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Cloning, Expression and Antifungal Activity of an Endochitinase Gene Derived from Barley (*Hordeum Vulgare*)

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Abstract: Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants, especially in the tropical and subtropical regions. Plants respond to fungal pathogens by producing various pathogenesis-related (PR) proteins, such as chitinases, which restrict the spread of the pathogen in incompatible interactions and allow for systemic acquired resistance. In this study, chitinase gene from barley (bec) was cloned and over-expressed in *E. coli*. Sequence analysis indicated that the *bec* gene consists of an open reading frame of 798 nucleotides, which encodes a protein of 266 amino acid residues. Amino acid sequence analysis revealed that BEC has 95% and 92% similarity with a class II chitinase from wheat (*Triticum aestivum*) and a seed chitinase from rye (*Secale cereal*e), respectively. The recombinant bec (29kDa protein) was produced as insoluble inclusion bodies and was released into active soluble form under alkaline pH and mild denaturing condition. The purified chitinase was assayed against the three economically important fungal pests, *Alternaria alternate*, the causative agent of leaf spot, *Fusarium oxysporium*, and *Fusarium solani*, the causative agents of leaf wilting and damping off diseases. BEC inhibits the growth of the three fungi at 100 µg- and 200 µg ml^{-1}. Data obtained from this study suggest that *bec*-coding sequence can be introduced into the genome of different crop plants to enhance its fungal-resistance property.

Key words: Barley endochitinase, inclusion bodies, *Hordeum vulgare*, antifungal activity.

INTRODUCTION

Fungal plant diseases are one of the major concerns to agricultural food production worldwide. Soil borne pathogenic fungi such as *Pythium, Fusarium*, *Rhizoctonia* and *Phytopthora* attack most of the economically important crop plants (either through seed root before germination or seedling after germination) resulting in loss of billions of dollars [1]. In order to protect themselves from pathogens, plants have evolved a number of defense responses that are elicited during their life cycle in response to developmental signals and pathogen attack [2, 3]. One of the best-studied plant defense responses is the expression of defense or pathogenesis-related proteins (PR) genes [4]. The best characterized genes belonging to this group are those that encode the lytic enzyme chitinase (EC 3.2.1.14), which is speculated to play a vital role in plant defense against fungal pathogens because of its ability to digest chitin, a major constituent of the cell walls of a number of plant pathogenic fungus [5,6,7]. Chitinases catalyse the hydrolytic cleavage of the β-1, 4-glycoside bond present in the biopolymer of *N*-acetylglucosamine, mainly in chitin [8]. Thus, chitinases that hydrolyze chitin-containing fungal cell walls are thought to play a major role in the defense response against phytopathogens.

Chitinases can be divided into two categories: exochitinases, demonstrating activity only for the non-reducing end of the chitin chain and endochitinases, which hydrolyse internal β-1, 4-glycoside bonds. Many plant endochitinases especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme-like activity [9, 10, 11, 12]. Chitinases have been reported from several species of plants as two isoforms, acidic and basic. The acidic and basic isoforms of chitinase are induced in plants, in response to pathogen attack and other environmental stimuli and are also expressed in certain tissues of plants during normal development [4, 5, 6, 7]. There are several reports, that chitinases isolated from monocot and dicot plants have been shown to inhibit the growth of fungi *in vitro* [13,1,14].

The fact that chitin is a structural component in cell walls of many fungi rapidly led to the proposal of chitinases as a defensive protein against pathogens. Various *in vitro* studies have demonstrated the

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inhibitory effect of chitinases against fungal growth [15, 16, 17, 18]. The purpose of this study was to clone and overexpress this barley chitinase and to evaluate its antifungal potential against major phytopathogenic fungi that attack potato.

MATERIALS AND METHODS

Bacterial Strains and Plasmids: Escherichia coli strain BL-21 (DE3) was used as host. The pET vector 19b+ (Novagen) was used for the overexpression of chitinase gene. PCR reagents, T4 DNA ligase and restriction endonuclease were purchased from New England Biolab Inc., 32Tozer Road, Beverly, MA, USA. *E. coli* cells with plasmids were grown aerobically in LB (Luria Bertani) medium or on LB agar plate at 37 °C, supplemented with Ampicillin (100ug/ ml) for the selection of transformants.

