Cloning and characterization of β-1, 3 Glucanase from blight resistant and susceptible potato (Solanum tuberosum L.) cultivars

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Abstract: Phytophthora infestans is the etiologic agent of late blight, a major disease that has a significant impact on global production of potato (Solanum tuberosum L.). The pathogen attacks tubers and foliage during any stage of plant development. In this study, RT-PCR was used to qualitatively analyze different potato cultivars for their overproducing ability of the pathogenesis-related (PR) protein, β-1,3-glucanase (Stgln), after exposing to P. infestans. Fungal assay revealed that cultivar Hanna was more resistant to the fungal attack than Lady Rosetta under the same experimental conditions. In addition, the Stgln-specific transcripts were significantly higher in Hanna when compared to Lady Rosetta. The full length cDNA encoding for Stgln-H and Stgln-L were cloned from Hanna and Lady Rosetta, respectively, and their nucleotide sequences were determined. The cDNA of Stgln-L and Stgln-H have open reading frame that extend to 1011 and 1014 bp, respectively. The encoding polypeptides for Stgln-L and Stgln-H composed of 337 and 338 amino acid residues with calculated Mr of 37.5 and 37.7, respectively. The deduced amino acid sequence of Stgln-H and Stgln-L shared 93% identity with the main differences located around amino acid residues 207 to 212 and 331 to 334. Stgln-H was expressed in E. coli and purified to homogeneity. In an in vitro assay, the recombinant Stgln-H and the highly purified enzyme produce direct inhibitory effect on the germination of sporangia of P. infestans.

Keywords: Potato (Solanum tuberosum), β 1,3-glucanase, phytophthora infestans

INTRODUCTION

The glucan endo-1, 3-β-D-glucosidases, classified as endo-1,3-β-glucanases (EC 3.2.1.58) and exo-1,3-β-glucanases (EC 3.2.1.58) are widely distributed among higher plants, fungi and bacteria. This class of enzyme catalyses the hydrolysis of 1,3-β-D-glucosidic linkages in 1,3-β-D-glucan which is the main constituent of the fungal cell walls and a major structural and storage polysaccharide (laminarin) of marine macro algae. In plants, it may be involved in the defense mechanism against pathogenic fungi through its ability to degrade fungal cell walls[6,10,29]. The 1,3-β-Glucanase has several potential applications in biotechnology including its utilization in the production of yeast extract[22], and exhibits antifungal activity for disease protection in plants[7,16,29]. Also, soluble 1,3-β-glucan act as immunoactivators[17,19]. The β-Glucanases are characterized by molecular mass of around 35 KDa and numerous isoforms that differ in their enzymatic activity, structural properties, cellular localization (vacular or apoplastic) and their regulation patterns[1]. β-1,3-Glucanases responded synergistically in vitro and as inhibitor of fungal growth[20]. β-1, they are believed to contribute to plant defense in two ways; responding directly by degrading the hyphal walls of the pathogen and rendering them susceptible to lyses or indirectly, by releasing oligosaccharide elicitors from the fungal cell wall which are detected by the plant host and cause an accumulation of phytoalexin[15,28]. The accumulation of phytoalexins may lead to the resistance following the infection by pathogens[12]. Phytophthora infestans (Mont.) de Bary is the causal agent of potato late blight (LB); one of the most devastating diseases on potatoes[9]. Indeed, both enzymes (glucanase and chitinase) have the capability to degrade the isolated fungal cell walls and it has been demonstrated that the 1,3-glucanase can effectively restrict growth of different fungi when tested in vitro[4,16,27]. For these reasons, considerable effort has been aimed at isolating and characterizing these plant hydrolases to evaluate their potential for improving disease resistance of plants against fungi, or fungi-like protists such as Phytophthora, which lack chitin in their cell walls. Two forms of genetic resistance to Phytophthora infestans in potato species have been described; either race-specific (vertical resistance) or race non-specific either horizontal or field resistance[24].

