INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1092/2019/22–6–1655–1662
DOI: 10.17957/IJAB/15.1247
http://www.fspublishers.org

Full Length Article

In Silico Studies and Functional Characterization of a Napin Protein from Seeds of *Brassica juncea*

Aqsa Munir[1], Sadaf Iqbal[2], Binish Khaliq[3], Qamar Saeed[4], Saber Hussain[1], Kausar Hussain Shah[1], Fiaz Ahmad[1], Sohaib Mehmood[1], Zahid Ali[5], Aisha Munawar[6], Muhammad Qamar Saeed[7], Seema Mehmood[1], M. Yasin Ashraf[3] and Ahmed Akrem[1*]

[1]*Department of Botany, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan*
[2]*Department of Chemistry, University of Karachi, Karachi-75720, Pakistan*
[3]*Institutes of Molecular Biology and Biotechnology (IMBB), University of Lahore, Lahore, Pakistan*
[4]*Department of Entomology, Bahauddin Zakariya University, Multan, Pakistan*
[5]*Department of Biosciences, COMSATS University Islamabad (CUI), Islamabad 44000, Pakistan*
[6]*Department of Chemistry, University of Engineering and Technology, Lahore, Pakistan*
[7]*Department of Microbiology, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan*
[*]For correspondence: ahmedakrem@bzu.edu.pk

Abstract

Pathogenicity related proteins and peptides have been utilized as natural substitute to control phytopathogenic microbes and insects. A 15 kDa Napin protein from seeds of *Brassica juncea* was identified and characterized. SDS-PAGE separated native 15 kDa protein into two peptidic molecular masses of approximately 10 kDa and 5 kDa under reduced conditions. Ammonium sulfate precipitation (70%) followed by gel filtration chromatography provided a highly purified protein. A homology model of *B. juncea* Napin (*Bj*N) was developed by using PDB ID: 1SM7 as the template structure. *Bj*N predicted model showed conserved topology and disulfide bond patterns similar to rapeseed napin; which however, possessed a long hyper variable region between helices III and IV. An extra internal repeat of residues 77-87 has been predicted as unique segment of *Bj*N predicted model. *Bj*N exhibited inhibitory activity against growth of different pathogenic bacterial and fungal strains assessed by disc diffusion method. A 60 µg/disc concentration of the purified protein exhibited strong antibacterial activity towards *Xanthomonas oryzae*, *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa*, *P. syringae* and *Bacillus subtilis*. Similarly, *Bj*N (90 µg/disc) significantly inhibited the mycelial growth of *Aspergillus flavus* and *Fusarium oxysporum*. *Bj*N also showed good insecticidal potential against *Sitophilus oryzae* and *Tribolium castaneum* at a dose of 1.5 mg. The strong antimicrobial as well as insecticidal activity shown by the protein signified its potential as arsenal of antimicrobial and insecticidal agents. © 2019 Friends Science Publishers

Keywords: *Brassica juncea*; Napin; Antibacterial; Antifungal; Insecticidal

Introduction

Brassica juncea, commonly known as vegetable mustard; has been cultivated in many parts of Eurasia as leafy vegetable and highly esteemed oil producer (Dasgupta *et al*., 1995). Other than oil, its seeds are rich in nutrient supplements of high vitality such as starches and storage proteins (Bewley and Black, 1994). Additionally, plants can cope with different abiotic and biotic issues by the expression of certain genes and the corresponding expressed molecules especially the proteins. The major seed storage proteins of *B. juncea* are 12S globulins about 25% and 2S albumins with 45–50% of the total protein content (Appleqvist, 1972). Many plants contain 2S albumins as storage proteins *e.g*., cotton, castor bean, cashew nut, oil seed rape, turnip rape and Brazil nut (Perera *et al*., 2016). 2S Albumins from few species were depicted as allergens such as sesame, cashew nut, Brazil nut and mustard seeds. Allergenic character of these proteins may be due to glutamine residues (Jyothi *et al*., 2007). The residual sequence responsible for allergenecity of *B. juncea* 2S albumin is reported as QGPHVISRIYQTAT (Monsalve *et al*., 1993).

The 2S albumins of family *Brassicaceae* are known as Napins and these storage proteins have attracted much interest as good resources for animal nutrition, industrial oils and for genetic manipulations (Sharma *et al*., 2017; Faraz *et al*., 2019). The napins are a rich source of reduced nitrogen because of their abundant arginine and amide residues formed during the early seedling stages (Ye and Ng, 2002). Napins have been reported to be expressed as a single polypeptide precursor (Müntz, 1998)

To cite this paper: Munir, A., S. Iqbal, B. Khaliq, Q. Saeed, S. Hussain, K.H. Shah, F. Ahmad, S. Mehmood, Z. Ali, A. Munawar, M.Q. Saeed, S. Mehmood, M.Y. Ashraf and A. Akrem, 2019. *In-silico* studies and functional characterization of a Napin protein from seeds of *Brassica juncea*. *Intl. J. Agric. Biol*., 22: 1655–1662

and mature napin is produced after the removal of signal peptide from the N-terminal and further proteolytic cleavage of single polypeptide into two daughter subunits as a part of post-translational modifications. The two chains are connected to each other by disulfide linkage in the mature protein and majority of napins are comprised of typical eight cysteine residues. These napins have been assigned many important functions such as nitrogen and sulfur storage, calmodulin antagonist capability as well as antimicrobial activity (Pantoja-Uceda *et al*., 2004). *B. juncea* also utilizes a preformed and inducible protective mechanism against different pathogens which is based on such defense proteins and peptides (Krebbers *et al*., 1998) as napins. The existence of multigene families and proteolytic processing together produce a high degree of polymorphism in napins.