Plasmid DNA Preparation and Digestion: A pGEM-T easy was used as the cloning vector for cloning the PCR product generated during this study. Plasmid DNA was prepared from *E. coil* cells, using Wizard plus DNA purification system. One microgram of the plasmids was digested using *Nde*I and *Bam*H1 restriction enzymes in 20 ml volume with *Nde*I and *Bam*H1 buffer. Digestion reaction was incubated at 37˚C for 2 hr.

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).

Cloning and Overexpression of Chitinase Gene in E. coli: Chitinase gene corresponding with 266 amino acids was amplified using forward primer (Bec-E1) 5- CCC GGG CAT ATG ATC GAT ATG AGA TCG CTC GCG GTG GTG-3 and reverse primer (Bec-E4) 5- CAT ATG GGA TCC TTA TTA GGC GAA GGG TCT CTG GCT GTA -3. In the forward and reverse primers, the underlined sequences represent the sites of *Nde*I and *Bam*H1, respectively. The conditions for PCR were 94 °C, 1min; 51 °C, 1min; 72 °C, 1min for 30 cycles. The PCR amplified DNA fragments were purified, double digested with *Nde*I and *Bam*H1, ligated into *Nde*I-*Bam*H1 linearized pET-19b+ with the corresponding enzymes then transformed into *E. coli* BL-21 (DE3) competent cells to construct the recombinant plasmid pET-37. The clone was streaked onto LB agar plate containing 100 µg/ml ampicillin and the plate was incubated at 37 °C for 16 h. A single colony was used to inoculate 5ml LB broth containing 100 µg/ml ampicillin followed by its incubation at 37°C with vigorous agitation in a shaking incubator. One milliliter of overnight culture was used to inoculate 100 ml of LB broth containing 100 µg/ml ampicillin in a 250 ml culture flask and the culture was grown at 37°C with vigorous agitation. When cells reached an optical density (OD) 0.6 at 600 nm, Isopropyl-b-D-thiogalactopyranoside (IPTG; 0.25mM) was added. After 2h of induction at 37°C, cells were harvested by centrifugation at 3000*g* for 10 min at 4°C and frozen.

Purification of Recombinant Protein: All steps were carried out at 4°C. After two freeze–thaw cycles, cells were resuspended in lysis buffer (50mM Tris/HCl, pH 7.8, 1mM EDTA) and homogenized by sonic disruption for a total of 20 min, with pulse and interval time of 1min and 30 s, respectively for each duty cycle. The mixture was centrifuged at 13,500*g* for 10min at 4°C to remove the unbroken cells and the supernatant. The pellet containing the inclusion bodies was washed in three volumes of washing buffer (10mM Tris–HCl, pH 7.5, 300 mM NaCl, 1mM EDTA and 1% Triton X-100), kept at room temperature for 5 min, and centrifuged as above. The detergent was removed by resuspending and washing the pellet with sterile distilled water 5 times and centrifuged as above.

Gel Electrophoresis: Protein analysis was performed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein samples were reduced by boiling for 5 min in loading buffer containing 5% β- mercaptoethanol then centrifuged at 10,000 *g* for 3 min and directly loaded the gel. Protein electrophoresis was performed in vertical sub-cells (Bio-RAD, England). Slab gels, containing 10% (w/v) resolving gel and 5% stacking gel concentrations of acrylamide, were run at a constant current of 80V for 2 h. The proteins on PAGE gels were fixed in 45% methanol and 10% acetic acid in distilled water, stained in 0.25% Coomassie brilliant blue R- 250 previously dissolved in 10% acetic acid, 50% methanol and water. The gels were destained for 3 h in 5% methanol and 7% acetic acid in distilled water.

Antifungal Assays: Cylinder plate method [15] was used to make wells on medium. The mycelium of the tested fungi was inoculated in the middle of the petri plates containing potato dextrose agar (PDA). Three days after inoculation, when the colony diameter reached 3–4 cm, wells were filled with various concentrations of chitinase protein (80 µl chitinase of 100 µg and 300 µg in PBS buffer pH 7.2). The plates were further incubated at 28 °C until the mycelial growth has

enveloped the peripheral well containing the control (buffer) and had produced crescents of inhibition around the wells loaded with antifungal protein. The major phytopathogenic fungi used in this study include; *Alternaria alternata, Fusarium solani* and *Fusarium oxysporium*.

