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ISOLATION AND CHARACTERIZATION OF THE CHITINASE FROM *TRICHODERMA ASPERELLUM*

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SUMMARY

Chitinases, a group of enzymes capable of degrading chitin directly to low molecular weight products, have been shown to be produced by a number of numerous bacteria, fungi, insects, plants, and animals. A chitinase producing strain was isolated from Thua Thien Hue, it was identified as *Trichoderma asperellum* SH16 using ITS sequence. Maximum activity of extracellular chitinase was observed after 96 h inoculation of micelia on 1% coloidal chitin medium. SDS - PAGE and native PAGE shown that molecular weight of this enzyme was 42 kDa and named as CHIT42-SH16. Characterization of the chitinase displayed an optimum pH at 7 and temperature at 40°C. The enzyme is stable between pH 3 - 9 and is able to retain its activity from 10 to 60^oC. The presence of Al^{3+}, Fe^{2+} and Ca^{2+} ions increased the CHIT42-SH16 activity up to 161%, 131%, and 165%, respectively. It is also stable towards detergents (5% DMSO and 1% Triton-X100), chelating reagent (1 mM EDTA) and denaturing agent (1 M urea). The results show that CHIT42-SH16 from *T. asperellum* SH16 is able for further research such as a biocontrol for plant pathogens and hydrolysis of chitinous wastes.

Keywords*: CHIT42, chitinase, ITS, biocontrol, Trichoderma asperellum.*

INTRODUCTION

The fungal genus *Trichoderma* (Ascomycetes, Hyprocreales) contains species that are of vast economic importance owing to their production of industrial enzymes (cellulase and hemi-cellulase), antibiotics, and their ability to act as biocontrol agents against plant pathogens since 1920s (Siddiquee *et al.*, 2007). Species of *Trichoderma* excrete hydrolytic enzymes such as chitinases, β-glucanases and proteinases, these extracellular enzymes are induced during interactions between *Trichoderma* spp. and cell - wall materials of phytopathogenic fungi. Although other lytic enzymes may be involved in the complete degradation of fungal cell walls, chitinase is generally considered a critical enzyme for mycoparasitism since its substrate, chitin, is the most abundant component in many fungal cell walls (Baek *et al.*, 1999).

Chitinases are the heterogeneous group of enzymes that catalyze the hydrolytic reaction of chitin, which is the second most abundant polysaccharide (after cellulose) on Earth and is long-chain polymer of N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) linked by β-1,4 bonds (Song *et al.*, 2005). Chitin is an important structural component of many organisms including fungal cell walls, shells of crustaceans, and insect cuticles. Therefore, chitin degradation and chitinases are speculated to play the vital role in a wide variety of biological and biotechnological processes, ranging from the exploitation and environmental clean-up of chitinous wastes to human therapy, plant defense systems and biological control. The chitinolytic system in *Trichoderma* consists of at least six distinct enzymes, two *N*-acetylglucosaminidases and four endochitinases (Huang *et al.*, 2007).

Due to the important biophysiological functions and applications of chitinase, a numerous literatures of research on fungal chitinases has been carried out in recent years (Li, 2006). In Vietnam, chitinase was obtained from soybean (Đinh Duy Kháng *et al.*, 1999), rice (Hai *et al.*, 2003), *Trichoderma* sp. (Nguyễn Thị Hồng Thương *et al.*, 2003), and sweet potato (Đặng Trung Thành, 2008)… In this work, we show the results of isolation and characterization of 42-kDa chitinase from *Trichoderma asperellum* SH16.

MATERIALS AND METHODS

Sampling, isolation and screening of chitinase - producing *Trichoderma* sp.

Trichoderma spp. strains were isolated from soil (field growing peanut and chilli) and compost samples (rice straw, rice husk, and waste) in Quang Dien, Thua Thien Hue in 2009. One gram of soil sample was suspended in 5 ml of sterile distilled water and vortexed for 10 min at room temperature. The suspensions were serially diluted and plated on *Trichoderma*-selective medium (Elad *et al.*, 1981) supplemented with propamocarb (Askew, Laing, 1993). Cultures were incubated at 28°C for 4 days, and then colonies of *Trichoderma* spp. were subcultured on potato dextrose agar (PDA) medium for purifying. Colloidal chitin was prepared from the chitin (Sigma, USA) according to Shanmugaiah *et al.* (2008). In order to detect chitinase production, 100 µl of diluted *Trichoderma* spp. suspension cultured on solid glucose - free Czapeck-Dox medium supplemented with 1% (w/v) colloidal chitin at 28°C for 36 h. Colloidal chitin hydrolysis was visualized by Lugol's solution (Orpin, 1977).