The antimicrobial activity has been accepted as general property of napins especially the broad-spectrum antifungal activity against different phytopathogenic fungi with all isoforms inhibiting the mycelia growth (Terras *et al*., 1992; Sharma *et al*., 2017). The demonstration of strong antimicrobial activities of these albumins/napins has broadened their scope from being a simple nitrogen providing source for the germinating embryo. Much effort has been spent on the characterization of these proteins because of the associated economic implications. To broaden the exploration spectrum of antimicrobial agents from natural resources, *B. juncea* seeds were chosen due to their traditional use as antimicrobial and insecticidal agents.

Materials and Methods

Extraction and Purification of *Bj*N

Seeds of *B. juncea* were obtained from Bio Park, Bahauddin Zakariya University, Multan. These seeds were washed with distilled water and placed on tissue paper to evacuate stickiness. Dried seeds were ground to get powder form using mortar and pestle. The powder was homogenized in 100 m*M* Tris at pH 7.0 and stirred for 2 h at 4°C. The extract was centrifuged at 13000 *g* for 20 min. The clear crude extract (50 mL) was subjected to precipitation by stepwise slow addition of ammonium sulfate to a saturation constant of 70% with constant stirring using a magnetic stirrer at 4°C. The precipitated protein was collected by centrifuging at 5000 rpm for 2 min. The supernatant was removed, and the resulting pellet was re-dissolved in 10 mL of the same and dialyzed (Millipore, MWCO 3.5 kDa) (Cat no: 9200092) overnight with gentle stirring. The desalted *Bj*N was further purified by loading the solution onto a pre-equilibrated Hi Load 16/60 Superdex® 200 column (GE Healthcare). The *Bj*N was eluted at a flow rate of 1.0 mL/min. Absorbance of the eluents was recorded at a wavelength of 280 nm. The fractions with maximum protein content were combined and analyzed by SDS-PAGE along the protein marker. 0.01% NaN_3 (Sodium Azide) was added to avoid any microbial growth. Protein quantification was done by Bradford reagent (Sambrook and Russell, 2001) using BSA (Bovine Serum Albumin) as standard.

Gel Electrophoresis (SDS-PAGE)

The standard gel preparation procedure (Laemmli, 1970) was used for the preparation of the 12% acrylamide gels and visualization of the protein samples. The protein samples were mixed with 2x sample buffer and heated at 95°C for 5 min to denature the protein. The gel was stained with coomassie brilliant blue R-250 dye (Sigma Aldrich) and molecular weight determination was done by using standard Pierce™ Unstained Protein MW Marker (Catalog no. 22610).

Homology Modeling

The amino acid sequence of the *Bj*N was downloaded from UniProtKB (www.uniprot.org) under accession number P80207 (Monsalve *et al*., 1993). The sequence was subjected to BLAST (Altschul *et al*., 1997) in protein databank (www.pdb.org) for a suitable structure template. The NMR structure of rapeseed napin (PDBID: 1SM7) (Pantoja-Uceda *et al.*, 2004) was chosen for the prediction of *Bj*N model. *B. napus* Napin (*Bn*N) was constructed as the best model by providing 49% identity, 64% similarity and an expect value of 2e-30 with the *Bj*N sequence (P80207). The amino acid # 8-121 of *Bj*N perfectly aligned with the amino acid # 4-106 of rapeseed napin with only one gap corresponding to the amino acids 77-87 of the primer. The aligned sequences were used to model building in Swiss-Model server (Guex and Peitsch, 1997; Arnold *et al*., 2006; Biasini *et al.*, 2014). ProMod3 was used to build the models on the base of the target –template alignment. (Benkert *et al*., 2011). For the Model construction the conserve coordinates between target and template were copied from the template. A fragment library was used to remodel the insertions and deletions. The geometry of the model was obtained by using the force field and side chains were rebuilt. The model quality was analyzed by representing Ramachandran plot using ProCheck (Laskowski *et al*., 2001). Structural comparisons were made by Chimera (Pettersen *et al*., 2004).

Antibacterial Assay

Antibacterial activity of purified napin was assessed against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Staphylococcus aureus* and *P. syringae*. Antibacterial activity of *Bj*N was evaluated by using Kirby-Bauer susceptibility disc diffusion method (Boyle *et al*., 1973). Bacterial strains were grown in Luria-Bertani medium. Each of the bacterial strains was inoculated into a 100 mL flask having 25 mL of the LB medium and

cultured at 37°C with shaking at 200 rpm. Streptomycin (50 μg/disc) was used as positive control and 100 m*M* Tris buffer was used as negative control. *Bj*N concentrations of 30 and 60 μg/disc were used for the growth inhibition of bacteria.

Antifungal Assay

Fungal strains *Aspergillus flavus* and *Fusarium oxysporum* were tested to assess the antifungal activity of *Bj*N. Fungal strains were maintained on YPSA medium. Sterile paper discs were placed at equi-distance on plate. Topsin® fungicide (50 μg /disc) was used as positive control with Tris buffer as negative control. *Bj*N concentration of 90 μg/disc was used for the growth inhibition of fungal strains.

Insecticidal Assay

Insect Culture

Stored grain insect pests *Tribolium castaneum* and *Sitophilus oryzae* were reared on wheat flour and rice grains in the laboratory of stored grain research, Department of Entomology, Bahauddin Zakariya University, Multan (+30° 11' 52"N, +71° 28' 11" E). To attain homogeneous population, fresh pupae of *T. castaneum* were retrieved, sexed and then sorted based on the abdominal characteristics, while adults of *S. oryzae* were sexed and found out on the size of snout and rostrum (Alam, 2006). Culture jars were kept undisturbed till the needed population level was achieved.

Toxicity Assay

Three concentrations of *Bj*N 0.5, 1.0 and 1.5 mg were prepared with a control in 100 m*M* Tris (pH 7.5). Each concentration was mixed with 150 grams of pre-sanitized wheat flour and a compact dough was kneaded which was air dried and again ground into powder. This powder was replicated 5 times for each concentration and control. Five pairs of *T. castaneum* were used in each experimental jar containing 30 grams of treated flour. Similarly, 100 grams of rice kernels were repeated with treatment of same concentrations and control. Each concentration was again replicated into 5 times for rice (20 gm each) and 5 pairs of *S. oryzae* were released in each experimental unit. After 10 days, the adults of both insect pest species were removed and data was collected weekly for number of eggs, larvae, puape and next generation's adults. The life cycle attributes of both pests were recoded (Athanassiou *et al*., 2010).

