

The antifungal activity of a serine protease and the enzyme production of characteristics of *Bacillus licheniformis* TG116

Lijun Ling (✉ 13919343210@163.com)

Northwest Normal University

Wenting Cheng

Northwest Normal University

Kunling Jiang

Northwest Normal University

Zhenglong Jiao

Northwest Normal University

Hong Luo

Northwest Normal University

Caiyun Yang

Northwest Normal University

Mingmei Pang

Northwest Normal University

Lu Lu

Northwest Normal University

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Abstract

The growth of *Phytophthora capsica*, *Rhizoctonia solani*, *Fusarium graminearum*, *Fusarium oxysporum* and *Botrytis cinerea* were all inhibited by the fermentation supernatant of *Bacillus licheniformis* TG116, a biocontrol strain isolated from *Typhonium giganteum* Engl previously with broad-spectrum resistance to plant pathogens. The fermentation supernatant of the TG116 has a great stability on temperature and UV, and shows the biological activity of serine protease and cellulase. The antifungal protease produced by *Bacillus licheniformis* TG116 was purified to homogeneity by ammonium sulfate precipitation, DEAE Sepharose Fast Flow column chromatography and Sephadex G-50 column chromatography. When *Colletotrichum capsici* was used as an indicator strain, the protease exhibited great antifungal activity at a pH range from 5.0 to 11.0 and temperature at no higher than 60°C. Gene amplification verified the presence of a gene fragment of serine protease in the strain TG116. The antimicrobial substance isolated from the fermentation broth of TG116 is a serine protease component. The serine protease isolated and purified from *B. licheniformis* TG116 has a great antifungal activity.

Introduction

Diseases caused by pathogenic fungi lead to serious wastes of resources and economic losses in the pre-harvest, post-harvest, transportation and preservation of fruits and vegetables, and in animal husbandry (Leiter et al. 2017). In agriculture, growers use broad-spectrum chemical fungicides to control plant diseases caused by pathogenic fungi, however, these fungicides not only make pollutions to environment but also may remain in plant products, which have been of the biggest concerns to human health. Therefore, the use of biological control agents may be a safe alternative to the control of plant diseases (Prapasri et al. 2018). Biological control is an increasingly successful and broad measure used to reduce the negative effects of plant pathogens on agricultural practices and environment protection (Ciancio et al. 2016).

Among these diverse and beneficial microorganisms, endophytes can protect plants from plant pathogens by entering and colonizing plants without causing any signs of plant disease (Huang et al. 2016). Endophytic fungi mainly enter the roots of plants from the soil and spread to leaves, flowers and fruits through the vascular system (Hardoim et al. 2008; S et al. 2011). These endophytes colonize during part of (facultative endophytes) or all of (obligate endophytes) the plant's life cycle and exploit their advantages through this strategic interaction (Hardoim et al. 2015). They promote plant growth and prevent plants from harmful bacteria and fungi.

Endophytic bacteria increase plant growth rate and biomass mainly by acting on plant hormone synthesis, nitrogen fixation, phosphate dissolution and ammonium ion production (Agarwal et al. 2020). As biocontrol agents, they protect their hosts by interacting directly with pathogens and producing many antifungal molecules or by competing for nutrients in colonized tissues (Ryan et al. 2010). Biocontrol endophytes also act indirectly by triggering Induced Systemic Resistance (ISR) in plant hosts, which is the

result of plethora metabolites, produced by endophytes, that activate the plant defense priming responses against pathogens (Verhagen et al. 2011).

The important characteristic of endophytes is the secretion of lytic enzymes that degrade many biopolymers in the environment. Those cellulase, protease and chitinase, allow endophytic bacteria to enter plant tissues and form stable colonies, providing a clear competitive advantage for bacteria with this ability (Compant et al. 2005).

The inhibition and killing effect of biocontrol microorganisms on pathogenic fungi are related to the structure of the fungal cell walls (Calonje et al. 2000). As the cell wall composition of pathogenic fungi is complex, the degradation of pathogenic fungi cell wall is an important aspect. However most fungal cell walls contain chitin, protein, β -1.3-Glucan and lipids (Bowman and Free 2010). Due to the complex composition of the fungal cell wall, its degradation may require the interaction of multiple enzymes. Antifungal proteins have a more pronounced effect on pathogenic fungi, and when the cell wall of the pathogenic fungus is degraded by hydrolases, it exhibits bacteriostatic or bactericidal effects.

