Purification and Characterization of Chitinase Produced by Endophytic *Streptomyces hygroscopicus* Against Some Phytopathogens

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**Abstract**  
Microorganisms, which secrete a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases. Since Chitinases are digestive enzymes that break down glycosidic bonds in chitin. In this present study, an industrial enzyme chitinase produced by endophytic *Streptomyces* was purified and its antifungal activity was investigated against phytopathogens *i.e.* Rhizoctonia solani, Fusarium oxysporum, Alternaria alternate, Aspergillus niger, Aspergillus flavus, Sclerotinia sclerotiorum, Phytophthora parasitica and Botrytis cinerea. A chitinase was produced by endophytic *Streptomyces hygroscopicus* in cultures containing chitin as the sole substrate that degrade chitin in 0.9 mm zone of clearance. The purification steps included ammonium sulfate precipitation, with columns of DEAE-Sepharose anion exchange chromatography and Sephacryl S-400 high resolution gel chromatography. The method gave a 5.4 fold increase of the specific activity and had a yield of 18%. The molecular weight of the chitinase was found to be around 80.8, 78 and 76 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. Chitinase was optimally active at pH of 6.0 to 7.0 and at 30°C. The chitinase were found to inhibit the growth of all phytopathogenic fungi.

**Keywords**  
Antifungal Activity, Chitinase, Plant Phytopathogens, *Streptomyces hygroscopicus*

1. Introduction

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants. Chemical fungicides are extensively used in current agriculture. However, excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution, and development of pathogen resistance to fungicide. Because of the worsening problems in fungal disease control, a serious search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environmentally friendly.

Microbial antagonists are widely used for the biocontrol of fungal plant diseases [1]. Endophytic micro-organisms have received considerable attention for their potential as biocontrol agents of fungal plant pathogens. Varied enzyme production may result in new biochemical characteristics and in part be responsible for the inherent biodiversity of endophytic micro-organisms. Among the lytic enzymes evaluated as a source of biocontrol agents, chitinases have been studied largely because these enzymes are produced by a variety of endophytic micro-organisms [2]. Endophytic actinomycetes have been cited as promising biocontrol agents, either acting directly on fungal cell walls or initiating increased plant responses against disease. In addition, research indicates that both mechanisms operate to control fungal pathogens [3]. Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental-friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme applications in many industrial processes [4]. Chitinase, a group of enzymes capable of degrading chitin directly to low molecular weight product and found in a broad range of organisms, including bacteria (*Bacillus, Aeromonas, Pseudomonas, Serratia, Enterobacter, Actinomycete*), fungi (*Trichoderma and Aspergillus*), and higher plants, insects, crustaceans, invertebrates and some vertebrates [5].

Chitinases have received increased attention due to their wide range of biotechnological applications, especially in the biocontrol of fungal phytopathogens [6]. Chitinases have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activities *in vitro*. In viruses, chitinases are involved in pathogenesis [4].
Due to multiple applications of chitinases in biocontrol, waste management, medicine and biotechnology they become interesting enzymes for study.

In the present study, chitinase production by endophytic actinomycetes, Streptomyces hygroscopicus and their anti-fungal activities against phytopathogens. Rhizoctonia solani, Fusarium oxysporum, Alternaria alternata, Aspergillus niger, Aspergillus flavus, Sclerotinia sclerotiorum, Phytophthora parasitica and Botrytis cinerea were evaluated.

2. Materials and Methods

2.1. Micro-organism Strains and Growth Conditions

Streptomyces hygroscopicus was previously isolated from peanut plants and identified in Plant Pathology Department, National Research Centre, Egypt. Culture was grown and maintained on solid starch medium[7] at 28°C.

The fungal pathogens Rhizoctonia solani, Fusarium oxysporum, Alternaria alternata, Aspergillus niger, Aspergillus flavus, Sclerotinia sclerotiorum and Botrytis cinerea were isolated originally from different naturally diseased plants collected from different agricultural fields. The isolated fungi were grown on potato dextrose agar (PDA) (Difco, USA) plates from different agricultural fields. The isolated fungi were then transferred into slant of PDA and kept at 4°C for further studies. Pure cultures of the isolated fungi were identified according to the cultural properties, morphological and microscopical characteristics of each fungus[8].