Statistical Analysis

Data was analyzed for toxicity through "Statistix 8.1" subjected to ANOVA and significance difference was calculated through Tukey LSD test at 95% confidence level.

Results

Purification of *Bj*N

*Bj*N was extracted in 0.1 *M* Tris-HCl buffer (pH 7.0) at 4°C was partially purified from the crude extract of *B. juncea* at 70% (w/v) ammonium sulfate. *Bj*N precipitates were dialyzed to remove salt traces and subjected to gel filtration chromatography. Gel filtration chromatography of the partially purified protein was performed in 0.1 *M* Tris-HCl buffer containing 0.15 *M* NaCl. It produces three peaks, two minor and one major peak which later turned out as 2S albumin (Fig. 1A). Protein fractions were collected at 280 nm highest absorbance. These were run on SDS-PAGE with maximum purity pooled together and stored at 4°C. Column chromatography provided more than 95% pure napin. SDS-PAGE exhibited intact polypeptide of 15 kDa in the absence of β-mercaptoethanol and production of two daughter bands of approximately 10 kDa and 5 kDa under reduced form (Fig. 1B).

Structural Features of *Bj*N

A homology model of *Bj*N (8-121) was constructed by using the NMR structure information of rapeseed napin (PDB-ID: 1SM7). Fig. 2 represents the structural superimposition between *Bj*N predicted model (grey and orange ribbons) and rapeseed napin *Bn*N (golden and green ribbons) proteins executed by Chimera. Rapeseed napin consists of a globular four-helical motif arranged in a right-handed super helix manner. The first helix is split in two small helices, residues 3-11 (helix Ia), and 16-25 (helix Ib). Remaining three helices constitute residues 44-54 (helix II), 57-71 (helix III) and 81-95 (helix IV). A long unstructured loop L2 was present between helix Ib and helix II from residues L26-S43. The modeled structure of *Bj*N showed similar topology where residues 9-16 encompass helix Ia and 21-28 comprise helix Ib, helix II residues contain 49-55, helix III enclose residues 67-73 and residues 96-110 constitute helix IV. The long unstructured loop, L2, encloses the residues Q29-L48, found between helix Ib and helix II. In the rapeseed structure, helices I and II are coupled to the bundle formed by helices III and IV, by means of two disulfide bridges, C_5 (helix Ia)-C_{59} (helix III) and C_{49} (helix II)-C_{96} (helix IV). A third disulfide bond is formed between C_{18} (helix Ib) and C_{48} (helix II). Helix IV is followed by a long loop and the fourth disulfide bridge between C_{61} (helix III) and C_{104} anchors this segment to the protein core. Structural comparison of *Bj*N and rapeseed napin discloses the perfect alignment of C_9, C_{22}, C_{52}, C_{53}, C_{63}, C_{65}, C_{111} and C_{119} of *Bj*N with C_5, C_{18}, C_{48}, C_{49}, C_{59}, C_{61}, C_{96} and C_{104} of the latter (Fig. 2). Four disulfide bond pairs of *Bj*N, C_9 (helix Ia)-C_{63} (L3), C_{22} (helix Ib)-C_{52} (helix II), C_{53} (helix II)-C_{111} (L5), C_{65} (L3)-C_{119} (L5) are also absolutely superimposed to rapeseed.

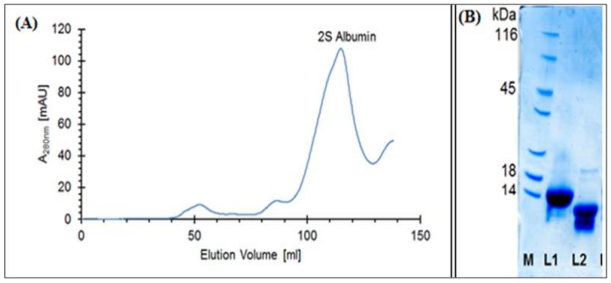

Fig. 1: Gel filtration chromatogram and SDS-PAGE analysis of the purified *Bj*N. (**A**) Partially purified *Bj*N was eluted as major peak in size-exclusion chromatography. (**B**) SDS-PAGE further confirmed the purity of the band and the existence of inter chain disulfide linkage which is typical to 2S albumins. Under non-reduced conditions (L1), *Bj*N showed molecular weight of 15 kDa while in the presence of β-mercaptoethanol (L2), it separated into two bands of 10 and 5 kDa approximately. M is the standard protein ledder

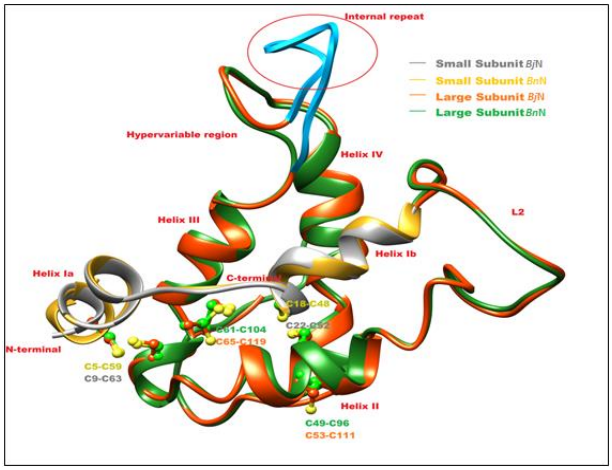

Fig. 2: Superimposed structures of *B. juncea* predicted model (*Bj*N) and rapeseed napin molecular structure (*Bn*N). Grey and orange ribbons correspond to small and large subunits of *Bj*N while golden and green ribbons show small and large subunits of rapeseed *Bn*N respectively. Light blue region is indicating the unique extended loop part of *Bj*N comprising of 11 amino acid residues (77-87)

The model quality was analyzed by ProCheck online tool (http://services.mbi.ucla.edu/PROCHECK/) which provide a Ramachandran plot for the modeled *Bj*N (Fig. 3). Out of 113 modeled residues, Ramachandran plot predicted 75.8% in the most favored region, 21.1% in allowed region, 2.1% in generously allowed and 1.1% in disallowed region. Therefore, the 3D *Bj*N modeled structure is good enough for further evaluation.