Studies have shown that most *Bacillus* can produce secondary metabolites with broad spectrum antibiotic activity and a very diverse structure. Therefore, *Bacillus* have broad application prospects in agricultural applications and medical applications (Moyne et al. 2004).

B. licheniformis TG116, an endophytic bacterium isolated from *Typhonium giganteum* Engl, has been reported in previous studies on its spectral inhibition against plant pathogens (Ling et al. 2014). In this study, the enzyme production of the aseptic filtrate of *B. licheniformis* TG116 were characterized. An important role that extracellular serine protease played in the process of biological control, and its enzymatic properties were also investigated in this study.

Materials And Methods

Strains and medium

B. licheniformis TG116, cultured as described previously study (Ling et al. 2014), which was used as a protease producer. The fermentation medium for TG116 was composed of peptone (0.5%), yeast powder (0.5%), and glucose (2%), with a pH value of 7.5. TG116 was cultured in a 1 L fermenter and incubated at 37 °C for 72 h. Pathogens *P. capsici*, *R. solani*, *F. graminearum*, *F. oxysporum*, *B. cinerea* and *C. capsici* were maintained on PDA medium.

Chemical reagents

The DEAE Sepharose Fast Flow, used in this research, was produced by Shanghai Rachel Biotechnology Co., Ltd. (Shanghai, China). Molecular primers were synthesized by The Beijing Genomics Institute (Beijing, China). Other reagents were purchased from Shanghai Watson Biotechnology Co. Ltd. (Shanghai, China)

Enzyme activity determination in fermentation broth

The activity of each enzyme such as cellulase, protease and chitinase was determined qualitatively by the ability of the bacterium to form clear zone on solid media. TG116 was cultured in LB liquid medium for 24 h, and sterilized by filtration to obtain a sterile fermentation broth (Youcef-Ali et al. 2014).

Cellulase activity

The activity of cellulase was assayed according to the method of Kumar et al. by measuring the formation of different hydrolyzed ring sizes (Kumar et al. 2014). Strain TG116 was grown in carboxymethyl cellulose (CMC) liquid medium at 30 °C for 2 d, and then cultures were centrifuged at 10,000 g for 20 min. Cellulase activity of bacterial cultures used in CMC agar medium and their supernatants against commercial cellulose. For cellulase secretion, CMC agar plates were incubated for 5 days at 37 °C to allow for the secretion of cellulase. Finally, the aqueous solution of Congo Red (1% w/v) was poured into the medium for 15 min then rinse extra Congo red with 1 mol·L⁻¹ NaCl solution for 15 min. The formation of different hydrolyzed ring sizes indicates the ability of cellulase to degrade cellulose in the medium.

Protease activity analysis of the TG116 antifungal protein

The activity of protease was detected by the Oxford Cup method. Based on the study of, the Oxford cups were put on the solid medium containing 1% skimmed milk powder, 200 µL crude enzyme solution were added to the Oxford cups. All the plates were incubated at 30 °C for 48 h.

Chitinase activity

The activity of chitinase was detected by the Oxford Cup method. Briefly, 200 µL crude enzyme solution in triplicate were added into the Oxford cups in solid medium containing 2% colloidal chitin. All the plates were incubated at 30 °C for 48 h.

Purification of protease

Culture solutions were centrifuged (17,400 g, 20 min) and ammonium sulfate was added to the supernatant to a final concentration of 40% to precipitate other components. After centrifugation, ammonium sulfate was increased to 70% to obtain a crude protease, which was then dialyzed in a dialyzer with a molecular weight cut-off of 1,000 Da. After the dialyzer was freeze-dried, the crude extract was redissolved in PBS, and the supernatant was filtered with a 0.22 µm filter and stored at -20 °C for use. All purification steps were carried out at 4 °C.