Antagonistic activity assay: Dual culture assay was performed to assess the potential biocontrol activity of Streptomyces hygroscopicus against pathogenic fungi. In the dual culture assay, conidial suspension of each pathogen and bacterial suspension of Streptomyces were streaked on the surface of LB agar at a distance of 4 cm. The growth of both was examined daily for the formation of inhibition zone and growth reduction.

2.2. Quantitative Determination of Extracellular Chitinase Activity

Streptomyces was grown in ISP-2 broth[9] with continuous shaking at 150 rpm at 27°C for 10 days. Cell-free supernatants were collected at 1-day intervals by centrifugation at 8,000 rpm for 20 min at 4°C. Cell pellets were dried at 80°C and weighed to determine the growth. Chitinase activity was quantitatively determined by measuring the reducing end group of N-acetylglucosamine (NAG) that degraded from colloidal chitin as substrate[10]. The estimation of chitinase activity was carried out by the procedure described by Wang et al.[11].

Purification of chitinase: The proteins from the culture were precipitated by ammonium sulphate (75%). The precipitate was collected by centrifuging at 8,000 × g for 20 min, and resuspended in an acetate buffer (50 mM, pH 5.0). It was dialyzed against the same buffer and freeze-dried. The sample was then loaded on a preequilibrated DEAE-cellulose column (2 × 20 cm), and washed with the acetate buffer. The proteins were eluted in a stepwise gradient on NaCl (0-1.0 M) at a flow rate of 24 ml/h. Fractions of 3 ml were collected, and the absorbance was read at 280 nm in a spectrophotometer. The fractions with chitinase activity were combined, dialyzed against the acetate buffer, and concentration by lyophilization. The concentrated sample was passed through a Sephadex G-100 column (2 × 40 cm) and eluted with the acetate buffer at the rate of 15 ml/h. The fractions of 3 ml were collected, and the absorbance and chitinase activity were measured.

Chitinase activity measurements A fluorometric assay was used to determine chitinase activity using 4-methylumbelliferyl-N,N,N'-chitotriose (Sigma, St. Louis, USA) as a substrate. The amount of 4-methylumbelliferyl-β-N-acetylglucosaminide (4-MU) released was measured spectrofluorometrically by using a fluorescence spectrophotometer (F-4500, Hitachi) (excitation 390 nm and emission 450 nm). One unit (U) of chitinase activity was defined as the amount of enzyme required to release one μmol of 4-MU per min at 37°C.

SDS-PAGE analysis Sodium dodecyl sulphate-polyacrylamid gel electrophoresis (SDS-PAGE) was performed according to Laemmli procedure[12] using the Mini-Protein II apparatus (Bio-Rad, Hercules, USA). A 10% separating gel containing 0.01% Calcofluor White M2R (Sigma, St. Louis, USA) in 0.5 M Tris-HCl (pH 8.9) and examined for the chitinolytic bands under UV transilluminator[13]. In another gel, proteins were stained with Coomassie Brilliant Blue G-250.

Protein concentration was determined by Bradford’s method[14] using bovine serum albumin as standard.

2.3. The Studies of Chitinases Characterization

Determination of Optimum Temperature and pH

The reaction was carried out at temperatures ranging from 20 to 80°C and the chitinase activity at different temperatures were measured to find the optimum temperature. A similar method was applied at varying pH levels from 3.0 to 8.0 to determine the optimum pH.

2.4. Inhibition of Fungal Growth by the Purified-Enzyme Extract

A spore suspension of pathogenic fungi was uniformly spread on plates of PDA (potato dextrose agar). Discs that were soaked with the purified enzyme extract (5 U, 10 U) were laid on the seeded plates; the control was disc-soaked in boiled-enzyme extract. Fungal growth was observed over 2 d of incubation at 30°C.
2.5. Statistical Analysis

All data (chitinase production and antagonistic isolates) were analysed for significance (P < 0.01) using the SAS software package.