Results: In this study, barley chitinase gene was subcloned, overexpressed in *E. coli* and the purified chitinase was evaluated for *in vitro* antifungal activity against the major phytopathogenic fungi. Barley chitinase gene (798 bp) was successfully amplified from a cDNA library of barley using the forward and reverse primers (Bec-E1and Bec-E4) with *Nde*I and *Bam*HI added sites, respectively and cloned into the expression vector pET 19b. The *bec* gene was subcloned in an expression vector in order to test its chitinolytic property against many pathogenic fungi. To that end, the *bec* gene was excised from the pCR-*bec*-1 *via* *Nde*I digestion and subcloned in the *Nde*I - linearized pET-19b. The cloning vector pET19b has a unique *Bam*H1 site (nucleotide 319) and the *bec* gene has a unique *Bam*H1 at the 3`-end, which was introduced during PCR amplification with the Bec-E4 reverse primer. *Bam*HI digestion was used to study the orientation of the cloned gene. Accordingly, two clones No. 5 and 8 were shown to harbor the *bec* gene in the right orientation relative to T_7 promoter (Fig.1.). The overexpression of the recombinant plasmid (pET19b-*bec*-5) was achieved at 37 °C in LB medium with 100 µg/ml ampicillin and induced for 2 h with IPTG (0.25mM). SDS–PAGE analysis revealed the accumulation of 29-kDa chitinase in only the IPTG induced culture. The uninduced control (Fig.2 Lanes 1) did not express chitinase (Fig.2 Lanes 1–5).

The insoluble nature of the recombinant protein synthesized in *E. coli* facilitated its purification. A single centrifugation of the bacterial lysate was used to isolate the inclusion bodies in which the recombinant protein was only slightly contaminated. In order to eliminate these contaminating bacterial proteins that co-purify with the recombinant protein, inclusion bodies were washed with washing buffer containing 10mM Tris HCl pH 7.5, 300mM NaCl, 1mM EDTA and 1% Triton X-100, which was effective and yields high purity of recombinant protein. In order to solubilize the recombinant protein several buffers were used and prepared at various pH tested for their ability to solubilize the BEC and release it from the inclusion bodies. A Tris-HCL buffer at pH 10.0, with low concentration of a reducing agent such as DTT was optimum for BEC solubilization. Inclusion bodies that were prepared from the clone pET19b-*bec*-5 consist mainly of BEC among other proteins. The BEC was released from the inclusion bodies at pH 10, and the remnant insoluble fraction was collected by centrifugation and an aliquot was separated in SDS-PAGE (Fig.3)

The effectiveness of BEC gene against phytopathogenic fungi including *Rhizoctonia solanii* and *Fusarium oxysporium* (which cause plant damping off disease) and *Alternaria alternate* (which cause leaf spot) have been studied using hyphal extention-inhibition assays. The results demonstrated that BEC gene is more effective in inhibition of fungal mycelium.

Fig.4. Antifungal property of barley endochitinase (BEC) against the fungus *Fusarium solani*. A local isolate of *F. solani* was plated on PDA medium in the absence (A) or presence (B) of different concentration of purified BEC. The BEC inhibits the growth of *F. solani* as indicated by the presence of clear zones surrounding the wells, white ovals.

Antifungal assays were performed using major phytopathogenic fungi. The purified chitinase showed a broad-spectrum antifungal activity at a concentration of 100ug and 200ug against a wide range of test fungi such as *F. solani*, *F. oxysporium* and *A. alternata* (Figs. 4, 5 and 6). The light microscopic examination from the zone of inhibition plate of *F. oxysporium* and *F. solani* revealed deformation (lysis and fragmentation) of the fungal mycelium and inhibition of mycelial branching (Figs. 4B and 5B) whereas the control showed highly branched and well developed mycelium (Figs. 4A and 5A). When the mycelium of *A. alternata* from the periphery of the zone of inhibition produced by the purified chitinase was examined, the hyphae appeared to have clear cut mycelial deformations such as lysis and fragmentation (Fig. 6B), whereas the mycelium from the control plate was normal well developed and intact without any distortion (Fig. 6A).

Discussion: Fungal diseases are one of the major concerns to agriculture production. It has been estimated that total loss as a consequence of plant diseases reaches 25% of the yield in western countries and almost 50% in developing countries. Of this, one third is due to fungal infections [19]. Roberts and SelitrennikoV [20] studied plant and bacterial chitinases for antifungal activity. According to them chitinases isolated from the grains of wheat, barley and maize functioned as endochitinases and inhibited hyphal elongation of test fungi.