In this study, the full length β-1,3-glucanase-encoding gene from the late blight resistant and
susceptible potato cultivars Hanna (H) and Lady Rosetta (L), respectively, was cloned and expressed. Further, the β-1,3-glucanase gene of cultivar Hanna was expressed in E. coli system and its antifungal effects of the purified recombinant glucanase against P. infestans was evaluated.

**MATERIALS AND METHODS**

**Potato Manipulation and Plantation:** Potato cultivars Hanna (H) and Lady Rosetta (L), used in this study were obtained from the International Potato Center (CIP), Kafr El-Zayat, Egypt. Potato plants were generated from high quality potato seed tubers and grown in clay pots containing mixtures of soil: sand: peat moth at a ratio of 4:4:1, respectively. Potato plants were kept in a growth chamber at 18°C for 16h photoperiod for 6 weeks prior to inoculation.

**Fungal Isolation and Pathogenicity Test:** P. infestans was isolated from the diseased leaves by the direct transfer of sporangia to Rye agar medium (60g of rye grain, 20g sucrose and 20g Bacto agar dissolved in 1 liter). Diseased leaves with actively progressing lesions were incubated at 18°C in a moist chamber overnight to encourage sporulation. Next day sporangia were transferred to Petri-dishes containing Rye agar medium by using the point of a wedge of agar medium on the tip of a scalpel to collect few sporangia. Care was taken to avoid contact with septic surfaces of the plant tissues and Plates were incubated at 18°C in the dark. Colonies of P. infestans arising after 7 days were then sub-cultured on Rye agar medium. The virulence property of P. infestans was maintained by successive inoculations on potato leaves and re-isolations every 3–4 months. For pathogenicity, sporangia suspensions were collected from 10–14-day-old P. infestans cultures by washing culture plates with 10 ml sterilized distilled water and harvesting sporangia the second day by flooding the plates with 10 ml dH₂O. For the inoculation, 100 µl of sporangia suspension (5x10⁵ sporangia/ml) was deposited on the primary leaflet of the fourth full grown potato leaf as multiple tiny droplets using a micropipette to prevent the inoculum from running off. After inoculation, potato plants were kept at 100% humidity for 48 h to maintain the humidity required for successful infection. The inoculation was done in three replicates pots. Every pot, with two stems each, was considered as one replicate and received one of the treatments and every treatment was sampled at different time points (0, 6, 12, 24, 48, and 72h).

**Sampling Procedures:** Potato leaflets were collected from three leaf strata including inoculated leaflets (local, L), un-inoculated leaflets in the same leaf where P. infestans was inoculated (proximal, P) and un-inoculated leaflets from the leaf adjacent to the inoculated leaf (distal, D). Samples were collected at five different time points (0, 12, 24, 48 and 72 h). RNA isolated from the potato plants (cv Lady Rosetta and Hanna) that were infected with *Phytophthora infestans*. As a control; the terminal leaflet of the fourth full grown leaf from an individual healthy potato plant was collected at each time point. The harvested leaf samples were ground to a fine powder in liquid nitrogen immediately after the sampling and then they were stored at -80°C until used for RNA extraction.

**Preparation of Total RNA:** Total RNA was extracted using SV Total RNA isolation system (Promega, Cat. No. Z3100). The UV absorbance at 260 nm and the A260/A230 ratio were used to determine the concentration and purity of the tested RNA samples. The integrity of total RNA was further confirmed by formaldehyde-agarose electrophoresis. Leaf samples collected from the two separate sets of inoculations were used separately for RNA extractions.

