Pak. J. Bot., 52(2): 515-518, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-2(41)

GENE CLONING, RECOMBINANT EXPRESSION AND PURIFICATION OF A RICE GERMIN LIKE PROTEIN (*OS*RGLP1)

SYED IRTIZA HUSSAIN SHAH GARDEZI[1], DURE SHAHWAR[1], ZAIGHAM ABBAS[1] AND SYED MUHAMMAD SAQLAN NAQVI[1, 2*]

[1]Department of Biochemistry, PMAS Arid Agriculture University Rawalpindi, Pakistan
[2]Bacha Khan University, Charsadda, Khyber Pakhtunkhwa, Pakistan
**Corresponding author's email: saqlan@uaar.edu.pk*

Abstract

Germin-like proteins (GLPs) are plant glycoproteins, which are included in cupin superfamily. These proteins show high similarity in sequence but diversity in function and are reportedly involved in conferring biotic and/or abiotic stress tolerance to plants. In order to fully characterize the protein and establish structure-function relationship, it is important to obtain purified protein. Here we report the molecular cloning and recombinant expression of a rice germin like protein 1 (*Os*RGLP1) in *E. coli*. Expression vectors pET20b and pET14b carrying C-terminus His tag were used for cloning, optimal expression, and subsequent purification. *Os*RGLP1 expression was confirmed through western blotting using anti-His monoclonal primary antibody. *Os*RGLP1 was further purified using Immobilized metal affinity chromatography (IMAC) and size exclusion chromatography. The recombinant protein will be useful for characterizing the protein and raising antibodies for further applications.

Key words: Germin-like proteins, Molecular cloning, Western blotting, IMAC, Size exclusion chromatography.

Introduction

Germin and germin like proteins (GLPs) make a large family of proteins. Around twenty five members of proteins are being expressed in almost all higher plants. Germin like proteins (GLPs) are glycoproteins which belong to cupin protein superfamily. The proteins contain oxalate oxidase and super oxide dismutase (SOD) activity. Although these proteins exhibit diverse functioning in different plant species but they also play important role of disease resistance against some pathogens. GLPs are reported to express in stress conditions either biotic or abiotic (Dunwell *et al*., 2008).

The proposed mechanism by which GLPs provide broad spectrum disease resistance relates to their SOD activity and localization in plant cell walls (Wei *et al*., 1998; Membre *et. al*., 2000; Christensen *et al*., 2004). Due to the SOD activity GLPs convert the superoxide anion into H_2O_2 which is the main player in early plant defense and has a role in cell wall reinforcement and papillae formation (Wojtaszek *et al*., 1997; Schweizer *et al*., 1999).

The most of the germins and GLPs are comprised of 220 amino acid residues. Both germins and GLPs show some conserved features like signal peptide and three conserved regions. Amino acids in conserved regions constitute the putative site for metal ion binding and glycosylation. In fact metal ion and glycan moiety have been observed in many germins and GLPs activity (Bernier & Berna, 2001).

Germin like proteins are synthesized as pre-proteins which contain the signal peptide sequence by which they expel out from the cell. On the other hand mature GLPs contain atleast one or two N-glycosylation sites and metal ion binding sites at conserved positions. The presence of the motif derived from the cupin superfamily is the important feature of the germins and germin like proteins. GLPs play a role in broad spectrum disease resistance as they function as a quantitative trait loci (QTL) (Banerjee & Maiti, 2010).

*Os*RGLP1 is a rice root expressed germin-like protein gene originally amplified from *Oryza sativa.*and expressed in transgenic tobacco (Yasmin, 2009). Most of the characteristics of this protein are either based on studies of barley purified germin or bioinformatics analyses, thus there was a need to obtain pure protein preparation for reliable characterizationn. Accordingly, the current study was designed to express the *Os*RGLP1 in prokaryotic system, which could be useful in production of larger quantities of protein sufficient for characterization and antibody production.