Antibacterial Activity of *Bj*N

*Bj*N showed the clear formation of growth inhibition zones against gram positive *B. subtilis*, *S. aureus*, *X. oryzae* and gram-negative *P. aeruginosa*, *P. syringae* and *E. coli*

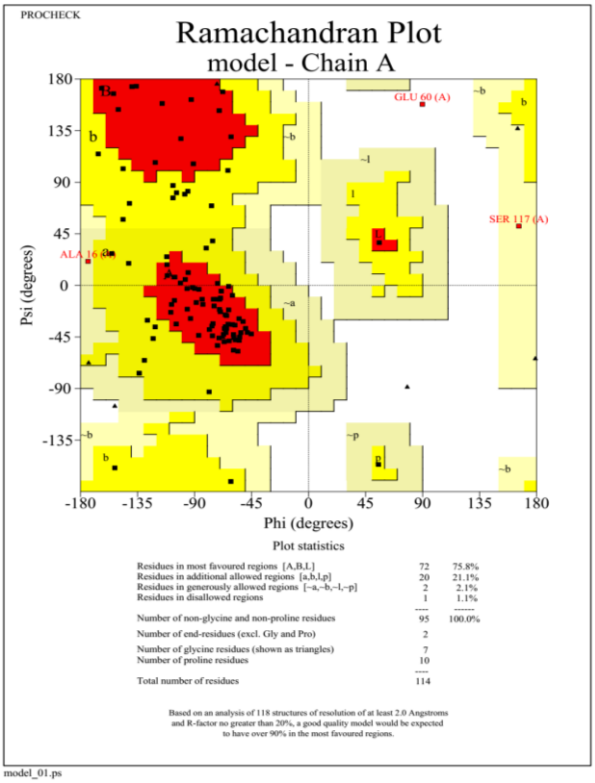

Fig. 3: Ramachandran plot for the modeled *Bj*N assessed by ProCheck online server

bacteria in disc diffusion assay (Fig. 4A, B, C, D, E and F). It showed lesser antibacterial activity at lower concentration of 30 μg against all bacterial strains, but significant growth inhibition was found at the concentration of 60 μg. Higher concentration of *Bj*N showed maximum growth inhibition zones against *X. oryzae* and *S. aureus* (Fig. 4B and E).

Antifungal Activity of *Bj*N

Similarly, *Bj*N strongly inhibited the mycelia growth of *A. flavus* and *F. oxysporum* (Fig. 5A and B). Maximum mycelia growth inhibition was observed at 90 μg/disc *Bj*N concentration.

Entomotoxin Activity against *T. castaneum*

The experimental data enlightens the fact that mortality of *T. castaneum* increases with increase in concentration of protein in the flour, while the highest concentration of 1.5 mg gives the maximum mortality which is significantly higher than the control after 10 days (Fig. 6A). The life cycle attributes of *T. castaneum* after treatment to *Bj*N proved its significant entomotoxic properties. The maximum number of eggs was recorded in control followed by 0.5, 1 and 1.5 mg concentration of treatments. Larvae, pupae and adult rates were also dependent on the concentration. The number of larvae pupae and adult was

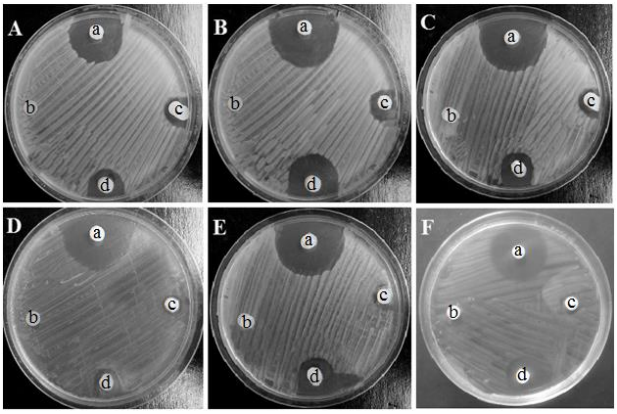

Fig. 4: Antibacterial assay of *Bj*N against different pathogenic bacterial strains. Disc a is positive control using Streptomycin antibiotic (50 μg/disc). Disc b is 20 μL buffer (0.1 *M* Tris; pH 7.0) as negative control. Discs c and d were indicating two concentrations of *Bj*N as 30 and 60 μg per disc respectively. Clear zones of inhibition were observed towards higher concentrations of *Bj*N (60 μg). (**A**) *P. aeruginosa* (**B**) *X. oryzae* (**C**) *E. coli* (**D**) *B. subtilis* (**E**) . *aureus* and (**F**) *P. syringae*

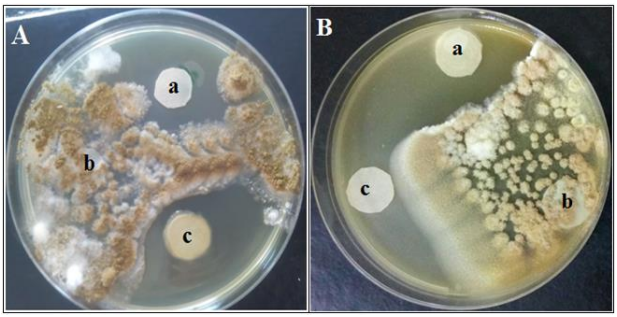

Fig. 5: Antifungal activity of purified *Bj*N against *A. flavus* (**A**) and *F. oxysporum* (**B**). Disc a is as positive control 50 μg/disc Topsin® fungicide, disc b is Tris buffer as negative control, Disc c is 90 μg concentration producing significant mycelia inhibition as compare to control disc b

least in 1.5 mg concentration but highest in control as shown in the (Fig. 6B). Entomotoxin *Bj*N was tested against adults as well as different life stages of *T. castaneum*. Number of adults, larvae, total eggs and pupae their male and female ratio were also compared against control. Adults of *T. castaneum* showed mean percent mortality at 1.5 mg dose (1.8 ± 0.2) after 10 days of treatment which is significantly higher than control.