**RT-PCR:** RT-PCR was performed using OneStep RT-PCR Kit (QIAGEN Cat. No. 210212). The message for GLU gene was amplified using two pairs of primers Stgln F GGA TCCATGGCTTTTCTAAG TTCTCTGTAGTC and Stgln R CTCGAGTTAA TGAAAATGAGTTGATACCTTT. RT-PCR amplification was carried out in 5x QIAGEN OneStep RT-PCR reaction buffers containing 50 pmol of each primer, 250µM dNTPs, and 5 units QIAGEN OneStep RT-PCR Enzyme Mix (AMV reverse transcriptase, and hot start Taq DNA polymerase). Total RNA isolated from the two potato cultivars (L, H) was used as a template to detect the induction for the messages of GLN gene. The RT-PCR profile was as follows, 1 cycle of 50°C for 30min, for first strand syntheses then for activation of hotstart Taq polymerase initial denaturation step of 95°C for 15 min, then 40 cycles of 94°C, 55°C and 72°C for 40, 50 and 60 sec, respectively followed by final extension step at 72 for 10 min. Ten µl from each sample was analyzed in 1.5% agarose gel.

**Cloning of PCR Fragments:** The purification of PCR products have been carried out using high pure PCR purification system. Ligation reaction consisted of: 1µl 10x ligation buffer, 2µl PCR vector (50 ng), 1µl T4 DNA ligase, 1 µg purified PCR product and 5d H₂O up to 10 µl. the reaction was mixed briefly and spined down to collect the contents in the tube, then incubated at 14°C for 16hr.
Plasmid DNA Preparation and Digestion: A pGEM-Teasy was used as the cloning vector for cloning the different PCR products generated during this study. Plasmid DNA was prepared from E. coli cells, using Wizard plus DNA purification system. One microgram of the plasmids was digested using EcoRI restriction enzymes in 20 ml volume with EcoRI buffer. Digestion reaction was incubated at 37°C for 2 hr.

Construction of Recombinant Protein, Expression in E. coli and SDS-PAGE: The expression of the recombinant StGLN protein was performed using GST Gene fusion system (Amersham Pharmacia Biotech). The coding region of the StGLN1 mature protein was amplified by a pair of primers, StGLN Forward; and StGLN Reverse. After PCR amplification, the DNA fragments were digested with BamHI and XhoI restriction enzymes and they were ligated into the BamHI and XhoI sites of pGEX4T-1 to produce pGST-StGLN1 vector. The vector pGST-StGLN was transformed into E. coli strain BL-21 (DE3) cells and was grown in LBA (Luria broth supplemented with ampicillin) at 37°C. One microgram of the plasmid DNA was added to fifty ml PCR reaction mix containing 250 mm each dNTPs, 25 pmol each primer and 1m Taq DNA Polymerase. The conditions for the PCR assay was; 94°C/3 min, 94°C/30 sec, 55°C/30sec and 72°C/30 sec. Five ml of overnight culture was diluted into 500 ml of LBA and was grown at 37°C for 2 hr. then 0.1 mM IPTG was added to induce the GST-St-glu fusion protein. Four hr post incubation at same temperature; cells were harvested by centrifugation and they were sonicated in phosphate-buffered saline (PBS) on ice. Celluar debris was removed by centrifugation and the supernatant was loaded on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). After extensively washing the column with PBS, the bound GST-recombinant protein was eluted with 100 mM Tris-HCl (pH 8.9) containing 10 mM glutathione according to the manufacturer’s instruction. Protein analysis was carried out by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoreses (PAGE) with 12 % (w/v) gels[19]. Protein concentrations were determined according to the methods of Bradford[20], using coomassie protein assay reagent (Pierce, Rockford) and Bovine serum albumin was used as a standard.

DNA Sequencing: DNA sequencing was carried out using the automated DNA sequencing method. The automated DNA sequencing reactions was performed with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA) using the ABI PRISM (310 Genetic Analyzer).