The enzyme solution was loaded onto a DEAE Sepharose Fast Flow column (16 mm x 400 mm) equilibrated with 10 mmol·L⁻¹ Tris-HCl buffer (pH 7.3), and the protease was eluted from the column with NaCl concentration of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 and 1 mol·L⁻¹ gradient solution. Each fraction was collected and the absorbance value was measured at 280 nm, and the elution curve

was made with the NaCl elution volume as the horizontal coordinate and the absorbance value as the vertical coordinate. The second peak sample was further separated by Sephadex G-50 molecular sieve chromatography. After elution with sterile water, the antifungal activity of each eluted peak was measured.

The positive fraction was evaporated and dissolved in 200 μL methanol, which was then divided into 20 μL aliquots for injection. A HPLC Agilent 1100 System (Agilent Technologies) was used with a SunFireTM C18 reverse phase column (250 x 4.6 mm, 5 μm ; Waters) and a methanol/water gradient at a flow rate of 0.2 $\text{mL}\cdot\text{min}^{-1}$ to perform the study (Zhao et al. 2012).

Antimicrobial activity assay

The diffusion plate method according (Shi et al. 2015) with slightly modified was used to determine the antimicrobial activity of the samples. Briefly, the plant pathogens *P. capsici*, *R. solani*, *F. graminearum*, *F. oxysporum*, *B. cinerea* and *C. capsici* were placed in the center of the Petri dish prepared agar medium and the sterile Oxford cup was then quickly placed in a petri dish 3 cm from the center of the pathogen, then 200 μL of sample was added after solidification of the medium. After incubation for 24 h at a temperature suitable for growth of the strain, the diameter of the zone of inhibition was measured in each case. Three parallel assays were performed for each sample.

Characterization of the purified antimicrobial protease

Molecular mass analysis

The purity and molecular mass of the antimicrobial protease were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% (w/v) separating gel and 4% stacking gel) as described by Pang *et al* (Pang *et al.* 2021) with some modification. The samples were boiled in the buffer for 5 min. Electrophoresis was performed at a constant current (20 mA) at 4 °C. After then, the gels were stained Coomassie Brilliant Blue R-250 for 30 min and decolorized with decolorizing solution for three times. The molecular mass of the purified protease was evaluated by comparing its electrophoretic mobility to marker proteins ran on the same gel.

Effect of crude protease solution on pathogenic fungi

The fungal hyphae from the periphery of the zone of inhibition produced by the *C. capsici* were observed under light microscope (400 $\times$) for morphological changes. Hyphae cultured under normal conditions as a control.

Enzyme assay

Detection of TG116 protein enzyme activity by revised Jin assay (Jin et al. 2011), one unit of enzyme activity is expressed as hydrolysis of casein per minute to yield 1 μg of tyrosine. A growing sample of *B.*

licheniformis broth (100 mL) was taken from a 500 L fermenter every 4 h and centrifuged at 1679 g for 10 min. The supernatant is used as a source of extracellular protease.

Mass spectrometry identification

The band corresponding to the antifungal protein was excised from an SDS-PAGE gel and sent to Huada Gene Company (Beijing, China) for the determination of its constituent peptides by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI- TOF-MS).

Cloning and amino acid sequence of the TG116 antifungal protein

The whole genome of *B. licheniformis* TG116 was used as a template to amplify the *Pase* using primer pairs *PaseF* (5'-GGTATTACGGGCGTCCAG-3') and *PaseR* (5'-AAGAAGTGCGGCATCAGG-3'). The PCR reaction conditions were: pre-denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 2 min before holding at 4 °C.

Agarose gel electrophoresis was used to detect the PCR products before they were sent for sequencing by Huada Gene Company (Beijing, China), to sequence and submit the obtained gene sequence to NCBI. Alignments of protein sequences were performed with the BLAST program from the NCBI (Thompson et al. 1994).

The nucleotide sequence of the gene encoding the extracellular serine protease gene has been submitted to the GenBank database.

Analysis of protein sequences

The protein encoded *Pase* were analyzed using the BLAST 2.0 program from the NCBI database. The presence and delimitation of protein domains were accomplished using the conserved domain database (CDD) (Marchler-Bauer et al. 2012).

Statistical analysis

Stability evaluation of the purified protein was performed in triplicate, and the mean diameters of inhibitory zones and standard deviations were determined. Data from different treatments were analyzed by ANOVA. $P < 0.05$ was considered statistically significant.