Materials and Methods

Source material: Transgenic tobacco (*Nicotiana tobaccum cv* Samsun) plants expressing *Os*RGLP1 were obtained from Plant Biotechnology LAB 1, Department of Biochemistry PMAS-AAUR and were used as a source of *Os*RGLP1 gene isolation.

Amplification and cloning of *Os*RGLP1 gene: Total DNA of tobacco plants was extracted by Cetyltrimethyl ethyl ammonium bromide (CTAB) method (Richards, 1997). The isolated DNA was used as a template in PCR reaction. The Primer pair (Forward: 5′AAGGCC ATGGCTTCGGATC 3′) and (Reverse: 5′GTCCTCG AGGTAATGGTTGTTCTCCCAG 3′) specific for *Os*RGLP1 gene were designed (www.ncbi.nlm.nih.gov/ primerBLAST) containing *NcoI* and *XhoI* restriction sites at 5′end of forward and reverse primers respectively. The synthesized primers were used to amplify the *Os*RGLP1 gene fragment (New England Biolabs). The PCR thermal profile consisted of 98°C for 30 seconds followed by 35 cycles each of denaturation at 95°C for 10 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 20 seconds, and a final extension at 72°C for 2 minutes. The PCR products were confirmed by running on 1% agarose gel.

The PCR products were purified (peqGOLD gel extraction kit, Peqlab) and ligated with pGEM-T Vector Systems, Promega. The transformed colonies were selected on LB agar media containing 50 µg/ml ampicillin. Selected colonies were used to isolate the plasmid (plasmid mini prep kit, Promaga). The isolated plasmids were subjected to PCR amplification with gene specific primers set and sent for sequencing to confirm the gene of interest (Macrogen).

The gene of interest was amplified with the above mentioned primers. The amplified product and pET20b expression vector were digested with *NcoI* and *XhoI* restriction enzymes. The reaction products were run on 1% agarose and desired band of both *Os*RGLP1 and expression vector was excised under UV light and purified by gel extraction kit. The digested pET20b and *Os*RGLP1 fragments were ligated and recombinant pET20b was transformed into DH5α *E.coli* competent cells by means of chemical transformation. Colonies were selected on LB media containing 50 µg/ml ampicillin. The selected colonies were used to isolate plasmid by miniprep and confirmed by PCR amplification with gene specific primers and restriction digestion with *NcoI* and *XhoI* enzymes.

The recombinant pET20b vector containing gene of interest and pET14b was digested with *Nco*I and *Blp*I enzymes. N-terminal His tag from pET14b was also removed by digesting with *Nco*I and *Blp*I restriction enzymes that may interfere with subsequent experiments of protein expression and its proper folding. The recombinant pET14b was cloned by the same process as in case of pET20b.

Expression and purification of *os*RGLP1: The recombinant pET20b was transformed into BL21 *E.coli* chemical competent cells. The transformed colonies were selected on selective LB media containing 50 µg/ml ampicillin. Miniprep was performed and the orientation of the cDNA in expression vector was verified with the help of restriction digestion with *Nco*I and *Xho*I restriction enzymes. After the confirmation of transformants, 500 µl of cultured cells were transferred to 500 mL LB media and incubated at 37°C with shaking at 250 rpm until the OD_{600} of 0.5 was achieved. Cells were induced with different concentrations of IPTG i.e. 0, 0.2, 0.4, 0.6, 0.8 and 1 mM. The cells were allowed to grow overnight at 25°C with shaking at 250 rpm. The cells were harvested and protein expression in cell pellet was observed using 12 % SDS-PAGE. Protein of interest was confirmed through western blotting by Anti-His monoclonal antibody (Novagen). The protein was purified by immobilized metal affinity chromatography (IMAC) and size exclusion chromatography.

Results and Discussion

The *Os*RGLP1 gene was amplified with gene specific primers containing *NcoI* and *XhoI* restriction sites, and the resultant product revealed 627 bp product which was purified (Fig. 1a). The purified product was ligated in to pGEMT vector, cloned and sequenced. The restriction digestion of amplified product and native pET20b vector was performed. The digested pET20b and *Os*RGLP1gene was ligated, cloned and confirmed by digestion with *NcoI* and *XhoI* restriction enzymes (Fig. 1b).