Many practices such as selection for natural resistant varieties *via* conventional breeding methods and application of various chemical fungicides are being applied to encounter this problem. Due to the environmental risks associated with the chemical fungicides, Egypt is now setting the base for the

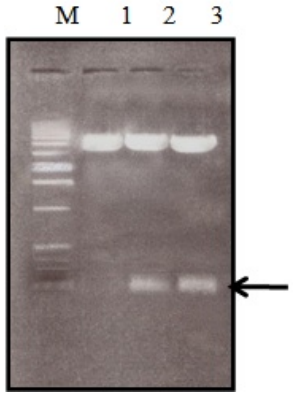

Fig. 1: Restriction digestion of recombinant clones using the restriction enzyme *Bam*HI lane 1 control (pET 19b) without insert, lanes 2 and 3 insert of 798 bp was released from the 5.7k vector and M = 1Kb DNA ladder.

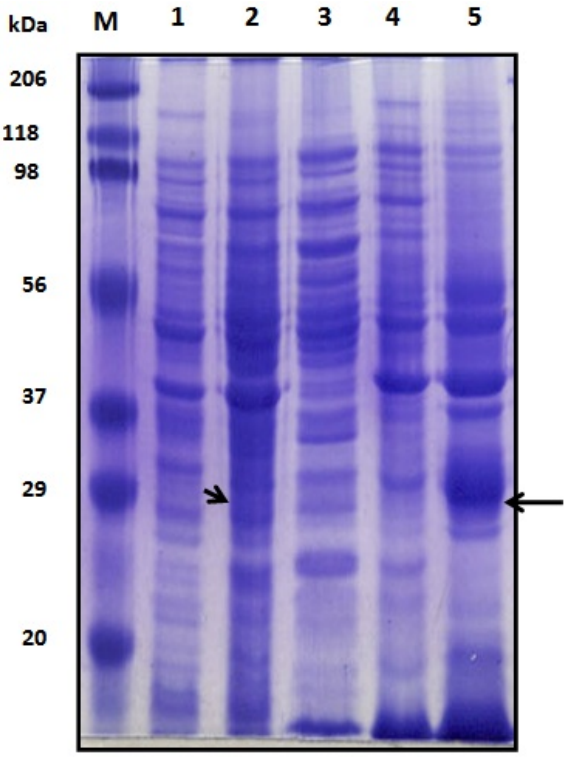

Fig. 2: SDS-PAGE showing expression of bec gene and production of recombinant BEC in *E. coli.* Lane 1, total cellular proteins of E. coli (BL21) harboring the non recombinant pET19b plasmid. Lane 2, total cellular proteins of *E. coli* (BL21) harboring the lyses by sonication of recombinant plasmid pET19b-*bec*-5. Lanes 3 and 4, aliquots from the soluble protein fraction after the cells that harbor the pET19b-*bec*-5 plasmid. Lane 5, insoluble protein fraction, which includes the BEC inclusion bodies, after removing the soluble fraction by centrifugation. Arrows are pointed to the expressed bec. M, high molecular weight protein standard.

development of environmentally friendly biotechnology industry. The aim is to produce genetically modified (GM) plants that are resistant to fungi. Different antifungal genes were selected and introduced into many economically important crops worldwide. In fact, most of the selected genes are naturally occurring in plants and considered as part of its defensive portfolio. Collectively, these genes code for Pathogenesis-related (PR) proteins such as chitinases [21].

In recent years, the cloning and expression of the chitinase gene and its introduction into the biologically susceptible species or the construction of recombinant