Results

Determination of activity and stability of sterile fermentation broth of TG116.

A qualitative study was conducted on the ability of TG116 to produce cell wall degrading enzymes. As the results show in Fig. 1AB, the strain produced protease and cellulase but no chitinase activity as showing Fig. 1C. The Fig. 1D showed crude protein of TG116 has good thermal stability, and its antifungal activity

remains basically unchanged with the increase of temperature. when the temperature reaches 100 °C, the residual activity of crude protein still maintains more than 95% of its original activity. With the increase of UV irradiation time, the bacteriostatic activity began to decrease slowly. After 6 hours irradiation, the antifungal activity began to decrease rapidly. After 10 hours of irradiation, the residual protease activity still retained 60% of the original bacteriostatic activity, so the TG116 crude protein had a certain UV tolerance in Fig. 1E.

Inhibitory spectrum of the crude protein

The crude protein showed broad-spectrum antimicrobial activity against some fungi as shown in Fig. 2, the protein has been de-genetically expressed against several pathogens such as *P. Capsici*, *C. Capsici*, *R. Solani*, *F. Graminearum*, *F. Oxysporum* and *B. Cinerea*. These data indicate that the protein encoded by *Pase* gene has broad application prospects in the prevention and control of agricultural microbial diseases.

As shown in Fig. 3, B and C compared with control A, the hyphae of *C. capsici* were distorted to bend, blend and break.

Purification and molecular weight determination of TG116 protease

The filtered culture supernatant of TG116 was separated and precipitated with saturated ammonium sulfate, and the result (Fig. 4) showed that the optimal saturation of the ammonium sulfate-precipitating antifungal protein was 70%. Then the protein precipitated by ammonium sulfate was concentrated and added to the DEAE-sepharose fast flow column. There were 12 peaks in the collection solution due to the different content of NaCl, and the five components (H, I, J, K, L) had antifungal effect (Fig. 5), and the antifungal effect is shown in Fig.6. Elution peak (I) was analyzed by SDS-PAGE. The fraction (I) was further purified by Sephadex G-50 molecular sieve chromatography to give a pure compound. (I) as shown in Fig. 7 lane C, the fraction included low impurity levels. After thorough dialysis, concentrate (I) and apply sterile water to the Sephadex G-50 molecular sieve column and elute the active peak (M) with sterile water (Fig. 8), the elution profiles were monitored by spectrophotometry at 280 nm, and its antifungal effect of is shown in Fig. 9. As shown in Fig. 7 and Fig. 10, the target band size was calculated to be approximately 86.17 kDa according to the different mobility of the standard proteins. The crude extract was concentrated and applied to a DEAE Sepharose Fast Flow (25 mm x 200 mm) column equilibrated with 0.02 mol·L⁻¹ Tris-HCl buffer (pH 8.5). After washing the column, active peak C was eluted using a stepwise gradient of 0.15-1 mol·L⁻¹ NaCl in 0.02 mol·L⁻¹ Tris-HCl (pH 8.5) buffer at a flow rate of 1.0 mL·min⁻¹. The elution profiles were monitored by spectrophotometry at 280 nm. Of the five active fractions, high performance liquid chromatography (I) was used to analyze the fraction, and acetonitrile of different concentrations was used as eluent. Three different components were added to show similar results to previous Sephadex G-50 molecular sieve chromatography as shown in Fig. 11.

Mass spectrometry analysis and identification of the TG116 antifungal protease

The results of mass spectrometry were shown in the Table 1. The top 10 proteins with the highest matching rate are oxalate decarboxylase, peptidase T, 2-cysteine peroxidase, aminopeptidase, extracellular serine protease, dihydrolipoyl dehydrogenase, putative cytosolic aminopeptidase, the putative aminopeptidase YSDC, the di-zinc aminopeptidase and the translation elongation factor TU. According to the migration rate of the standard protein, the relative molecular weight of M component was 86.17 kDa, which was the closest to the molecular weight of extracellular serine protease in the mass spectrometry analysis results, and the group was determined to be divided into extracellular serine protease.