Classification of *Trichoderma* sp.

Mycelia of *Trichoderma* spp. from PDA medium were grown in liquid potato dextrose broth (PDB) medium at 28°C for 2 days. Mycelia were then collected by filtering through a double layered of Whatman paper, washed with distilled water and frozen at -20°C. Fungal genomic DNA was extracted using the phenol-chloroform method according to Siddiquee *et al.* (2007). Genomic DNA was then used as template in PCR with ITS1 and ITS4 primers which specifically designed to the internal transcribed spacer (ITS) region of the rDNA of fungi (Manter, Vivanco, 2007). PCR product was sequenced and searched for the homology on the GenBank using BLAST tool.

Chitinase production and purification

Two mL of conidial suspension (10^6 conidia/ml) was grown in 100 ml of fresh Czapeck-Dox medium supplemented with 10% (w/v) glucose in 250 ml flask at 28°C for 96 h with shaking 180 rpm. Harvested mycelia were washed several times with sterile 2% $MgCl_2$ and distilled water, then transferred into liquid glucose - free Czapeck-Dox medium supplemented with 1% (w/v) colloidal chitin as a carbon source. The culture was incubated at 28°C on shaker at 100 rpm. The broth was harvested after 1 to 7 days and filtered through Whatman paper to use as crude enzyme (Harighi *et al.*, 2007). For purification, crude enzyme was precipitated by ammonium sulphate (70% saturation) at 4°C for 2 h, centrifuged at 15,000 rpm at 4°C for 10 min. The pellet was resuspended in suitable buffers and used for characterization of enzyme.

Chitinase characterization

Molecular weight determination

After ammonium sulphate precipitation, the pellet was resuspended in 0.1 M acetate buffer (pH 5) and dialyzed overnight against 0.05 M acetate buffer (pH 5) (Tsujibo *et al.*, 1998). The protein samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following standard procedure (Laemmli, 1970). For native PAGE, colloidal chitin was added to the gel preparation to give a final concentration of 0.1%. Electrophoresis was performed at 4°C for 3 h and gels were then incubated at 37°C for 2 h with gently shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 (Trudel, Asselin, 1989). Gels were stained with Lugol's solution.

Chitinase assay and protein quantification

Chitinase activity was assayed by Tsujibo's method (Tsujibo *et al.*, 1998) with a slight modification. Reaction was performed by mixing a 70 µl of diluted enzyme with 140 µl of 2.5 mM *p*-nitrophenyl-β-*N*-acetylglucosaminide (PNP-β-GlcNAc) in 50 mM acetate buffer (pH 5.0). After incubation at 50°C for 10 min, the reaction was terminated by adding 1.4 ml of 0.2 M Na_2CO_3. Chitinase activity was measured at 420 nm with *p*-nitrophenol standard curves. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol in 1 min under the conditions described above.

Total protein concentration was determined by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). The chitinase specific activity is obtained by dividing units of enzyme by total proteins in the sample.

Effect of some factors on the chitinase activity and stability

The optimal temperature and pH for enzyme activity was investigated with a range of temperature from 10 to 90°C and pH from 3 to 11. Buffers for optimal pH determination were 20 mM citrate buffer (pH 3-6), 20 mM phosphate buffer (pH 6-8), and 20 mM glycine-NaOH buffer (pH 9 - 11).

The temperature and pH stability of enzyme were investigated by incubating enzyme for 30 min at temperatures of 10-90°C and pHs of 3 - 11 without the substrate, the enzyme solution was then immediately cooled to 4°C (Yu, Li, 2008).

Effect of metal ions and some reagents on enzyme activity were tested by incubating enzyme 37°C for 30 min 5 mM metal ion (Na^{+}, Al^{3+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, or Fe^{3+}) or reagents such as 1% SDS, 1 mM EDTA, 1 M urea, 5% DMSO and 1% Triton X-100. Boiling enzyme solution was used as the blank.

