^{142}, $ASP^{144}$and HIS^{159}. In case of legume lectin the presence of metal ions, i.e. Mn^{2+} and Ca^{2+} was documented to be very important. This is signified by the evolutionarily conserved amino acid residues that bind to the metal ions. For example, ConA requires Mn^{2+} and Ca^{2+} for its activity (Hardman and Ainsworth 1972).

Conclusion

The complete coding sequence of *Va*lectin from *Vigna aconitifolia* was cloned and aphid bioassay by using expressed *Va*lectin protein in artificial diet against *L. erysimi* revealed its insecticidal nature. The structural attributes like secondary structure, physicochemical properties and the amino acid residues participating in the catalytic and docking activity of 3D model was envisaged via homology-based modelling using various bio-computational tools which can help in understanding the structural and functional characteristics of the protein. From the phylogeny tree, it is clear that functional domain of *Va*lectin and other legume lectin are conserved during evolution. Present study has successfully demonstrated that *Va*lectin can be a potent insecticidal against aphid and can be used to develop aphid resistant transgenic crops.

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