Entomotoxin Activity against *S. oryzae*

Insecticidal data of *Bj*N against *S. oryzae* showed mean percent mortality after 9.0 days at maximum concentration of 1.5 mg which is 2.67 ± 0.67, 6.33 ±0.33 and 8.66 ± 0.33 with no mortality in control (Fig. 7A). The life table attributes of *S. oryzae* were also parallel to that of *T. castaneum* when subjected to concentration of *Bj*N. The population of rice

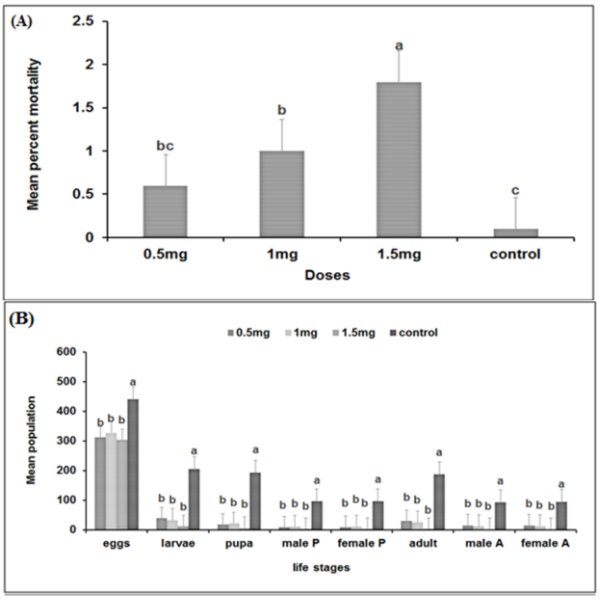

Fig. 6: Insecticidal toxicity of *Bj*A against *T. castaneum.* (**A**) Mean percent mortality (±SE) of *T. castaneum* adults in response to three doses of *Bj*A protein. (**B**) Effect of *Bj*A protein on different life stages of *T. castaneum*. Strong insecticidal activity of *Bj*A was observed against all life forms in comparison to control experiment

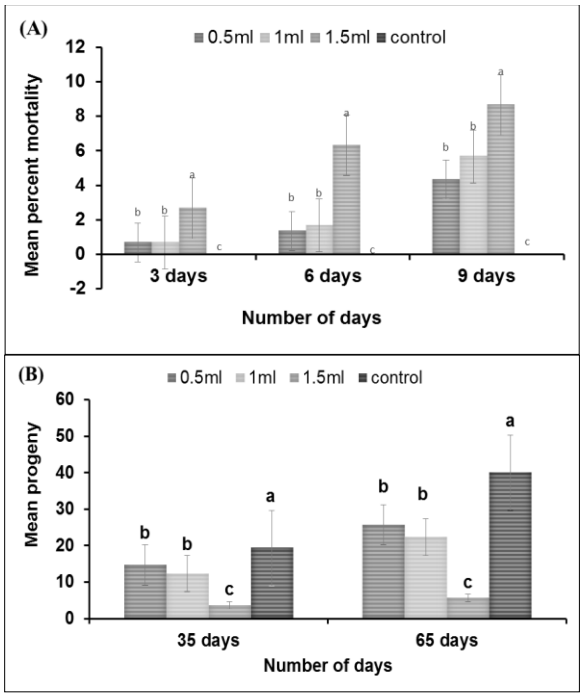

Fig. 7: Insecticidal toxicity of *Bj*A against *S. oryzae.* (**A**) Mean percent mortality (±SE) of S. *oryzae* adults in response to three doses of *Bj*A protein after nine days of exposure. (**B**) Comparative protein efficacy of *Bj*N against *S. oryzae* progeny after 35 (first generation) and 65 (second generation) days. Strong insecticidal activity of *Bj*N was observed against two progenies of *S. oryzae* in comparison to control experiment

weevil was least of its number in the highest concentration of 1.5 mg and it was at the highest number in the control, followed by 1.0 and 0.5 mg (Fig. 7B).

Discussion

A 15 kDa *Bj*N protein was identified and characterized from the *B. juncea* seeds. Under reduced conditions, the 15 kDa band was separated in daughter bands of 10 and 5 kDa showing disulfide linkages which is the basic character of all albumins. Two polypeptides are characteristic of mature napin produced by proteolysis of single polypeptide precursor (Crouch *et al*., 1983; Kinnunen *et al*., 2003). Both units are substrate for calcium dependent protein kinase and calmodulin antagonistic in plants (Yu *et al*., 2003). The protein pattern was obtained by SDS–PAGE of SiAMP2 showed two different bands, one with below 14 kDa other approximately 6 kDa (Maria-Neto *et al*., 2011).

Molecular structure determination of any protein is always an important step towards the understanding of relationship between the structure and functions attributed to that protein. Isolation of napin from the seeds renders a micro heterogeneous material because of their origin from multigene families. Consequently, napins being polymorphic protein; pose difficulties for three-dimensional structure determinations either by X-ray diffraction or NMR. Only one *B. juncea* napin (*Bn*N) structure is available from *Brassicaceae* family. Two different solution structures of rapeseed napin have been reported till now, one is global fold (PDB-ID: 1PNB) (Rico *et al*., 1996) and the other is pronapin (PDB-ID: 1SM7) (Pantoja-Uceda *et al*., 2004). Structurally, they have four α-helices and four disulfide bonds as found in the α-amylase/trypsin inhibitors and nonspecific lipid transfer proteins (Cândido *et al*., 2011).

