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CHARACTERIZATION AND EXPRESSION OF THE CHITINASE *CHIT* II GENE FROM *Lecanicillium lecanii* IN SOLID-STATE FERMENTATION

CARACTERIZACIÓN Y EXPRESIÓN DEL GEN QUITINASA *CHIT* II DE *Lecanicillium lecanii* EN CULTIVO SÓLIDO

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Abstract

Hydrolytic enzymes like chitinase are key factors in entomopathogenic fungi infections of insects. Chitinolytic activity of *Lecanicillium lecanii* was detected when growing in chitin and in grasshopper cuticle (*Sphenarium purpurascens*) in solid-state fermentation (SSF); the highest levels were reached when the fungus grew in chitin. Expression levels of *chit*II gene in *L. lecanii* depend on the carbon source present in the medium, and an increment in *chit*II gene expression was observed through fermentation time. Among the carbon sources evaluated, chitin showed the highest levels of *chit*II gene expression. When grown in glucose, basal levels of expression of *chit*II gene were detected, suggesting that the *L. lecanii* chitinolytic system is subject to an induction/repression mechanism. Electrophoresis in SDS-PAGE of a partially purified extract obtained from the growth on chitin and *S. purpurascens* cuticle revealed a band of 23 kDa approximately with β-N-acetyl-glucosaminidase activity in a zymogram analysis.

Keywords: chitinases, solid-state fermentation, RT-PCR, *Lecanicillium lecanii*, *chit*II gene.

Resumen

Se determinó la actividad quitinolítica de *Lecanicillium lecanii* al crecer en quitina y cutícula de chapulin (*Sphenarium purpurascens*) en fermentación en estado sólido. Así mismo, se midieron los niveles de expresión del gen *chit*II de *L. lecanii* observando que estos dependen de la fuente de carbono utilizada y se presentó un aumento de la expresión en función del tiempo de fermentación. Los más altos niveles de expresión del gen se observaron al crecer en quitina, sin embargo, en glucosa se detectaron niveles basales lo que sugiere que el sistema quitinolítico de *L. lecanii* está sujeto a un mecanismo de inducción/represión. Extractos parcialmente purificados obtenidos del crecimiento en quitina y cutícula de saltamontes se analizaron en zimogramas y se observó una banda de aproximadamente 23 kDa con actividad de β-N-acetil-glucosaminidasa.

Palabras clave: quitinasas, fermentación en estado sólido, RT-PCR, *Lecanicillium lecanii*, gen *chit*II.

1 Introduction

The development and application of biological agents that preserve natural resources and the environment in pest control products are an important alternative in agriculture. Entomopathogenic fungi have been used in pest biological control; during the infective process they synthesize hydrolytic enzymes like protease and chitinase which degrade the cuticle of insects (St Leger *et al*., 1986a). This opens the possibility of using these fungi for other applications such as a biotechnological integrated process for transforming shrimp shell waste

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and insect cuticle into high valued products (like chitosan in the pharmaceutical industry) which are the basis for the production of biological products used as micopesticides, and in the production of spores as bioinsecticides. In recent years, there has been a renewed interest in solid-state fermentation (SSF) due to its high productivity of bioactive compounds (Ruiz-Leza *et al*., 2007). Studies on entomopathogenic fungus *Lecanicillium lecanii* in SSF showed proteolytic and chitinolytic enzymes (Barranco *et al*., 2002). Chitin is hydrolyzed to its monomer by the synergistic and consecutive action of two enzymes, namely endo chitinases (EC 3.2.1.14), which hydrolyze polymer in random manner, and N-acetylglucosaminidase (chitobiase, EC 3.2.1.30) which preferentially attacks lower chitooligomers to produce N-acetyl-D-glucosamine (GlcNAc) monomers (Patil *et al*., 2000).