The resistance to surfactant active agents

Fig.14B shows that the effect of different surfactants on the activity of the protease. The results showed that the inhibitory effect of the tested surfactants on protease activity decreased, and the inhibitory effect became stronger with the increase of concentration. Among these surfactants, EDTA has the strongest inhibitory effect, and when the concentration reaches $1.1 \text{ mmol}\cdot\text{L}^{-1}$, the protein activity is only 25%.

***Pase* gene PCR results**

According to the PCR amplification of the previous primer pairs, the length of the target cloned fragment was 1600 bp. The product was sent to Beijing Huada Gene Sequencing Company for analysis, and 1617 bp gene fragment was obtained. The results showed that the entire TG116 gene contained the gene sequence of the protease, named as *Pase*, and submitted to Genbank (MK659579). Compared with the gene sequence encoding extracellular serine protease (KZD86682.1), the similarity was 98.20%. The use of ORF to predict the protein primary structure of nucleic acid information indicated that the sequence is 1506 bp in length and encodes 501 amino acid residues with a complete protein coding region.

Through blast homology alignment, the protein sequence was found to belong to the protease gene superfamily, which was similar to the expected result. The protein encoded by the *Pase* gene has three conserved sequences (Fig 8): from 8-94 bp, associated with a peptidase inhibitor i9 (peptidase inhibitor i9) with an e value of $1.61\text{e-}10$, acting as a molecular chaperone and promoting Folding of mature peptidase; from 131-497 bp, a peptidase s8 family member (peptidase s8 family) with an e value of $1.28\text{e-}82$, as a lytic protein, can exist both inside and outside the cell and can be used at extreme temperatures and pH. from 308-450 bp, is a serine protease. On the surface of cells with an e value of $5.86\text{e-}45$, the domain participates in substrate binding, promotes conformational changes, and affects the stability of the site to the substrate.

Discussion

In this paper, we describe the use of *Bacillus licheniformis* TG116 isolated from *Typhonium giganteum* Engl. as well as the secondary metabolites secreted as antifungal products against plant pathogens. This strain showed strong antifungal and antagonistic activities, and the cell-free culture supernatants significantly inhibited yeast growth (inhibition zone $\geq 19 \text{ mm}$). As described by Barakate (Barakate et al.

2002), this strong activity expressed by the large repression zone on agar plates indicated that the isolate produced water-soluble antimicrobial metabolites. It is well known that several *Bacillus spp.* can produce biologically active molecules, including antifungal substances (Caldeira et al. 2011; Qi-Qin et al. 2012; Zhao et al. 2010) and the present study was in a good coordination with the previous findings.

The main effect of the protease on plant pathogens occurred during spore germination. It completely inhibited spore germination by preventing the appearance of normal germ tubes and led to abnormal branching and expansion of hyphae (Chitarra et al. 2010; Deepak et al. 2003). The protein showed effective inhibition of plant pathogen fungal mycelial growth (Ryan et al. 2010). Analysis of the functional domains of the TG116 antifungal protein identified many domains, but the main domain is the Peptidase_S8 domain, consisting of amino acids 131–497. The peptidase_S8 domain is a characteristic catalytic structural domain in the *B. subtilis* protease family. As this family is part of the serine protease family, this again demonstrates that our protein is a serine protease (Ji et al. 2020). The inhibitor I9 region or commonly referred to as the proto-domain, was found to have specific inhibitory activity towards the adjacent peptidase S8 structural domain until it was autocatalytically cleaved during maturation of the enzyme (Baker et al. 1992; Huang et al. 1997). This structural domain has also been found to act as a molecular chaperone, assisting the mature serine peptidase to fold correctly into its active conformation (Li et al. 1995; Shinde et al. 1997; Yabuta et al. 2001). The PA domain consists of 120 to 160 amino acids inserted between the His and Ser active site residues (Luo and Hofmann 2001). In addition, for the I9 domain (I9), its main function is to maintain the inactive state of the zymogen and prevent the substrate from entering the active site (Bryan 2002). The interaction between the PA and I9 domains leads to N-terminal cleavage, which will open the way for the substrate to enter the catalytic site and promote protease activity (Bergeron et al. 2000).