Structural details of predicted *Bj*N model have already been described in the result section, however; a unique hypervariable region of *Bj*N was predicted in comparison to *Bn*N molecular structure. Helices III and IV of *Bn*N are connected by a short segment constituted by residues R72-F80, known as the "hypervariable region" in 2S albumins (Krebbers *et al*., 1998). This is due to the high variability in length and composition between the different members of the family. Hypervariable region in *Bj*N comprises of a long loop containing residues 74-95 and the residues 77-87 of *Bj*N "QQIRQQGQQQG" as extended part of this segment, highlighted in light blue ribbon (Fig. 2).

In future, it would be interesting to observe such unique loop part in napins of other *Brassica* species as more and more molecular structures are going to be solved either through X-ray crystallography or Nuclear Magnetic Resonance (NMR) techniques. Additionally, the amino acid sequence analysis of *Bj*N by using the software RADAR, has led to the identification of two internal repeats in the large subunit with 40% similarity (Jyothi *et al*., 2007). The two segments span from 31–60 and 73–109 residues in *Bj*N. Multiple sequence alignment with *Brassica* family 2S proteins has revealed that these internal repeats are highly conserved. The presence of these internal repeats may contribute to the enhanced stability of all napins by interacting with each other to form additional hydrophobic cores. The functional characterization of *Bj*N was done by performing different bioassays. Purified *Bj*N (60 μg) showed strong antibacterial activity against different pathogenic bacteria. Similarly, 2S albumin of *Raphanus sativus* seeds has shown growth inhibition of *B. megaterium* (Zheng and Sicnclair, 2000). A 2S albumin from kernels of *Sesamum indicum* (*Si*AMP2) inhibited specifically the growth of *Klebsiella* spp. at a lower MIC value of 3.1 μM. *Si*AMP2 was supposed to interact with the synthesis of acidic polysaccharide containing capsular complex of *Klebsiella* spp. which is essential for its virulence (Maria-Neto *et al*., 2011).

Most of the antimicrobial proteins being cationic are able to interact with anionic cell wall and/or cell membrane phospholipids of pathogenic bacteria (Brown and Hancock, 2006). It has been reported that positive electrostatic potential (mainly due to arginine) of the napin interacts strongly with the negative part of the membrane (such as dioleoyl phosphatidylglycerol; DOPG). The strong binding influences the ion flux and membrane permeation of gram-negative bacteria. The purified *Bj*N has inhibited the spores/ mycelia growth of *A. flavus* and *F. oxysporum* at a concentration of 90 μg (Fig. 5). Similar antimicrobial activities of 2S albumins from *Passiflora edulis*, *Malva paviflora* and *Brassica* species have already been reported (Wang *et al*., 2001; Agizzio *et al*., 2003). A peptide (5 kDa) from seeds of *Passiflora edulis* with high sequence homology to 2S albumins has shown antifungal activity against *A. fumigatus*, *F. oxysporum* and *Trichoderma harzianum* with an IC of 8.0, 6.8 and 6.4 μM respectively. However, this peptide showed no activity against *Candida albicans*, *Paracoccidioides brasiliensis* and *Rhizoctonia solani* (Pelegrini *et al*., 2006). Furthermore, 2S albumin from chili peppers has shown clear inhibitory activity towards yeast, showing that proteins belonging to this class could also inhibit non-mycelia fungi (Ribeiro *et al*., 2007). A high thermostable napin-like polypeptide exhibited antifungal properties (Lin and Ng, 2008). The antimicrobial properties of napins have been attributed towards two structural requirements including a cationic charge and an amphipathic shape; the rapeseed napins fully comply with criteria because of their high arginine and lysine content as well as amphipathicity. Moreover, this protein can also work synergistically with Thionins towards the destruction of fungal membrane (Terras *et al*., 1993). Another report has indicated the involvement of amphipathic α-helical structure of *Brassicaceae* napins in CaM antagonism, thus producing the pores in fungal membrane (Neumann *et al*., 1996a). Some workers have also suggested the possible involvement of napins towards protease inhibition which causes a change in protein metabolism of fungal mycelia

and growth is affected (Neumann *et al*., 1996b).

*Bj*N was analyzed against most detrimental pests of stored insects such as *T. castaneum* and *S. oryzae*. It clearly decreased the number of inhabitants of *S. oryzae* and *T. castaneum*. These results are in favour of earlier reports on different stored grain insect pests (Gressent *et al*., 2003; Jouvensal *et al*., 2003; Gressent *et al*., 2007; Rahioui *et al*., 2007; Muench *et al*., 2014). The reason of reducing population of insects is due to the insect's V-ATPase inactivity. V-ATPase subunit e and c in insects is binding site in *Bj*N. The lethality of the pea seed albumin 1b (PA1b) against grain weevils (*S.. oryzae*, *S. granarius* and *S. zeamais*) has already been evaluated (Gressent *et al*., 2003; Muench *et al*., 2014). However, its entomo-toxic mechanism of activity is not clear and possibly includes an objective protein in insect tissue. When the larvae and pupae were exposed to the treated *Bj*N flour, they did not develop to adult and their population got reduced. Their life stages were delayed in time duration which supports a previous study (Rahioui *et al*., 2007), where *D. melanogaster* was fed on the albumin treated diet. It showed a delayed development with 100% mortality. Mortality and delayed developmental process in different life stages is due to the cysteine knot binding of insects V-ATPase (Rahioui *et al*., 2007; Muench *et al*., 2014). The 2S albumin plant protein could be used against the different economically important plant insect pests. Due to the insecticidal activity of albumin against the insect reduces the progeny of red flour beetle and rice weevil. 3D structure and its binding site of *Bj*N will be helpful to understand of its mechanism against the insect.