3. Results

Chitinolytic and antifungal activities

The endophytic *S. hygroscopicus* was screened for chitinase production and inhibition of fungi some phytopathogens i.e. *R. solani, F. oxysporum, A. alternata, A. niger, A. flavus, S. sclerotiorum, B. cinerea*. *S. hygroscopicus* produced a high concentration of chitinase and inhibited the phytopathogens. *S. hygroscopicus* generally induced larger fungal growth inhibition zones for *R. solani, S. sclerotiorum, B. cinerea* and *F. oxysporum* than for *A. alternata, A. niger, P. parasitica* (Fig. 1).

The production of extracellular chitinase enzyme by a potent antagonist

The production of extracellular chitinolytic enzyme in the strain was determined at different growth phases. The level of chitinase was sharply increased during the exponential phase and dramatically declined when the cells entered the stationary phase (Fig. 2). The strain produced relatively high levels of chitinase (5.8 U/mg protein) at day1 of the incubation period.

![Antifungal activity](image)

Figure 1. Chitinolytic and antagonistic activities of *Streptomyces hygroscopicus*

![Time-course experiment related to growth, chitinase activity in the *Streptomyces hygroscopicus* strain](image)

Figure 2. Time-course experiments related to growth, chitinase activity in the *Streptomyces hygroscopicus* strain
Purification of the chitinase

The purification procedures of the chitinase secreted by the \textit{S. hygroscopicus} are summarized in Table 1. The results showed that, the enzyme was purified 1.9-fold with a specific activity of 38.7 U/mg protein after ammonium sulfate fractionation. The enzyme was then purified with DEAE-sepharose fast flow and showed 4.7-fold enzyme purification with a specific activity of 7.5 U/mg protein. The final purification step presented 5.4-fold enzyme purification with a specific activity of 3.1 U/mg protein. These results indicated that the effectiveness of the purification method applied in this research and the chitinase was purified to 5.4-fold with a 18% yields (Table 1).

The extract was purified through fractional precipitation with ammonium sulphate, column chromatography, and non-denaturing PAGE, and was then checked using SDS-PAGE. The result indicates high chitinase activity, which showed as a three band, at a molecular weight of about 80.8, 78 and 76 kDa (Fig. 3).

Effect of temperature

The chitinase activity was most active at 30°C. Above 40°C, the activity decreased and was lost completely at 60°C. When the enzyme was kept at various temperatures for 30 min in an acetate buffer (pH 5.0), it was significantly inactivated above 50°C and completely at 65°C (Fig. 4).

Effect of pH

The optimal pHs for chitinase activity and stability of the chitinase were examined. The enzyme was most active between pH 5.0 and 6.0. It was relatively stable at pHs between 4.0 and 8.0, when kept at 4°C. However, beyond these pH ranges, it rapidly lost its activity (Fig. 5).

| Step                      | Total protein (mg) | Total activity (Unit) | Specific (U mg⁻¹) | Purification (fold) | Yield (%) |
|---------------------------|--------------------|-----------------------|-------------------|---------------------|-----------|
| Culture supernatant       | 81.3               | 87.6                  | 14.6              | 2                   | 100       |
| (NH₄)₂SO₄                 | 38.7               | 64.1                  | 29.8              | 1.9                 | 76        |
| DEAE-Sepharose fast flow  | 7.5                | 33.5                  | 56.9              | 4.7                 | 41        |
| Sephadex                  | 3.1                | 12.4                  | 66.9              | 5.4                 | 18        |

Table 1. Purification of thermal chitinase from \textit{Streptomyces hygroscopicus}

The activity assay was carried out at different temperatures using 1% (w/v) colloidal chitin (dissolved in 50 mM pH 7.2 Tris-HCl buffer) as substrate

Figure 3. Activity gels of extracellular lytic chitinase enzyme secreted by \textit{Streptomyces hygroscopicus} showing detection of lytic enzyme activities on Native gel; M = Marker; Streptomyces = Step

Figure 4. Effects of temperature on the chitinase activity of \textit{Streptomyces hygroscopicus}. 
The activity assay was carried out at 60°C using 1% (w/v) colloidal chitin (dissolved in 50 mM different pH buffer) as substrate.