Microbial proteases secreted by bacteria breakdown proteins into their constituent monomers, accounting for approximately 40% of the total global enzyme production (Gupta et al. 2002). Most microbial proteases from the genus *Bacillus* are the most widely developed industrial enzymes, mainly used in uncertain agents (Gupta et al. 1999). All of these enzymes exhibited stability in the presence of various detergent components and were active under different conditions. Since the commercialization of the first alkaline protease Carlsberg from *B. licheniformis* in the 1960s used as an additive in detergents, proteases that are more alkaline have been purified and characterized, and significant biological activity and stability, extensive substrate specificity, short fermentation times and downstream flow application techniques have been demonstrated (Haddar et al. 2009).

Endophytic bacteria and host plants coexist to manage various environmental stresses. The rich biodiversity and biosynthetic diversity of endophytes make them a treasure trove of undeveloped natural products (Gaiero et al. 2013). Microbial production of a variety of chemical scaffolds with extensive plant interactions or protective properties may be desirable to provide a preference for endogenous life. The identification of these promising isolates can be of great use in a variety of fields, including clinical, industrial, and agricultural fields (Jasim et al. 2016).

Conclusion

In this study, *B. licheniformis* TG116 was verified that it has strong antifungal activity, especially against *C. capsici*. *B. licheniformis* TG116 had the potential to produce cell-wall-degrading enzymes but did not show any chitinase activities. Therefore, these bioactive ingredients may provide an alternative resource for the drug control of plant pathogens. The results of this study provided evidence of the potential presence of the protein encoded by the *Pase* gene in *B. licheniformis* TG116. This protein played an important role in protecting plants from harmful pathogens. Thus, the antifungal properties of proteins may be beneficial against plant pathogen diseases, demonstrating their ability as effective biocontrol agents for the effective management of these plant pathogen diseases. Further research is needed to determine the ability of this *Bacillus* strain to control diseases caused by other plant pathogens.

Declarations

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Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions:

Lijun Ling: Put forward experimental ideas and provide funds; Wenting Cheng, Kunling Jiang: Complete the experiment and write the first draft of the paper; Zhenglong Jiao, Hong Luo, Caiyun Yang, Mingmei Pang: The processing of Experimental data and the making of related Chart; Lu Lu: revise the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Table 1

Table 1 The blast result of the protein sequence in NCBI

Description	Mass
Oxalate decarboxylase	43582
Peptidase T	45566
2-cys peroxiredoxin	20548
Aminopeptidase	39249
Extracellular serine protease	85510
Dihydrolipoyl dehydrogenase	49873
Probable cytosol aminopeptidase	53863
Putative aminopeptidase YsdC	39544
Double-zinc aminopeptidase	49446
Translation elongation factor Tu	22084

Figures

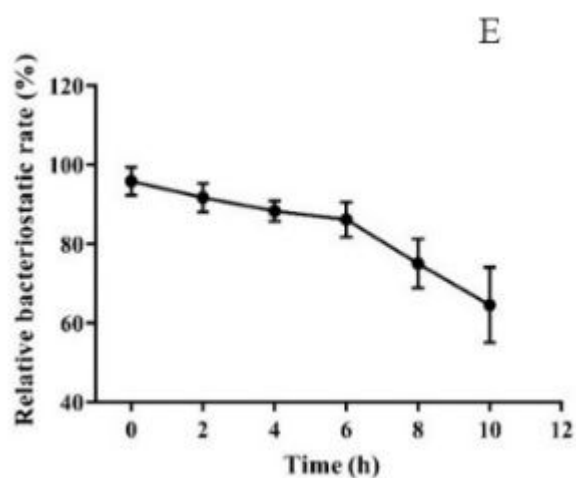
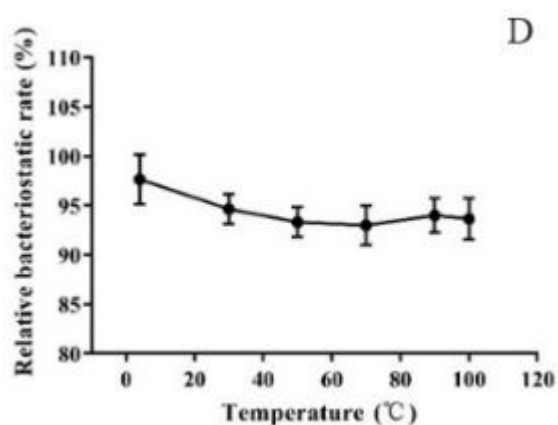
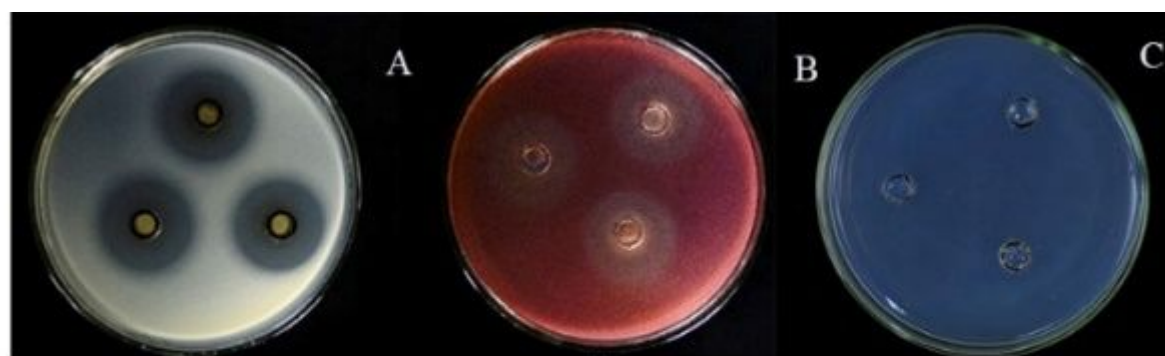