During growth, fungi synthesize chitinolytic enzymes its production is induced by adding chitin to the culture medium (Taylor *et al*., 2002; Li *et al*., 2004; 2005). Gene expression of chitinases in fungi is regulated by an induction/repressor system in which chitin or the GlcNAc waste products act as inductors, while glucose or any easily metabolizable carbon sources act as repressors (Blaiseau *et al*., 1992). Repression through glucose is a common phenomenon in chitinase gene expression in fungi grown in submerged fermentation culture (SmF) (De las Mercedes *et al*., 2001). In the presence of glucose, regulator protein CreA/Cre1 binds to the consensus sequence (SYGGRG) and represses chitinase genes transcription (Strauss *et al*., 1995; Ilmen *et al*., 1996; Stapleton and Dobson, 2003). Most studies about the expression of chitinolytic genes in fungi have been carried out only in SmF and few have been done in SSF. This system offers economical advantages over SmF, such as the use of a simple medium of production and growth as it uses agro industrial waste, as well as low cost equipment (Pandley *et al*., 2000).

The use of *L. lecanii* is an alternative to chemical pesticides, although its utility is limited due to the relatively low death rate of insects (St Leger *et al*., 1995). In order to improve virulence, a detailed knowledge of pathogenic molecular mechanisms of the fungus is necessary. *L. lecanii* is currently being evaluated as a pathogenic agent in insects. Implied genes in fungus pathogenesis towards insects have been isolated and characterized (Zhen-Xiang *et al*., 2005). Gene expression studies are crucial to elucidate pathogenic function and fungus development. In this study, the effect of an easily assimilated carbon source and complex substrates as chitin and grasshopper cuticle (*Sphenarium purpurascens*) on β-N-acetylglucosaminidase activity, as well as the expression of *chit*II gene of *L. lecanii* grown in SSF have been studied.

2 Material and methods

2.1 Microorganism

L. lecanii ATCC 26854 was grown in potato dextrose agar (PDA), at 25°C during seven days. Spores were collected in a solution of Tween 80 at 0.05%.

2.2 Solid-state fermentation

The culture was carried out in 500 mL Erlenmeyer flasks using bagasse in a mineral medium with (gL^{-1}): $(NH_4)_2SO_4$, 6; $MgSO_4$, 1.2; NaCl, 1; KH_4PO_4, 15; $FeSO_4 \cdot 7H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.028; and $MnSO_4 \cdot H_2O$, 0.032 as support. Glucose (Sigma), chitin and *S. purpurascens* (60 gL^{-1}) were used as carbon sources. Flasks contained 35 g of humid matter, and SSF conditions were: initial humidity 75%, pH 6, temperature 25°C. Inoculation was carried out with 1×10^7 spores/g humid weight. Enzymatic extract was obtained after five days of fermentation, weighing the contents of each flask and adding distilled water in a proportion 1:1. Afterwards, this extract was homogenized and compressed and the supernatant was centrifuged 10 min at 5000 rpm. Samples of fermented fungi in stationary phase were taken for extraction and purification of total RNA.

2.3 Chitin and *S. purpurascens* cuticle treatment

Chitin and *S. purpurascens* flakes were treated with NaOH 0.1 N for 30 min; they were washed two times with distilled water, then with HCl 0.2N for 30 min and with distilled water again. The material was dried at room temperature (RT) and sieved through a 32 mesh. Later, it was sterilized at 121°C, 15 min, and kept at RT for later use.

2.4 Chitinolytic activity

Chitinolytic activity was determined according to Coudron *et al*. (1984) for each carbon source, using p-nitrophenol N-acetyl-β-D-glucosamine (Sigma Chemical Co.) as substrate. To a mixture of 150

μL of deionized water, 200 μL of citrate-phosphate buffer 0.2M (pH 5.6), 200 μL of substrate (1.0 mg mL^{-1}), and 50 μL of enzymatic extract were added. The reaction was incubated for 1 h at 37°C and 180 rpm. Reaction was stopped by adding 1 mL of NaOH 0.02 M. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min at 400 nm. All experiments were triplicated.