RESULTS AND DICUSSION

***Trichoderma* spp. isolation**

Total 22 strains isolated from Thua Thien Hue were examined for their chitin hydrolytic activity on agar plates. Three isolates named CH2, SH16, and WH28 were found to produce high level of chitin hydrolytic activity (data not shown). The best chitinase producing strain SH16 (Fig. 1) was selected for further study. The ITS sequence of strain *Trichoderma* spp. SH16 contains 602 nucleotides (accession number HM545080). Searching the current GenBank database against this gene sequence revealed 100% identity with the ITS sequence of *Trichoderma asperellum* ZJPH0810 (accession number GU318216). Hence, the strain SH16 was classified as *T asperellum*.

Production of chitinase

T. asperellum SH16 was cultured in glucose-free Czapeck-Dox medium supplemented with 1% (w/v) colloidal chitin. Extracellular chitinase activity was already present after 24 h and reached the maximum value of 11.45 units/mg protein after 96-h cultivation (Fig. 2). Crude chitinase solution was harvested at this time for further study.

Chitinase characterizations

Molecular weight

SDS-PAGE and native PAGE of total extracellular protein were analysed by GS-800 scanner system (Bio-rad, USA) (Fig. 3). The results showed that a colloidal chitin hydrolytic band of approximate 42 kDa was observed on native PAGE (clear band). Hence, this enzyme was designed as CHIT42-SH16. The 42 kDa chitinases were also reported in some publications such as CHIT42 from *T. harzianum* (De La Cruz *et al.*, 1992), *Trichoderma* sp. (Yu, Li, 2008), and *T. virens* Gv29-8 (Baek *et al.*, 1999), or Ech1 from *T. virens* UKM-1 (Alias *et al.*, 2009).

Effects of temperature and pH on chitinase activity

The CHIT42-SH16 activities at the various temperatures were analyzed using PNP-β-GlcNAc as substrate, of which enzyme exhibited high level of chitin-hydrolysis in the temperature range of 30-70°C, and the optimal temperature was found at 40°C. More than 90% of enzyme activity remained after 30 min at less than 60°C. However, the enzyme activity decreased dramatically when the temperature increased over 60°C (Fig. 4). The 42-kDa endochitinase from another fungus such as *T. harzianum* also exhibited the optimal temperature of 40°C (Ulhoa, Peberdy, 1992; De La Cruz *et al.*, 1992), which was similar with present result. However, Ech1 showed the optimal temperature at 50°C (Alias *et al.*, 2009).

The effect of pH on the CHIT42-SH16 activity and stability was also determined. The enzyme exhibited the optimum activity at pH 7, which was higher than that of Ech1 (Alias *et al.*, 2009), and stable in the pH range of 3-10 (Fig. 5), The pH value of 3-6 seems not suitable for CHIT42-SH16 activity, which was similar with CHIT42 from *Trichoderma* sp. (Yu, Li, 2008).

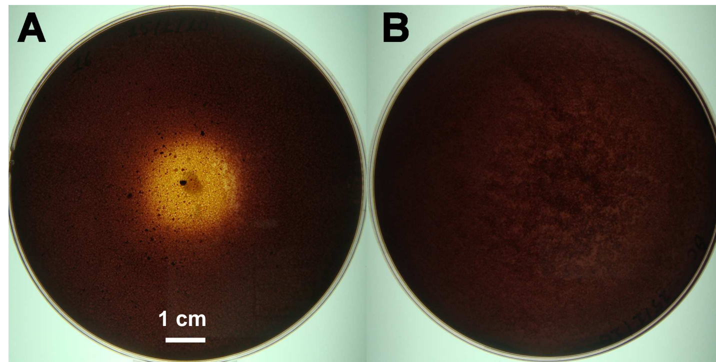

Figure 1. (A) Hydrolytic activity of strain SH16 on 1% coloidal chitin agar plate (clear zone), and (B) Control plate without sample.