Inhibitory Activity Assay: Total and purified proteins were sterilized by filtration through a 0.22 µm membrane filter (Millipore, Bedford, MA). The fungal growth bioassay was conducted according to the method of Ali and Reddy[21]. Sporangia were harvested from 3 weeks-old cultures of P. infestans by rinsing the plates with 10ml sterilized distilled water. The sporangial suspension concentration was estimated using hemoctytometer to 1 × 10³ sporangia ml⁻¹. Fifteen µl of sporangia suspension and 50µl of liquid pea broth media were added to each well of a sterile 96-well titer plate. Fifteen µl of the extracted proteins were applied in replicates of four wells for each treatment. Samples treated with 50 µl sterilized distilled water were served as control. Plates were covered with sterile lids and placed in a sealed polystyrene box with moistened filter paper to maintain humidity and incubated for 48hrs at 18°C.

Optical Density (OD) readings were taken at 630 nm over a 48hrs time course by Universal automated microplate reader E1, 80.

\[
\text{Fungal growth} = \frac{\text{Final OD reading} - \text{Initial OD reading (0 times)}}{\text{Initial OD reading}} \times 100
\]

RESULTS AND DISCUSSIONS

RNA isolation and RT-PCR: B-1,3-Glucanases are members of the PR protein family, which are either induced in response to pathogen attack or by releasing oligosaccharides from the fungal cell wall during invasion. Previously, it was shown that glucanases have the capability to degrade isolated fungal cell walls and can effectively restrict growth of different fungi when tested in vitro[27, 16].

Furthermore, Tonon et al. [25] reported that a purified β-1,3-glucanase from potato has a direct inhibitory effect on the germination rate of P. infestans sporangia. Total RNA was isolated from infected potato leaves ( cvs Lady Rosetta and Hanna) with phtophthora infestence (0, 12, 48, 72 hr). RT-PCR was performed using (OneStep RT-PCR Kit obtained from QIAGEN Cat. NO. 210212). The message for gln gene was amplified from both cultivars using the specific primers gln-F and gln-R at 48 and 72h post infection. Total RNA from two potato cultivars (L & H) were used as templates to detect the induction for the messages of GLU gene. Ten µl from each sample was analyzed using 1.5 % agarose gel (Fig.1).

Cloning and Sequence Analysis of β-1,3-glucanases from Two Potato Cultivars: The specific primers GLN-F and GLN-R were used in the RT-PCR amplification. Accordingly, 1.05 kb DNA segment was amplified (Fig. 1). DNA segment from susceptible and
resistant potato varieties (L & H) was cloned in pGEM-T easy vector and transformed into E. coli strain JM109 competent cells. The recombinant clones were selected based on blue/white color on X-gal/IPTG-LB plates. The white colonies were subsequently screened for recombinant plasmids using the rapid phenol/chloroform method. Ten clones were screened and four was selected based on its relative migration on the agarose gel compared to negative control (non recombinant pGEM-T easy). The selected clone was further confirmed by PCR using GLU F & R primers and EcoRI digestion (data not shown).

The complete nucleotide sequences for gluconase gene were determined, then Blasted at GenBank. The results showed that the search for this DNA segment showed closest matches for Lady Rossetta and Hanna cultivars were obtained from mRNA of beta-1, 3-glucanase (accession # AJ009932.1) Solanum tuberosum producing a similarity percentage of 98% and 96%, respectively. Sequence analyses of the potato beta-1,3-glucanase showed that this glucanase was closely related to glycosyl hydrolases family.

Stopn-L and Stgl-H have open reading frame that extend to 1011 and 1014 bp and encode polypeptides of 337 and 338 amino acid residues with calculated Mr of 37.5 and 37.7, respectively. The deduced amino acid sequence of StGLN-LR and StGLN-H shared 93% identity with the main differences located around amino acid residues 207 to 212 and 331 to 334 (Fig. 2). Based on the deduced amino acid sequence, StGLN-L is acidic (pI 6.45), whereas StGLN-H is slightly basic (pI 7.45). Both 1,3-beta-glucanase proteins have identical N-terminal signal peptide, which targets the nascent polypeptides into the endoplasmic reticulum and finally secreted into the apoplast.