2.5 Electrophoresis and zymogram

In order to determine the protein profile of *L. lecanii* growing in glucose, chitin and *S. purpurascens* cuticle, SDS-PAGE on 11% (w/v) polyacrylamide gels was performed according to Laemmli (1970). Gels were stained with coomasie blue. Zymograms were run according to Guthrie *et al*. (2005), proteins were separated in polyacrylamide gel electrophoresis (PAGE) in native conditions. Crude protein samples were prepared in 125 mM Tris- HCl (pH 6.8), 20% glycerol (v/v) and 0.2% bromophenol blue. Native gel was simply washed in distilled water for 5 min before being placed in the agarose-substrate solution previously prepared by heating 20 mL of 100 mM sodium acetate (pH 5.6), 1% agarose at 50°C. The substrate 4-methyllumbelliferyl N-acetyl-β-D-glucosaminide [4-MU(GlcNAc)] (Sigma) was added to final concentration of 0.025 $mg \cdot mL^{-1}$; gel was agitated gently in this solution for 5 min at 37 °C prior to detection under UV light.

2.6 Extraction and purification of total RNA in L. lecanii grown in SSF

Total RNA extraction and purification was carried out with $Trizol^R$ (Invitrogen). Samples of fungi grown in glucose, chitin and *S. purpurascens* cuticle (stationary phase), were placed in a mortar with liquid N2 and then 1 mL $Trizol^R$ was added. The samples were centrifuged at 14,000 rpm for 5 min at RT. Supernatant was transferred into another flask with 200 μL chloroform and vigorously stirred for 15 s; then incubated at RT for 3 min. Then it was centrifuged at 4°C, 12,000 rpm for 15 min. The watery phase was collected and precipitated with 500 μL isopropanol, then centrifuged at 4°C, 12000 rpm during 30 min. To this precipitation 1 mL ethanol 75% was added, then centrifuged at 7,500 rpm, 4°C for 5 min. Total RNA was resuspended in 40 μL H_4O-DEPC. Total RNA was kept at -20°C for later use. RNA concentration was determined by measuring its absorbance at 260 nm and total RNA quality was analyzed in electrophoresis in 1% agarose gel with a UV light.

2.7 RT-PCR

5S Ribosomal RNA (control) and mRNA from *chit*II gene of *L. lecanii* were analyzed through one step reverse transcription (Qiagen One Step RT-PCR kit, CA. USA) following manufacturer instructions. A total of 0.5 μg total RNA was used for reverse transcriptase with Superscript II at 50°C for 30 min, followed by specific oligonucleotides amplification of *chit*II gene of V. lecanii (Zhen-Xiang *et al*., 2005). Conditions for amplification were: 15 min at 95°C, followed by 30 amplification cycles at 94°C 30 s, 60°C 30 s, and 72°C 1 min. After amplification, RT-PCR products were analyzed in 1.5% agarose gels, and stained with ethidium bromide for luminescence in a UV transilluminator (Syngene).

2.8 Densitometric analysis

Pictures of gel with RT-PCR products were taken under exposition to UV light using Kodak EDAS 290 system. Amplicons (cDNA bands) were determined as the integrated area (pixels) in band intensity through densitometric analysis with Kodak Digital Science 1D 3.6 software. Numeric values for cDNA bands intensity were corrected with the values of 5S RNA, as these express themselves at a relatively constant level in cells and are generally used in semi-quantitative systems of RT-PCR for analyzing relative efficiency of each individual PCR. Decimal dilutions of the cDNA mixture were used to verify lineal correlation among band intensity (pixels) and the initial cDNA.