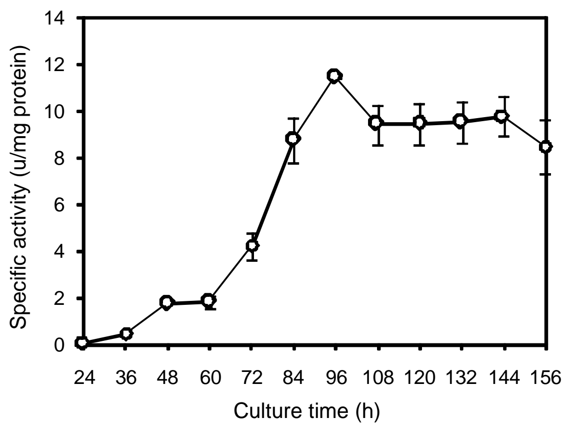

Figure 2 Time course chitinase production of *T. asperellum* SH16**.**

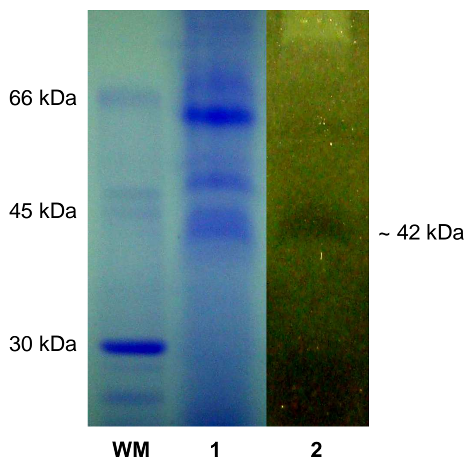

Figure 3. Analyses on 12% polyacrylamide gel electrophoresis for extracellular protein from *T. asperellum* SH16. WM: protein weight marker (14.4 - 97 kDa); 1. SDS-PAGE; 2. native PAGE.

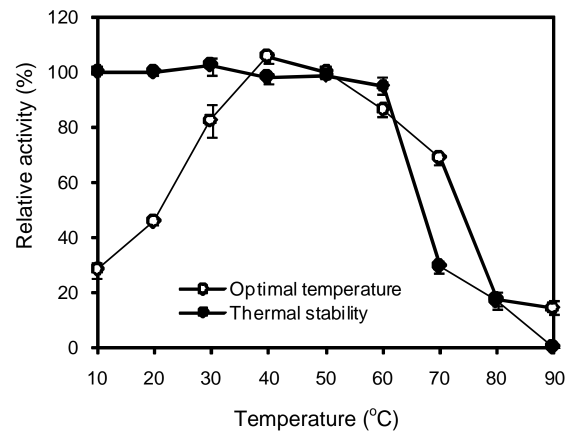

Figure 4. Effect of temperature on the chitinase activity and thermostability.

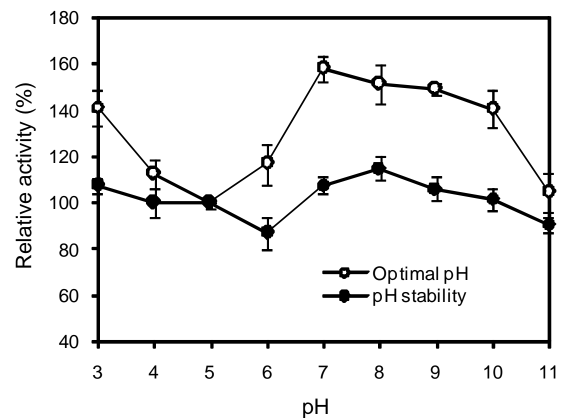

Figure 5. Effect of pH on the enzyme activity and stability.

Effects of metal ions and some reagents on chitinase activity

The effect of metal ions and some reagents on the CHIT42-SH16 activity was summarized in Fig. 6. In this study, Zn^{2+}, urea, DMSO, SDS, and Triton X100 showed the significantly inhibitory effects on the enzyme activity. The same effect was also observed for Ech1 (Alias *et al.*, 2009). The present results showed that Al^{3+}, Fe^{2+}, and Ca^{2+} significantly enhanced the activity of the CHIT42-SH16 upto 161%, 131% and 165% respectively. While other ions including Mg^{2+}, Co^{2+}, and Fe^{3+} slightly increased chitinase activity upto 105%, 105% and 107%. Results of Alias *et al.* (2009) showed that Ca^{2+} and Mg^{2+} increased Ech1 activity to 120% and 115%, respectively, while Yu, Li (2008) reported that Fe^{2+} and Mg^{2+} raised CHIT42 activity to 145% and 144%, respectively.