The predicted hexameric structure of *Os*RGLP1 revealed that the N-terminus of all monomers lies inside the hexamer so any tag at N-terminus would interfere with the hexameric structure of the protein (Fig. 2). For purification of *Os*RGLP1 with intact hexameric structure, it was desirable to have His-tag at the C-terminus of recombinant protein. For this purpose we selected pET20b expression vector which has pelB signal peptide for potential periplasmic localization and C-terminus His tag.

PET20b/OsRGLP1 vector was transformed into BL21 DE3 strain of *E. coli* and induction was optimized by using different concentrations of ITPG and not a big difference was observed in the protein expression with 0, 0.2, 0.4, 0.6, 0.8 and 1Mm IPTG concentration. The induced fraction was run on 12 % SDS-PAGE which confirmed the presence of 26 kDa fragment size corresponding to induced recombinant protein of interest (Fig. 3a). The binding of anti-His antibody to desired protein band in western blot is a confirmation of presence of recombinant *Os*RGLP1 protein in the target band (Fig. 3b).

Transformed cell culture was subjected to osmotic shock to release periplasmic proteins including *Os*RGLP1 due to pelB signal sequence at its N-terminus. Immobilized metal affinity chromatography (IMAC) was used to purify the protein having His-tag at its C-terminus. After IMAC, the fractions containing proteins were run on 12% SDS PAGE that showed the presence of two bands of approximately 26 kDa and 46 kDa (Fig. 4a). Protein samples were concentrated using Vivaspin concentrators and subjected to size exclusion chromatography that separated these bands on the basis of their size in two different fractions that were run on 12% SDS PAGE (Fig. 4b).

The amount of protein that comes in soluble fraction was insufficient to be used for characterization experiments which may be due to the presence of pelB signal sequence at N-terminus that may hinder its proper folding. To avoid this possible issue *Os*RGLP1 was again expressed in BL21 cells without any tag/signal sequence at its N-terminus. pET14b expression vector was used for this purpose.

After the expression of protein in pET14b, it was observed that protein was precipitated as inclusion bodies as insoluble fraction (Fig. 5). In order to extract the protein from insoluble fraction, cell pellet was resuspended in 8 M urea and supernatant obtained after centrifugation was purified using IMAC and run on 12% SDS PAGE. Results indicated that pET14b is more suitable to express this recombinant protein.

Similar study was conducted by Casslanda *et al*., (2004) in which they expressed one oxalate oxidase encoding cDNA from barley and two from wheat. They observed most of the protein in insoluble fraction. We are in agreement with their results because most of our protein was in insoluble fraction.

The method described in current study elaborated the expression of *Os*RGLP1 at large scale which would be helpful in characterization of this protein including secondary structure analysis, heat stability, enzyme kinetics, metal ion analysis and study of three dimensional structure. As this protein shows SOD activity and is involved in plant defense so the mutagenic analysis of the expressed protein would also be useful in enhancing its activity in plant. The expressed protein would also be useful in raising antibodies in model animals.

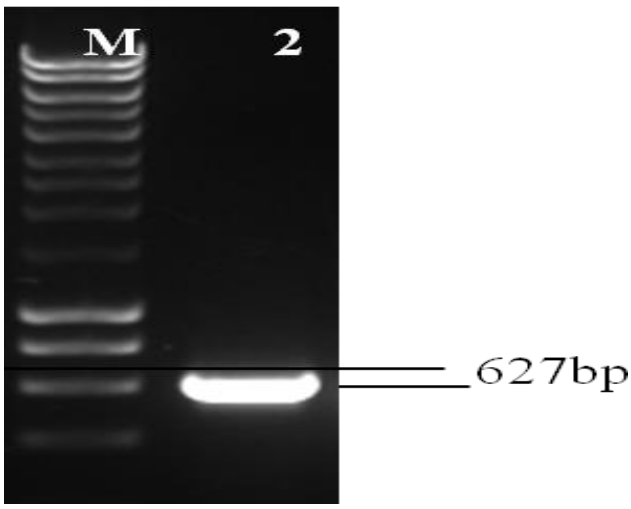

Fig. 1a. PCR amplification and purification of PCR Product.