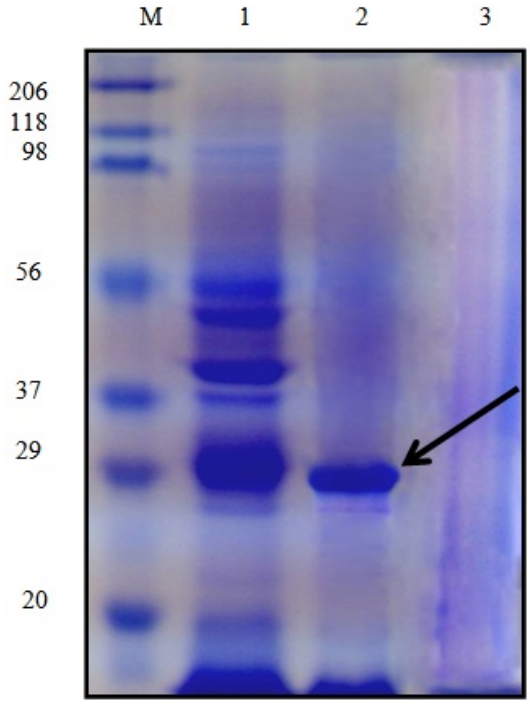

Fig. 3: SDS-PAGE showing partial purification of recombinant BEC from inclusion bodies that was shown in lane 5 in the previous figure. Lane 1, inclusion bodies that were prepared from *E. coli* BL21(DE3) harboring the recombinant plasmid pET19b-*bec*-5 showing BEC among other proteins. Lane 2, soluble fraction showing release of BEC (arrow) from inclusion bodies after subjecting to pH10. Lane 3, insoluble fraction collected by centrifugation. M, high molecular weight protein standard.

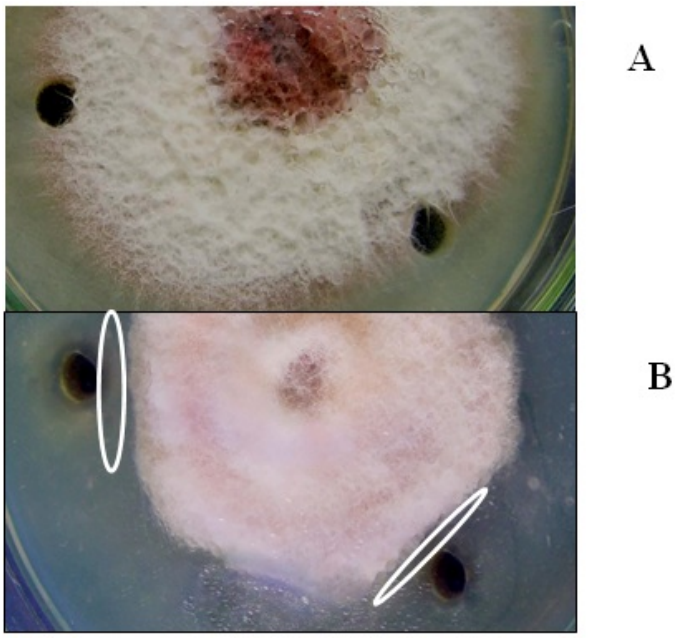

Fig. 4: Antifungal property of barley endochitinase (BEC) against the fungus *Fusarium solani*. A local isolate of *F. solani* was plated on PDA medium in the absence (A) or presence (B) of different concentration of purified BEC. The BEC inhibits the growth of *F. solani* as indicated by the presence of clear zones surrounding the wells, white ovals.

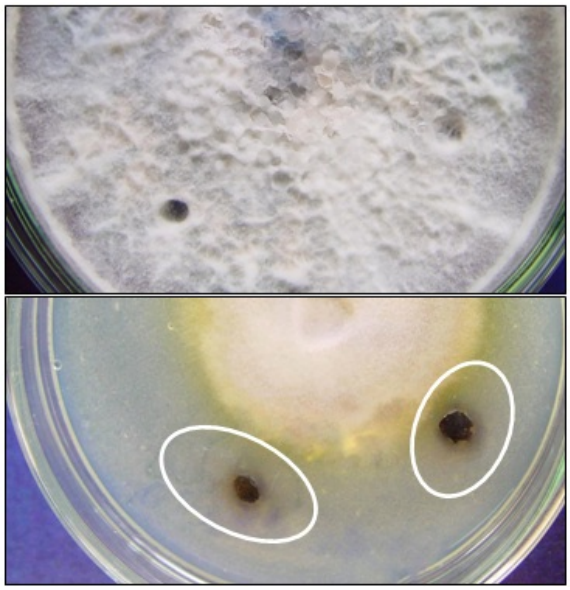

Fig. 5: Antifungal property of barley endochitinase (BEC) against the fungus *Fusarium oxysporium*. A local isolate of *F. oxysporium* was plated on PDA medium in the absence (A) or presence (B) of different concentration of purified BEC. The BEC inhibits the growth of *F. oxysporium* as indicated by the presence of clear zones surrounding the wells, white ovals.