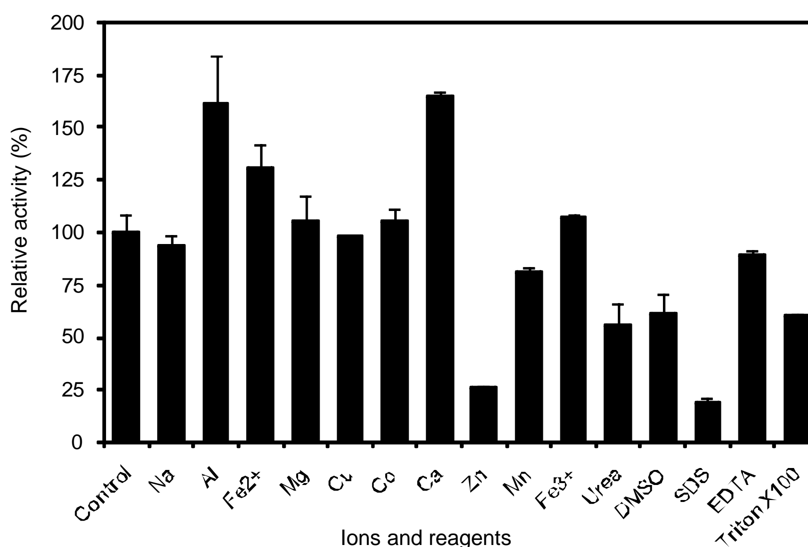

Figure 6. Effects of metal ions and reagents on the CHIT42-SH16 activity.

The enzyme CHIT42-SH16 was also partially inhibited to the denaturation caused by 1 M urea, 5% DMSO and 1% Triton X-100, of which about 56%, 61%, and 60% of original activity were retained, respectively. According to Alias *et al.* (2009), the recombinant Ech1 showed that 60% of its activity was retained when reacted with 1 M urea and 1% Triton-X100. In addition, the enzyme was also found to be unstable towards organic solvent 5% DMSO. Thus, this may indicate that hydrophobic interactions are significantly important for enzyme activity, similar to previous report for chitinase from *B. licheniformis* Mb-2 (Toharisman *et al.*, 2005).

CONCLUSSION

An chitinase - producing strain was isolated from Thua Thien Hue province, which was identified as *Trichoderma asperellum* SH16 by analysis of fungal ITS sequence. Extracellular chitinase was harvested after 96 h of mycelia culture in 1%-colloidal-chitin supplemented medium and the SDS-PAGE analysis showed that molecular weight of enzyme was approximately 42 kDa. This enzyme was designed as CHIT42-SH16. The CHIT42-SH16 exhibited highly thermo- and pH-stability and was stable towards detergents (5% DMSO, and 1% Triton-X100), chelating reagent (1 mM EDTA) and denaturing agent (1 M urea).

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PHÂN LẬP VÀ ĐÁNH GIÁ MỘT SỐ ĐẶC TÍNH CỦA CHITINASE TỪ *TRICHODERMA ASPERELLUM*

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TÓM TẮT

Chitinase là nhóm enzyme có khả năng phân giải trực tiếp chitin tạo sản phẩm có khối lượng phân tử thấp, được sinh ra bởi một số vi khuẩn, nấm, côn trùng, thực vật và động vật. Chủng nấm SH16 có khả năng sinh chitinase mạnh đã được phân lập ở Thừa Thiên Huế và định danh là *Trichoderma asperellum* SH16 thông qua phân tích trình tự ITS. Nuôi cấy sợi nấm trên môi trường có bổ sung coloidal chitin 1% cho hoạt tính chitinase ngoại bào tối đa sau 96 giờ. Điện di SDS và điện di không biến tính có bổ sung cơ chất cho thấy enzyme này có khối lượng phân tử khoảng 42 kDa và được gọi là CHIT42-SH16. Nghiên cứu một số đặc tính cho thấy cho thấy CHIT42-SH16 hoạt động tốt nhất ở pH 7 và nhiệt độ 40°C. CHIT42-SH16 bền trong khoảng pH 3-9 và có khả năng duy trì hoạt tính trong khoảng nhiệt độ 10-60°C. Sự có mặt của các ion kim loại Al^{3+}, Fe^{2+} và Ca^{2+} làm tăng hoạt tính của CHIT42-SH16 lên lần lượt là 161%, 131% và 165%. Ngoài ra, CHIT42-SH16 cũng bền đối với các tác nhân hoạt động bề mặt (5% DMSO và 1% Triton-X100), chất kìm hãm (1 mM EDTA) và tác nhân biến tính (1 M urea). Những đặc tính trên cho thấy enzyme CHIT42-SH16 của chủng *T. asperellum* SH16 có tiềm năng ứng dụng cao trong kháng bệnh ở thực vật hay phân giải chitin trong phế thải các ngành chế biến.

***Từ khóa**: CHIT42, chitinase, đối kháng sinh học, ITS, Trichoderma asperellum.*

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