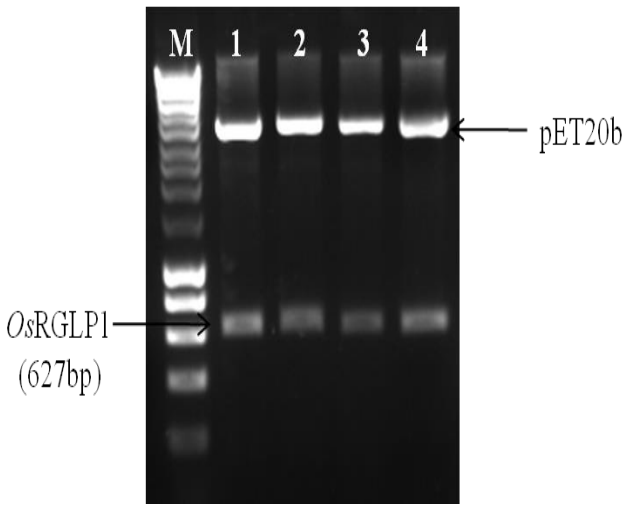

Fig. 1b. Confirmation of pET20b:*Os*RGLP1 isolated from *E.coli* (DH5 α) through Restriction Digestion by *Nco*I and *Xho*I.

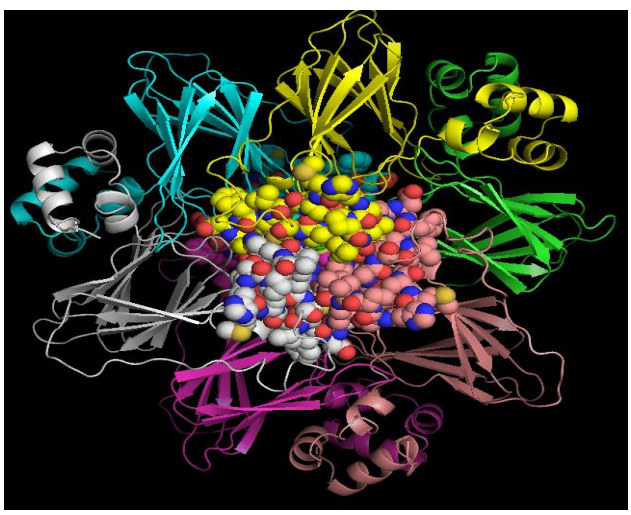

Fig. 2. Predicted Hexameric Structure of *Os*RGLP1 showing ribbon presentation of hexamers. Each monomer is in different color. N-terminus shown in ball presentation lies in the centre of hexamer.

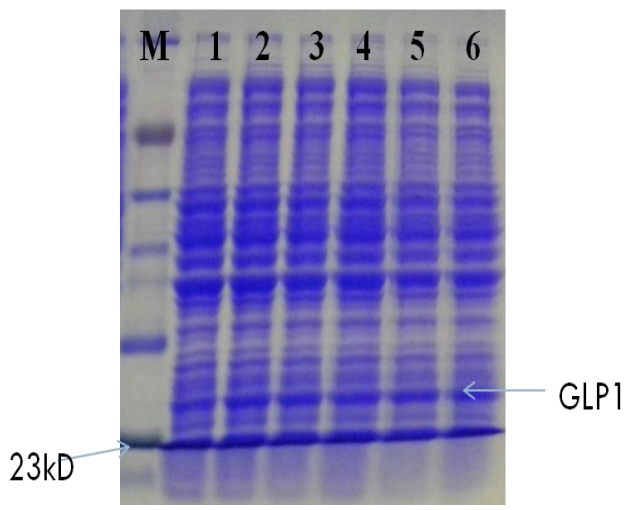

Fig. 3a. 12%SDS-PAGE of the induced cells with different IPTG concentrations. The size of 26 kDa protein of interest is shown. Lane M: pre-stained protein marker. Lane 1-6: whole cell pellet induced with 0, 0.2, 0.4, 0.6, 0.8 and 1 mM IPTG concentrations.