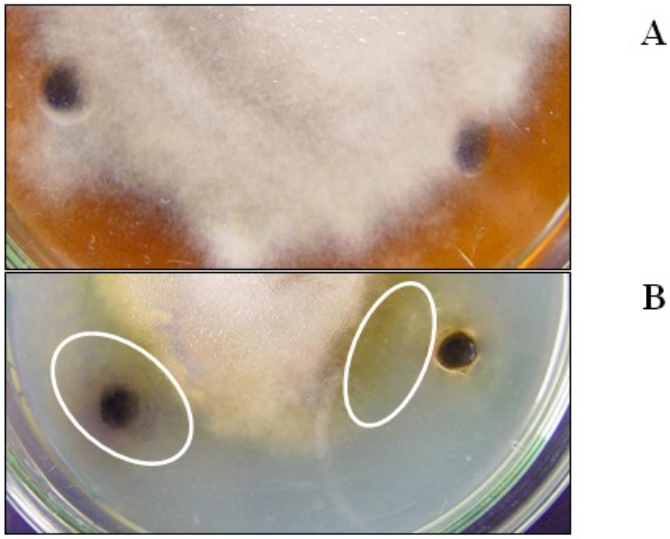

Fig. 6: Antifungal property of barley endochitinase (BEC) against the fungus *Alternaria alternata*. A local isolate of *A. alternata* was plated on PDA medium in the absence (A) or presence (B) of different concentration of purified BEC. The BEC inhibits the growth of *A. alternata* as indicated by the presence of clear zones surrounding the wells, white ovals.

strains with new capacities have been one of the interesting areas of chitinase studies and applications. Chitinase genes have been cloned and characterized from many microorganisms [22,23,24,25], some of which were either transformed into plants and bacterial strains to increase their ability to control phytopathogens [26,27] or were high level expressed in *Escherichia coli* cells to enhance the activity of *Bacillus thuringiensis* to control pests [28,29].

Barley endochitinase (BEC), a class II chitinases, is a 29-kDa monomeric enzyme. BEC has some topological similarities with animal lysozymes [30]. Therefore, similar catalytic mechanisms have been suggested for both proteins.

The first step in this study was targeted cloned to the *bec* coding sequence in an *E. coli* pET19b for high level gene expression and efficient purification of BEC protein (Fig. 2). One major problem associated with over production of recombinant proteins in *E. coli* in general is the improper folding and formation of highly insoluble inclusion bodies. High molar concentration of denaturing agents such as Urea and Guanidine HCL are routinely used to dissociate the inclusion bodies and release protein of interest into the solution. Treating the inclusion bodies with such denaturing agents truly affect the secondary and tertiary structural integrity of recombinant proteins. Subsequently, successive steps of dialysis in the presence of decreasing concentration of denaturing reagents are essential to renature the recombinant protein. Despite these laborious and time consuming successive dialysis steps, obtaining properly-folded recombinant proteins are always not guaranteed. Since the antifungal activity of bec protein should be restored during purification, it was necessary to minimize the damage of recombinant protein during purification by using mild and less harsh condition. A Tris-HCL buffer at pH 10.0 with low concentration of a reducing agent such as DTT was optimum for BEC solubilization.

It is known that recombinant proteins overexpressed in bacteria often form insoluble proteins that contain most of the expressed protein [31]. In our study the recombinant chitinase was produced as insoluble inclusion bodies. The purified protein reported in this study (29 kDa) showed *in vitro* antifungal activity against three major plant pathogenic fungi that reside in Egypt was investigated. The three fungal species tested (*Alternaria alternata, Fusarium solani* and *Fusarium oxysporium*) among the phytopathogenic fungi tested at a concentration of 100 µg and 200 µg, respectively. The light microscopic examination disclosed chitinase-induced lysis and fragmentation of the mycelium and hyphal distortion in the fungus.

A. alternata has been recorded as the causative agent of leaf spots, rots and blights diseases on over 380 host species. In addition to its plant pathogen property, *A. alternata* has emerged as opportunistic pathogens particularly in immunocompromisd patients. In immunocompetent patients, *Alternaria* colonizes the paranasal sinuses, leading to chronic hypertrophic sinusitis. The pathogen colonization may end up with development of invasive disease. *A. alternate* is among the causative agents of Otitis media in agricultural field workers.

F. solani and *F. oxysporum* are the causative agents of wilt disease in more than a hundred species of plants. They colonize the water-conducting vessels (xylem) and cause blockage and breakdown of xylem tissue. Symptoms include leaf wilting, yellowing and eventually plant death.

Barley chitinase exhibited a broad-spectrum antifungal activity against phytopathogenic fungi and therefore, can be used to develop fungal resistant crop plants such as potato. Results presented in this study will be useful to design appropriate strategies for transgenic resistance in crop plants.

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