Figure 1

(A) Protease activity of fermentation broth. (B) Cellulase activity of fermentation broth. (C) Chitinase activity of fermentation broth. (D) (E) Activity and Stability of Sterile Fermentation Broth of TG116, Vertical bars represent standard deviation (n = 3)

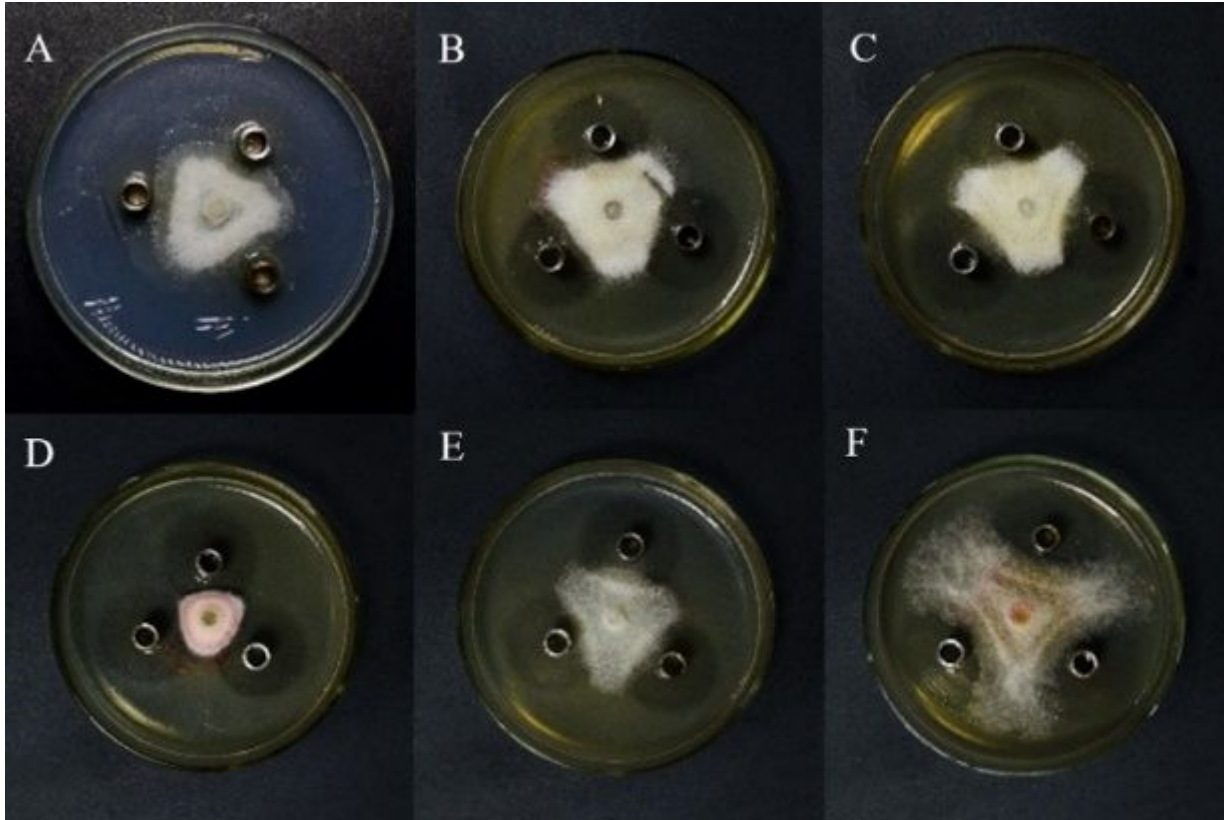


Figure 2

Bacteriostatic effect of plant pathogen by the crude protein (100 mL) of TG116

Note: A: *Phytophthora capsici*, B: *Colletotrichum capsici*, C: *Rhizoctonia solani*, D: *Fusarium graminearum*, E: *Fusarium oxysporum*, F: *Botrytis cinerea*