3 Results and discussion

3.1 Chitinolytic activity from L. lecanii

L. lecanii was grown in three SSF mediums containing different sources of carbon and energy: glucose, chitin and *S. purpurascens* cuticle during 144 h. Maximum chitinolytic activity was observed when fungi was grown in chitin media probably to the greater bioavailability of the chitin polymer (β-(1,4)-N-acetyl-D-glucosamine) compared to *S. purpurascens* cuticle. This could probably be due to the fact that the complex substrates used were not soluble. This later media is a more complex polymeric structure that consists of predominantly of proteins and chitin

chains and in a lesser quantity minerals and lipids (Kramer *et al*., 1988). This makes the synergetic action of protease, chitinase and lipase necessary for its metabolization (Clarkson and Charnley, 1996; Beys *et al*., 2005). Chitinolytic activity was four times lower with *S. purpurascens* cuticle when compared to chitin media (Fig. 1). Assuming that a basal production of limited hydrolyzing enzymes for the polymer exists liberating soluble complexes assimilated by the microorganism, which induce enzymes synthesis. Chitinase production is ruled by an induction/repression system in which chitin degradation products like N-acetyl-D-glucosamine work as inductors (St. Leger *et al*., 1986a). Some enzymes are synthesized only in presence of a specific substrate. This can be observed in *L. lecanii* chitinases where there were high levels of chitinolytic activity in response to chitin presence, its usual substrate and in a lesser degree in complex substrates such as *S. purpurascens* cuticle. Bidochka and Khachatourians, (1988) determined a high chitinolytic activity when *Beauveria bassiana* was grown in insects cuticle, liberating N-acetyl-D-glucosamine in enough quantity to be used as a carbon an nitrogen source for its growth. In the same way, Sureh and Chandrasekaran, (1999) determined an inductor effect from colloidal chitin in a SSF system using *B. bassiana*.

Also Barranco *et al*. (2009) determined the inductive capacity of shrimp shell in the synthesis of chitinolytic enzymes from *L. lecanii* in SSF. Bing-Lang *et al*. (2003); Barreto *et al*. (2004) showed the inductive effect of chitin in extracellular chitinolytic activity of *L. lecanii* and Metarhizium anisopliae in a submerged culture system. When *L. lecanii* was cultured in a glucose medium as the only carbon source, chitinolytic activity was minimum (basal level) (Fig. 1). Invertase and tannase basal activity were determined in *Aspergillus niger* Aa-20 using glucose as the only carbon source in SSF (Aguilar *et al*., 2001; Aranda *et al*., 2006). Microbial chitinases are generally detected in low levels during growth in simple substrates (Shirai, 2006). Results obtained show that synthesis of chitinase from *L. lecanii* is subject to regulative mechanisms when grown in different carbon sources under the conditions mentioned above.

3.2 *Electrophoresis and zymogram*

Fungi extracellular hydrolytic enzymes are important for host cuticle degradation during infection because they provide nutrients during their growth (St. Leger

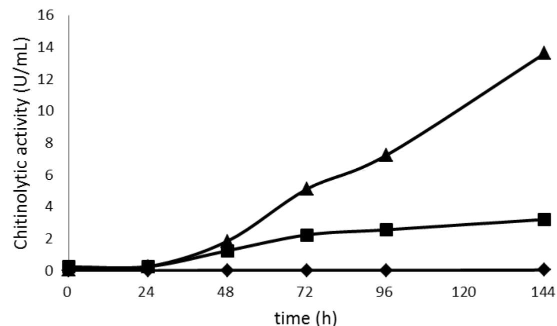

Fig 1. Chitinolytic activity of *L. lecanii* grown in different carbon sources in a SSF system. (♦) Glucose, (■) *S. purpurascens* cuticle, (▲) Chitin.