**Sequence of Hanna (Resistance) and Lady Rosetta (Susceptible) Cultivars:** Many endo-1, 3-beta-glucanase genes have been isolated from potato[23] cDNA sequence has an ORF that encodes endo-1, 3-beta-glucanase protein of 314 amino acid residues (Fig. 2). Accordingly, Akiyama et al.,[3] working on rice and Hrmova & Finchert[11] working on barley obtained an endo-1,3-beta-glucanase protein of 318 amino acid. It is believed that the four amino acid residues at N-terminal of OsGLN1 protein was eliminated during processing steps to mature endo-1,3-beta-glucanase protein[21]. It is also believed that most of the potato endo-1,3-beta-glucanases proteins so far identified have an N-terminal signal peptide Tonon et al.,[23] which targets the nascent polypeptides into the endoplasmic reticulum and the mature protein is finally secreted into the apoplast. To cope with our results, Romero et al.,[3] reported the isolation of an OsGLN1 mature protein with 314 amino acid residue are 34 kDa with a PI of 8.38.

**Construction of B-1,3-glucanases in Bacterial Expression Vector:** The restriction sites BamHI and XhoI were added to the primers that were used to isolate beta-1,3-glucanase, to facilitate Ligation into the BamHI and XhoI sites of pGEX4-T-1 in order to produce pGST-StGLN1 vector using E. coli strain BL21 (DE3) and was prepared for GST-POLN1 fusion protein induction by using the IPTG. The cloned beta-1,3-glucanase gene isolated from the resistant Hanna cultivar was subcloned in the pGEX4T-1 bacterial expression vector at BamHI, XhoI sites. Recombinant plasmids were screened by PCR (Fig. 3) and restriction digestion assay (Fig. 4).

**GST-fusion and expression of StGLN1:** The endo-1, 3-beta-glucanases secreted into apoplast have been thought to be implicated in the first level of plant defense by releasing elicitors from cell walls of fungal pathogens[9]. In agreement with these observations, our experimental evidence clearly indicated that oriza sativa OsGLN1 mature protein also fall into this range. To confirm whether the StGLN1 protein has endo-1, 3-beta-glucanase activity, the mature protein of StGLN1 encoding 34-kDa was expressed in E.coli cells as GST-StGLN1 recombinant protein. The accumulation of 29-kDa GST was observed when E. coli BL21 cells containing pGEX-4T-1 without the insert was induced with IPTG (Fig. 5, lane 2). On the other hand, the induction of the E. coli cells containing the pGST-STGLN1 plasmid by IPTG resulted in the accumulation of a large amount of the 63-kDa recombinant protein in the cell soluble fraction (Fig. 5, lane 4-7).
(A) Sequence
atggcttttctaatgacctttgttacctgtaaggatttaacgaggacagttcagttaaagagctttctgttgtaagtctttttactttgctgtgcagttcgcctgcataacgagttcagtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
(B) Sequence

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TGAAQPGVCGYKIANNLPSD
caagtgtctataaaattatataatagctataaacaatcagaaactgaaatttactatcca
QDVILKNYIKKLRYY
gatacaaatgtcatttaatgccttcaaatgaagaataataattttgtatgtccca
DTNVMNALKGSNIEIIILDVYP
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FINPIIgLARHNLPLLANI
atccctattatgtcaccatagctgtgatataatgtctctctttttatgtgacttttcaagcaca
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attaactcatgtgaaagggggtgagaaaccaaaagaacctggaagaacatagaact
INHVKGCCAGTFKPKFKGKTITET
atatattccggagatgtttagaataagaagatttgaaaccaagcttgtgacacatttt
YLPAMFDENRKDGPKPSEOHPF
gggtctttttattcaccgtaagagcccacmagtacacacacacatttaa
GLFYPDKPVPKVSPTQFQL
1965
(C) Amino acid sequence alignment