Conclusion

Plant antimicrobial proteins play an important role as first line defense of plant immune system. Moreover, screening and exploration of plant originated proteins is important for treatment against different pathogenic agents. In this study, structural and functional characterization of the purified *Bj*N protein from the seeds of *B. juncea* has been done. *Bj*N showed activity against pathogenic bacteria, fungi and insects and may serve as new antimicrobial agent in future.

Acknowledgements

We are thankful to the Institute of Pure and applied Biology, Bahauddin Zakariya University Multan, Pakistan for help in providing the fungal and bacterial species for antimicrobial activity assays.

References

Agizzio, A.P., A.O. Carvalho, F.R.S.D. Fátima, O.L. Machado, E.W. Alves, L.A. Okorokov, S.S. Samarão, C. Bloch, M.V. Prates and V.M. Gomes, 2003. A 2S albumin- homologous protein from passion fruit seeds inhibits the fungal growth and acidification of the medium by *Fusarium oxysporum*. *Arch. Biochem. Biophys.*, 416: 188–195

Alam, S., 2006. Efficacy of some plant indigenous plant extracts against rice weevil *Sitophilus oryzae* under laboratory conditions. *Master thesis*. Bangladesh Agriculture University, Mymensingh, Bangadesh

Altschul, S.F., T.L. Madden and A.A. Schäffer, 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl. Acids Res.*, 25: 3389–3402

Appleqvist, L.A., 1972. Chemical constituents of rapeseed. *In: Rapeseed Cultivation, Composition, Processing and Utilization*, pp: 147–175. Appleqvist, L.A. and R. Ohlson (Eds.). Elsevier, New York, USA

Arnold, K., L. Bordoli, J. Kopp and T. Schwede, 2006. The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics*, 22: 195–201

Athanassiou, C.G., F.H. Arthur and J.E. Throne, 2010. Effects of short exposures to spinosad-treated wheat or maize on four stored-grain insects. *J. Econ. Entomol.*, 103: 197–202

Benkert, P., M. Biasini and T. Schwede, 2011. Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics*, 27: 343–350

Bewley, J.D. and M. Black, 1994. *Seeds: Physiology of Development and Germination*, 2nd edition. Plenum Press, New York, USA

Biasini, M., S. Bienert and A. Waterhouse, 2014. SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. *Nucl. Acids Res.*, 42: 251–258

Boyle, V.J., M.E. Fancher and R.W. Ross, 1973. Rapid, modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial discs. *Antimicrob. Agents Chemother.*, 3: 418–424

Brown, K.L. and R.E. Hancock, 2006. Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol.*, 18: 24–30

Cândido, E.D.S., M.F.S. Pinto, P.B. Pelegrini, T.B. Lima, O.N. Silva, R. Pogue, M.F. Grossi-de-Sa and O.L. Franco, 2011. Plant storage proteins with antimicrobial activity: novel insights into plant defense mechanisms. *FASEB J.*, 25: 3290–3305

Crouch, M.L., K.M. Tenbarge, A.E. Simon and R. Ferl, 1983. cDNA clones for *Brassica napus* seed storage proteins: evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. *J. Mol. Appl. Genet.*, 2: 273–283

Dasgupta, J., S. Dasgupta, S. Ghosh, B. Roy and R.K. Mandal, 1995. Deduced amino acid sequence of 2S storage protein from *Brassica* species and their conserved structural features. *Ind. J. Biochem. Biophys.*, 32: 378–384

Faraz, A., M. Faizan, F. Sami, H. Siddiqui and S. Hayat, 2019. Supplementation of salicylic acid and citric acid for alleviation of cadmium toxicity to *Brassica juncea*. *J. Plant Growth Regul*., https://doi.org/10.1007/s00344-019-10007-0

Gressent, F., G. Duport and I. Rahioui, 2007. Biological activity and binding site characteristics of the PA1b Entomotoxin on insects from different orders. *J. Insect Sci.*, 7: 1–10

Gressent, F., I. Rahioui and Y. Rahbé, 2003. Characterization of a high-affinity binding site for the pea albumin 1b entomotoxin in the weevil *Sitophilus*. *Eur. J. Biochem.*, 270: 2429–2435

Guex, N. and M.C. Peitsch, 1997. SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis*, 18: 2714–2723

Jouvensal, L., L. Quillien, E. Ferrasson, Y. Rahbe, J. Gueguen and F. Vovelle, 2003. PA1b, an insecticidal protein extracted from pea seeds (*Pisum sativum*): 1H-2-D NMR study and molecular modeling. *Biochemistry*, 42: 11915–11923

Jyothi, T.C., S. Sinha, S.A. Singh, A. Surolia and A.A. Rao, 2007. Napin from *Brassica juncea*: Thermodynamic and structural analysis of stability. *Biochim. Biophy. Acta Prot. Proteom.*, 1774: 907–919

Kinnunen, T., C. Buhot, A. Närvänen, M. Rytkönen-Nissinen, S. Saarelainen, S. Pouvelle-Moratille, J. Rautiainen, A. Taivainen, B. Maillere, R. Mäntyjärvi and T. Virtanen, 2003. The immunodominant epitope of lipocalin allergen Bos d 2 is suboptimal for human T cells. *Eur. J. Immunol.*, 33: 1717–1726

Krebbers, E., L. Herdies, A.D. Clercq, J. Seurinck, J. Leemans, J.V. Damme, M. Segura, G. Gheysen, M.V. Montagu and J. Vandekerckhove, 1998. Determination of the processing sites of an *Arabidopsis* 2S albumin and characterization of the complete gene family. *Plant Physiol*., 87: 859–866

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685

Laskowski, R.A., M.W. MacArthur and J.M. Hornton, 2001. *PROCHECK: Validation of Protein Structure Coordinates*, pp: 722–725. International Tables of Crystallography, Vol. F. Crystallography of Biological Macromolecules. Kluwer Academic Publishers, The Netherlands