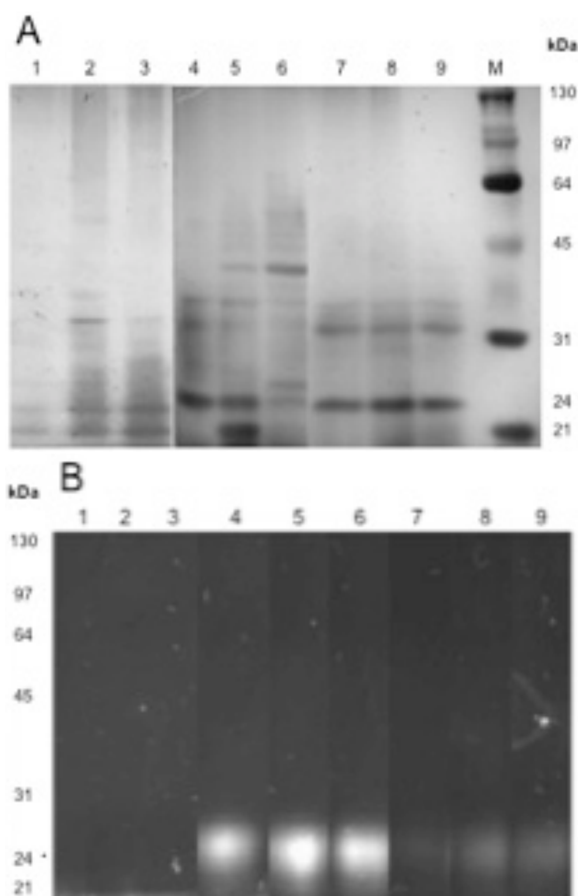

Fig 2. (A) SDS-PAGE and (B) zymogram of proteins of N-acetyl-glucosaminidase activity from *L. lecanii* crude extract grown in glucose, chitin and *S. purpurascens* cuticle. 1) Glucose 48h 2) Glucose 96h 3) Glucose 144h 4) Chitin 48h 5) Chitin 96h 6) Chitin 144h 7) *S. purpurascens* cuticle 48h 8) *S. purpurascens* cuticle 96h 9) *S. purpurascens* cuticle 144h. M) Protein marker.

et al., 1995). The determination of proteic profile of *L. lecanii* was carried out with the crude extract of fungi grown in glucose, chitin and *S. purpurascens* cuticle at 48, 96 and 144 h of fermentation in SSF. As shown in Fig. 2A, extracellular total protein analysis through SDS-PAGE showed that *L. lecanii* synthetized a great number of proteins when grown in a simple medium as well as in a complex medium. Nevertheless, proteic profile among glucose, chitin and grasshopper cuticle are different, certain proteic bands at approximately 24 kDa and 33 kDa appear only in chitin and *S. purpurascens* cuticle. These bands might be proteins with chitinolytic activity similar to that reported in *Lecanicillium fungicola* (Ramirez *et al*., 2006). Fang *et al*. (2005) reported a chitinase CHIT1 of *B. bassiana* with a molecular mass of about 33 kDa. Fig 2B shows fluorescence of 4-MUGlcNAc with enzymatic extract in SSF as consequence of β-N-acetyl-glucosaminidase activity. When fungus was growth in chitin, lysis zones with higher intensity were detected in the gel as fluorescence intensity raised (chitinolytic activity) when the fungus was reaching exponential growth phase (96 h) and stationary phase (144 h). The same phenomenon, but with lesser fluorescence intensity was observed when fungus was grown in *S. purpurascens* cuticle. In both cases, band intensity was increased when fungus was grown in chitin and *S. purpurascens* cuticle during the fermentation process. When fungus was growth in glucose, no lysis zone was detected, probably due to the null chitinolytic activity (Fig. 2B). According to these results, we can observe the presence of only one β-N-acetyl-glucosaminidase isoform. Analysis of the respective bands indicated that chitinase has a molecular mass of approximately 23 kDa (Fig. 2A and B). Molecular characterization is necessary in order to forecast the potential role that chitinase could have in insect lysis because low molecular mass enzymes have the advantage that they can easily penetrate chitin fibers. Chitinolytic enzymes with low molecular mass have been previously reported in several microorganisms like *Neurospora crassa*, 20 kDa (Zarain and Arroyo, 1983), *Pycnoporus cinnabarinus*, 38 kDa (Ohtakara, 1988), and *Trichoderma harzianum*, 33 kDa (De la Cruz *et al*., 1992).