H: H  
M: M

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<th>Sequence</th>
<th>Hanna cultivar (H)</th>
<th>Lady Rosetta cultivar (L)</th>
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<td>Fig. 2.</td>
<td>Nucleotide and deduced amino acid sequence of resistance and susceptible Stgln1 cDNA and alignment of both deduced amino acid sequence.</td>
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<td>(A)</td>
<td>Sequence of Hanna cultivar (H)</td>
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<tr>
<td>(B)</td>
<td>Sequence of Lady Rosetta cultivar (L)</td>
<td></td>
</tr>
<tr>
<td>(C)</td>
<td>Alignment of both deduced amino acid sequence</td>
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</tbody>
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Fig. 3: PCR screening to selected pGST-Stgln1 recombinant plasmids. Lane M, 1 Kb ladder; lanes 1 and 2 are pGEX-Stgln1; lane 3, pGEX only.

Fig. 4: Restriction digestion conformation assay of pGST-STGLN1 expression

Lanes (1 & 2): BamHI / XhoI insert 1.50k+vector 4.9k.
Lane (3 & 4): BamHI / EcoRV 700b+1.8k+3.5k
Lane (5 & 6): EcoRV / NotI 2.5k+3.5k
Lane 7: Marker (1Kb DNA ladder)

Fig. 5: Expression of GST-StGLN recombinant protein in E. coli and after purification. All protein samples were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R250. Lane 1, soluble fraction of BL21 cells containing pGEX-4T-1 without an insert minus IPTG; Lane 2, cell soluble fraction of BL21 cells containing pGEX-4T-1 without an insert plus IPTG; Lane 3, soluble fraction of BL21 cells containing pGST-StGLN1 minus IPTG; Lane 4 to 7, soluble fraction of BL21 cells containing pGST-POGLN1 plus IPTG at different time (1,2,3,4 hours); Lane P, purified pGST-StGLN1 recombinant protein; Lane M, protein molecular mass markers.
The recombinant protein was further purified from the soluble fraction by affinity chromatography on glutathione-Sepharose 4B. The GST-POGLN1 recombinant protein was eluted from the affinity column as a single band of 63 kDa by 10 mM glutathione, as judged by SDS-PAGE (Fig. 6, lane P). Same results were obtained by Akiyama et al.\(^{(1)}\) working on *Oryza sativa*. They isolated OsGLN1 recombinant protein that has a molecular weight of 34KDa. Also, Akiyama and Arumugam\(^{(2)}\) isolated the same molecular weight OsGLN1 recombinant protein.

**Antifungal Activity of the Acidic β-1,3-glucanase:**

Antifungal activity of the acidic β-1,3-glucanase using the total pGST-StGLN1 recombinant protein and purified protein b-1,3-glucanase was assayed for its antifungal activity against *P. infestans*. The degree of inhibition of sporangia germination was measured *in vitro* as described in Materials and Methods, and it was found that the germination of sporangia can be inhibited by as little as 0.3 µg/ml of b-1,3-glucanase. Results in (Table, 1) shows that the inhibition of sporangia germination was not significant between control (medium) and pGEX-4T-1 without an insert, but it was significant with the total pGST-StGLN1 recombinant protein and purified protein.

<table>
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<th>Treatments</th>
<th>*Growth rate</th>
<th>**Difference %</th>
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<tr>
<td>Control</td>
<td>24.4 a</td>
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<tr>
<td>pGEX-4T-1 without an insert</td>
<td>23.6 a</td>
<td>0.3</td>
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<td>pGST-StGLN1</td>
<td>21.5 b</td>
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<tr>
<td>Purified enzyme</td>
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</tbody>
</table>

* Mean of 5 replicates.
** Difference % compared with control.
- Values between brackets are the arcine square root of the transformation percentage of fungal growth rate.
- L.S.D.\(_{0.05}\) for interaction=1.05.
- Values followed by the same letter (s), are not significantly different at \(P<0.05\).

The direct fungicidal action reported herein suggests that this hydrolytic enzyme could act directly by inhibiting the growth of the invading fungi. The obtained result is constitute as a new finding about the antifungal activity of an acidic, extracellular isoenzyme of a β-1,3-glucanase.

**REFERENCES**