Lin, P. and T.B. Ng, 2008. A novel and exploitable antifungal peptide from kale (*Brassica alboglabra*) seeds. *Peptides*, 29: 1664–1671

Maria-Neto, S., R.V. Honorato, F.T. Costa, R.G. Almeida, D.S. Amaro, J.T. Oliveira, I.M. Vasconcelos and O.L. Franco, 2011. Bactericidal activity identified in 2S albumin from sesame seeds and *in silico* studies of structure–function relations. *Prot. J.*, 30: 340–350

Monsalve, R.I., M.G.D.L. Pena, L. Menendez-Arias, C. Lopez-Otin, M. Villalba and R. Rodriguez, 1993. Characterization of a new oriental-mustard (*Brassica juncea*) allergen, Bra j IE: detection of an allergenic epitope. *Biochem. J.*, 293: 625–632

Muench, S.P., S. Rawson, V. Eyraud, A.F. Delmas, P.D. Silva, C. Phillips, J. Trinick, M.A. Harrison, F. Gressent and M. Huss, 2014. PA1b inhibitor binding to subunits C and E of the vacuolar ATPase reveals its insecticidal mechanism. *J. Biol. Chem.*, 289: 16399–16408

Müntz, K., 1998. Deposition of storage proteins. *Plant Mol. Biol.*, 38: 77–99

Neumann, G.M., R. Condron, I. Thomas and G.M. Polya, 1996a. Purification and sequencing of multiple forms of *Brassica napus* seed napin small chains that are calmodulin antagonists and substrates for plant calcium-dependent protein kinase. *Biochim. Biophys. Acta Prot. Struct. Mol. Enzymol.*, 1295: 23–33

Neumann, G.M., R. Condron and G.M. Polya, 1996b. Purification and sequencing of yellow mustard seed napin small and large chains that are phosphorylated by plant calcium-dependent protein kinase and are calmodulin antagonists. *Plant Sci.*, 119: 49–66

Pantoja-Uceda, D., O. Palomares, M. Bruix, M. Villalba, R. Rodríguez, M. Rico and J. Santoro, 2004. Solution structure and stability against digestion of rproBnIb, a recombinant 2S albumin from rapeseed: relationship to its allergenic properties. *Biochemistry*, 43: 16036–16045

Pelegrini, P.B., E.F. Noronha, M.A. Muniz, I.M. Vasconcelos, M.D. Chiarello, J.T. Oliveira and O.L. Franco, 2006. An antifungal peptide from passion fruit (*Passiflora edulis*) seeds with similarities to 2S albumin proteins. *Biochim. Biophys. Acta*, 1764: 1141–1146

Pettersen, E.F., T.D. Goddard and C.C. Huang, 2004. UCSF Chimera-a visualization system for exploratory research and analysis. *J. Comput. Chem.*, 25: 1605–1612

Perera, S.P., T. McIntosh and J.P.D. Wanasundara, 2016. Structural properties of cruciferin and napin of *Brassica napus* (canola) show distinct responses to changes in pH and temperature. *Plants*, 5: 36–60

Rahioui, I., C. Laugier and S. Balmand, 2007. Toxicity, binding and internalization of the pea-A1b entomotoxin in Sf9 cells. *Biochimie*, 89: 1539–1543

Ribeiro, S.F.F., A.O. Carvalho and M.D. Cunha, 2007. Isolation and characterization of novel peptides from chilli pepper seeds: Antimicrobial activities against pathogenic yeasts. *Toxicon*, 50: 600–611

Rico, M., M. Bruix, C. González, R.I. Monsalve and R. Rodríguez, 1996. 1H NMR assignment and global fold of napin BnIb, a representative 2S albumin seed protein. *Biochemistry*, 35: 15672–15682

Sambrook, J. and D.W. Russell, 2001. *Molecular Cloning*: *A Laboratory Manual*. Cold Spring Harb. Lab. Press, Cold Spring Harbor, New York, USA

Sharma, A., P. Kumar, P. Kesari, M. Katiki, M. Mishra, P.K. Singh, B.R Gurjar, A.K. Sharma, S. Tomar and P. Kumar, 2017. Purification and characterization of 2S albumin from seeds of *Wrightia tinctoria* exhibiting antibacterial and DNase activity. *Prot. Pept. Lett.*, 24: 368–378

Terras, F.R., H.M. Schoofs, K. Thevissen, R.W. Osborn, J. Vanderleyden, B.P. Cammue and W.F. Broekaert, 1993. Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. *Plant Physiol.*, 103: 1311–1319

Terras, F.R., H.M. Schoofs, M.F.D. Bolle, F.V. Leuven, S.B. Rees, J. Vanderleyden, B.P. Cammue and W.F. Broekaert, 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.*, 267: 15301–15309

Wang, X., G.J. Bunkers, M.R. Walters and R.S. Thoma, 2001. Purification and characterization of three antifungal proteins from cheeseweed (*Malva parviflora*). *Biochem. Biophys. Res. Commun.*, 282: 1224–1228

Ye, X.Y. and T.B. Ng, 2002. Isolation of a new cyclophilin-like protein from chickpeas with mitogenic, antifungal and anti-HIV-1 reverse transcriptase activities. *Life Sci.*, 70: 1129–1138

Yu, J.C., Z.T. Jiang, R. Li and S.M. Chan, 2003. Chemical composition of the essential oils of *Brassica juncea* (L) coss grown in different regions, Hebei, Shaanxi and Shandong of China. *J. Food Drug Anal.*, 11: 22–26

Zheng, X.Y. and J.B. Sinclair, 2000. The effects of traits of *Bacillus megaterium* on seed and root colonization and their correlation with the suppression of *Rhizoctonia* root rot of soybean. *Biocontrol*, 45: 223–243

[Received 05 Jul 2019; Accepted 26 Aug 2019; Published (online) 22 Dec 2019]