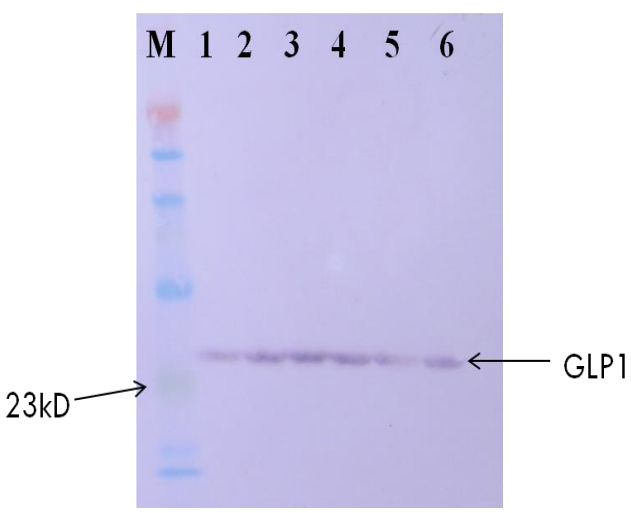

Fig. 3b. Confirmation of expression of *Os*RGLP1 using anti-His monoclonal primary antibody. Lane M: pre-stained protein marker.

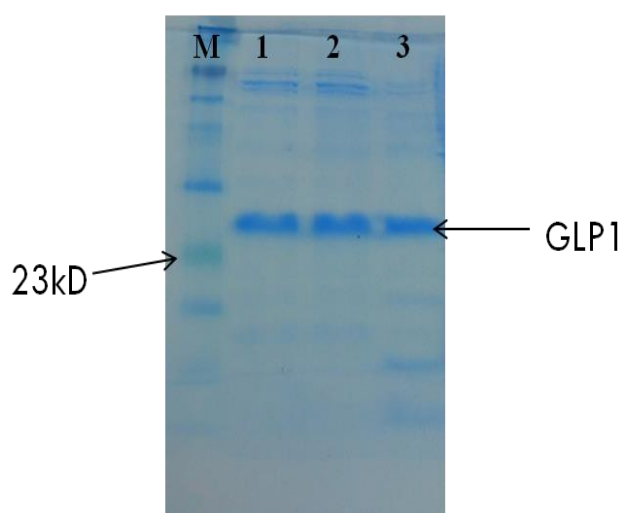

Fig. 4a. Partial purification of *Os*RGLP1 protein using affinity chromatography. Lane M contain pre-stained protein marker. Lane 1-3 show partially purified protein after affinity chromatography.

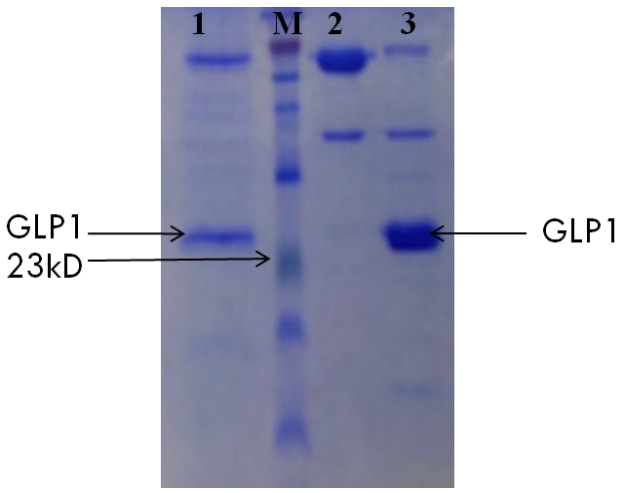

Fig. 4b. Purification of *Os*RGLP1 protein using size exclusion chromatography. Lane 1 is fraction after anion exchange chromatography. Lane M contain pre-stained protein marker. Lane 2-3 is concentrated fractions after size exclusion chromatography.