**Figure 5.** Effects of pH on the chitinase activity of *Streptomyces hygroscopicus*

- **Figure 6.** Antifungal activity of chitinase from *Streptomyces hygroscopicus* against some phytopathogens

**Inhibition of fungal growth by purified chitinase**

The inhibition of the fungi phytopathogens i.e. *R. solani, F. oxysporum, A. alternate, A. niger, A. flavus, S. sclerotiorum* and *B. cinerea* growth was observed on agar plates with paper discs that were coated with the purified chitinase enzyme (Fig. 6). Antifungal activity of the purified chitinase of *Streptomyces* was confirmed. The inhibition diameter of the 10 U enzyme was greater than that of the 5 U enzyme, and the boiled enzyme had no inhibitory activity against all pathogens.

**4. Discussion**

Microbial production of chitinase had great attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method. Microorganisms, which secrete a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases.

Endophytes are microorganisms that inhabit plant interior tissues. These microorganisms cause no harm to the host and do not develop external structures, which exclude nodulating bacteria and mycorrhizal fungi as endophytic micro-organisms [15]. Chitinolytic activity has been implicated in the biocontrol activity of several bacteria, including *Streptomyces* spp. Owing to the innocuous nature of these organisms, delivery systems to introduce endophytic bacteria, such as *Streptomyces* spp., into plants have been developed, and the potential for endophytes as biocontrol agents has been explored [16]. In a recent study,
an endophytic actinomycetes suppressed fungal pathogens, including *R. solani*, *Fusarium oxysporum*, *A. alternata*, *Aniger*, *A. flavus*, *S. sclerotiorum* and *B. cinerea*, indicating their potential use as biocontrol agents[1]. Shekhar et al.[1] purified a bioactive compound from endophytic *Streptomyces violaeeusniger* that showed a strong antagonism towards various wood-rotting fungi, and chitinase enzymes were associated with this inhibition. In general, the higher chitinase activity was correlated with higher fungal inhibition.

In the present study, the chitinolytic *S. hygroscopicus* showed high inhibition levels against fungi, and the fungal hyphae exhibited a degraded appearance after chitinolytic strain culture treatment. In this study on chitinase production, extracellular chitinase activity was determined. The purification steps included ammonium sulfate precipitation, with columns of DEAE-Sepharose anion exchange chromatography and Sephacyrl S-400 high resolution gel chromatography on AKTA purifier 100 protein liquid chromatography. The method gave a 5.4 fold increase of the specific activity and had a yield of 18%.

Enzyme production was increased in potential phase and more amounts were detected in the stationary growth phase. Chitinases are fairly stable over broad pH range. Chitinase was optimally active at pH of 6.0 to 7.0 and at 30℃. The molecular weight of the chitinase was found to be around 80.8, 78 and 76 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. The chitinase were found to inhibit the growth of all phytopathogenic fungi i.e. *R. solani*, *F. oxysporum*, *A. alternata*, *Aniger*, *A. flavus*, *S. sclerotiorum* and *B. cinerea*, respectively. Since the cell walls of fungi consist largely of chitin[17], it was postulated that chitinase produced by the antagonists could be involved in disease control. The production of these enzymes was therefore used as the criteria for selection of potential biocontrol agents against pathogens.

*Streptomyces* strains are a likely choice as potential biological control agents. Antagonistic activity of several *Streptomyces* sp. against a number of fungal pathogenic species has been known for a long time[18, 19 & 20]. Also, in greenhouse experiments, *Streptomyces* sp. has conferred various degrees of protection on different plant species against soil-borne pathogenic fungi[2]. Very high protein content of clam mydospores of *Fusarium* sp. (7 - 28%) may be responsible for their ability to resist lysis in soil.

Studies on chitinolytic microorganisms have yielded a large increase in knowledge regarding their role in inhibition of growth of fungal plant pathogens (20 & 21). Still this knowledge is not sufficient enough to formulate a preparation based on these agents that can work efficiently in all different environmental conditions. The biocontrol agents are also affected due to the geological and environmental conditions. Studying the environmental conditions of the area where biological control agent has been employed and statistical based formulation approach techniques will increase life span of microorganisms in different ecological conditions. The results of this study led us to conclude that chitinase produced by endophytic Streptomyces has the potential for control of plant pathogenic fungi. This study provides a quantitative assessment of *Streptomyces* chitinolytic and antifungal activities.

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