3.3 Expression of chit II gene from L. lecanii

Studies on the expression of chitinolytic genes in fungi have been generally done in SmF (Baratto *et al*., 2006; Choquer *et al*., 2007) or in surface adhesion fermentations (SAF) (Villena *et al*., 2009). There are not enough reports in the literature about studies on regulation of chitinolytic genes in fungi in SSF and for that reason we chose to study the effect of different substrates in the expression of *chit*II gene of *L. lecanii*. Fig. 3 shows differential expression of *chit*II gene detected when *L. lecanii* was grown in glucose, chitin and *S. purpurascens* cuticle, in a SSF system. When fungus was grown in non-inductive conditions using glucose as only carbon source, under conditions stated before (Material and methods), a minimum expression of *chit*II gene was detected. Same results were observed by Baratto *et al*., (2006) in *M. anisopliae* when glucose was used and it acted as repressor in the expression of *chit*II gene using RT-PCR. The catabolic repression effect caused by glucose has been previously described for different routes in carbohydrates degradation (Ronne, 1995).

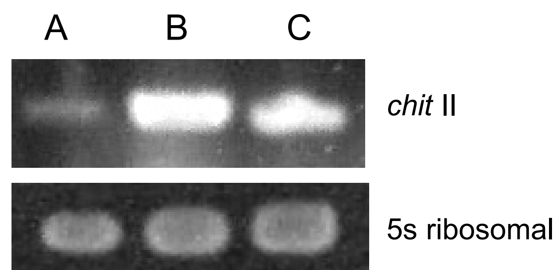

Fig 3. Expression of mRNA of *chit*II gene of *L. lecanii* grown in SSF, determined by RT-PCR, using glucose (A), chitin (B) and *S. purpurascens* cuticle (C) as carbon sources. Underneath gel on each panel represents 5S ribosomal transcript, used to normalize RNA total quantity.

In regards to complex substrates like chitin and *S. purpurascens* cuticle, expression of *chit*II gene of *L. lecanii* resulted in a 33% increase in chitin compared to the expression when fungus grew on *S. purpurascens* cuticle. These results confirmed that chitin and insect exoskeleton act as inducers of chitinolytic gene of *L lecanii*. Garcia *et al*. (1994) showed that chitin and residues of cellular wall fungal strongly induce expression of *chit*42 gene of *T. harzianum*. The results in Fig. 4 show the *chit*II/5S ribosomal gene proportion, measured by densitometry (pixels). Numeric values for band intensity of cDNA were corrected with 5S ribosomal band values expressed as constant values in cells, commonly used in RT-PCR semi-quantitative systems

in order to analyze relative efficiency of each PCR product. RT-PCR analysis indicates that expression of *chit*II gene is controlled by the nature of the carbon source in the culture medium.

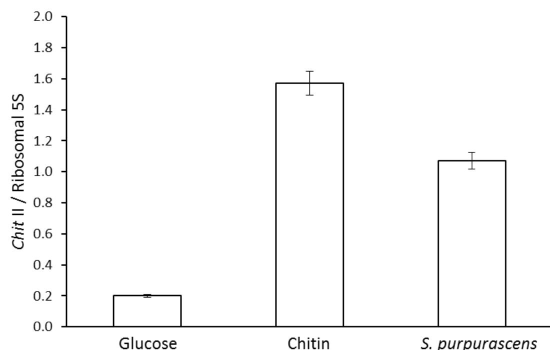

Fig 4. Effect of the carbon source in expression of mRNA that codifies *chit*II gene from *L. lecanii* grown in SSF. Results are shown as the proportion of *chit*II/5S ribosomal RNA, measured by densitometry and are the average of two different experiments carried out by triplicate. Lines over bars represent standard deviations.

Conclusions

We can suggest that when the *L. lecanii* grew in glucose, the chitinolytic activity was at basal level, this matches the results obtained in the zymographic analysis and when we detected the transcripts of *chit*II gene. We also observed that chitin complex substrates and *S. purpurascens* cuticle induced the expression of chitinolytic genes, where chitin was the best inductor substrate of chitinolytic enzymes in the fungus. This suggests that chitinolytic activity in *L. lecanii* is subject to an induction/repression mechanism.

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