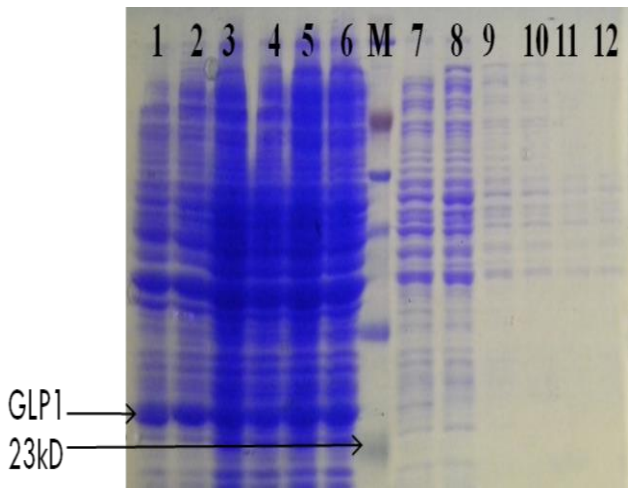

Fig. 5. 12% SDS-PAGE of supernatant and pallet fractions after sonication (cell lysis). Lane 1-6 contain supernatant while lane 7-12 contains protein from pallet fractions. Lane M is protein marker.

Acknowledgement

Authors are thankful to higher education commission (HEC) Pakistan for providing funds for this study.

References

Banerjee, J. and M.K. Maiti. 2010. Functional role of rice germin like protein 1 in regulation of plant height and disease resistance. *Biochem. Biophys. Res. Comm.,* 394: 178-83.

Bernier, F. and A. Berna. 2001. Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly? *Plant Physiol. Biochem.,* 39: 545-554.

Casslanda, P, S. Larssonb, N. Nilvebrantb and L.J. Jönsson. 2004. Heterologous expression of barley and wheat oxalate oxidase in an *E.coli* trxBgor double mutant. *J. Biotechnol.,* 109: 53-62.

Christensen, A.B., H. Thordal-Christensen, G. Zimmermann, T. Gjetting, M.F. Lyngkjaer, R. Dudler and P. Schweizer. 2004. The Germin-like protein GLP4 exhibits superoxide dismutase activity and is an important component of quantitative resistance in wheat and barley. *Mol. Plant Microbe Interact.,* 17: 109-117.

Dunwell, J., J.G. Gibbings, T. Mahmood and S.M.S. Naqvi. 2008. Germin and Germin-like Proteins: Evolution, Structure, and Function. *Crit. Rev. Plant Sci.,* 27: 342-375.

Membre, N., F. Bernier, D. Straiger and A. Berna. 2000. *Arabidopsis thaliana* germin like proteins: Common and specific features point to a variety of functions. *Planta,* 211: 345-354.

Schweizer, P., A. Christoffel and R. Dudler. 1999. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease. *Plant J.,* 20: 541-552.

Wei, Y., Z. Zhang, C. Andersen, E. Schmelzer, P.L. Gregersen, D.B. Collinge, V. Smedegaard-Petersen and H. Thordal-Christensen. 1998. An epidermis/papilla-specific oxalate oxidase-like protein in the defence response of barley attacked by the powdery mildew fungus. *Plant Mol. Biol.,* 361: 01-112.

Wojtaszek, P., M. Stobiecki and G.P. Bolwell. 1997. Changes in the composition of exocellular proteins of suspension-cultured *Lupinusalbus* cells in response to fungal elicitors or $CuCl_2$. *J. Exp. Bot.,* 48: 2015-2021.

Yasmin, T. 2009. Cloning and over-expression of a germin-like protein gene for its functional analysis. (Unpublished) Ph.D. thesis, Univ. Rawalpindi, p. 24-27.

(Received for publication 28 August 2018)