Figure 3

Effects of the antifungal protein E2 from endophytic *B. licheniformis* TG116 on hyphal growth of *Colletotrichum capsici* observed by the optical microscope (400×)

Note: (A) Hypha from untreated *Colletotrichum capsici* colony; (B) abnormal hyphal growth of *Colletotrichum capsici* treated with the antifungal protein

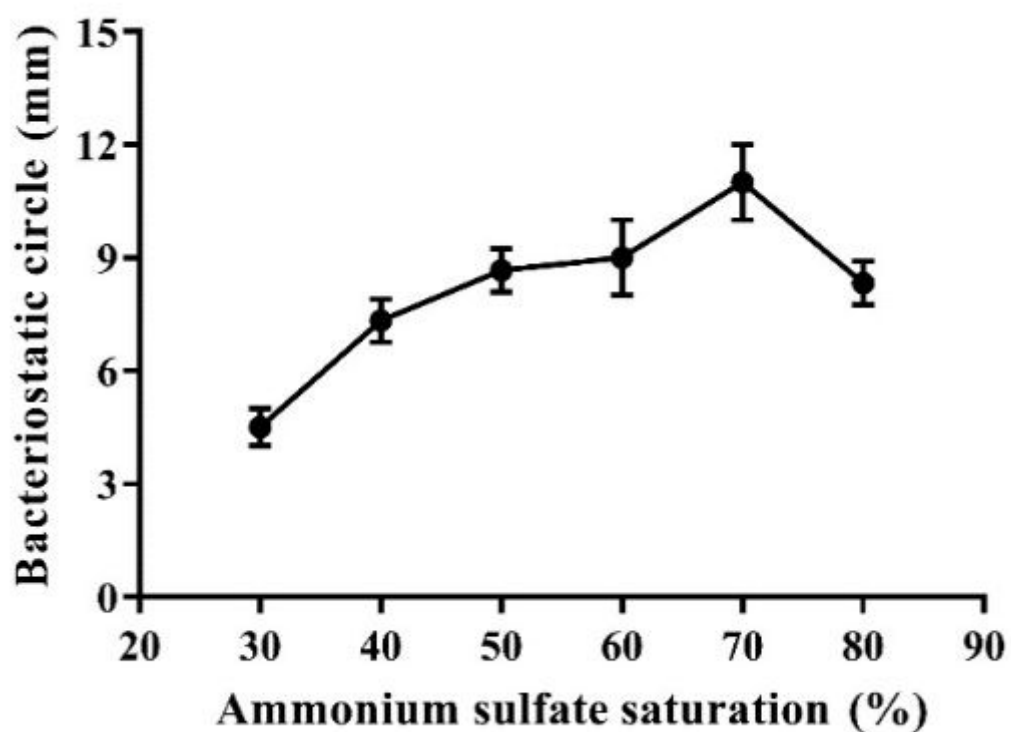


Figure 4

The effect the saturations of ammonia sulfate on the antifungal protein precipitation of TG116

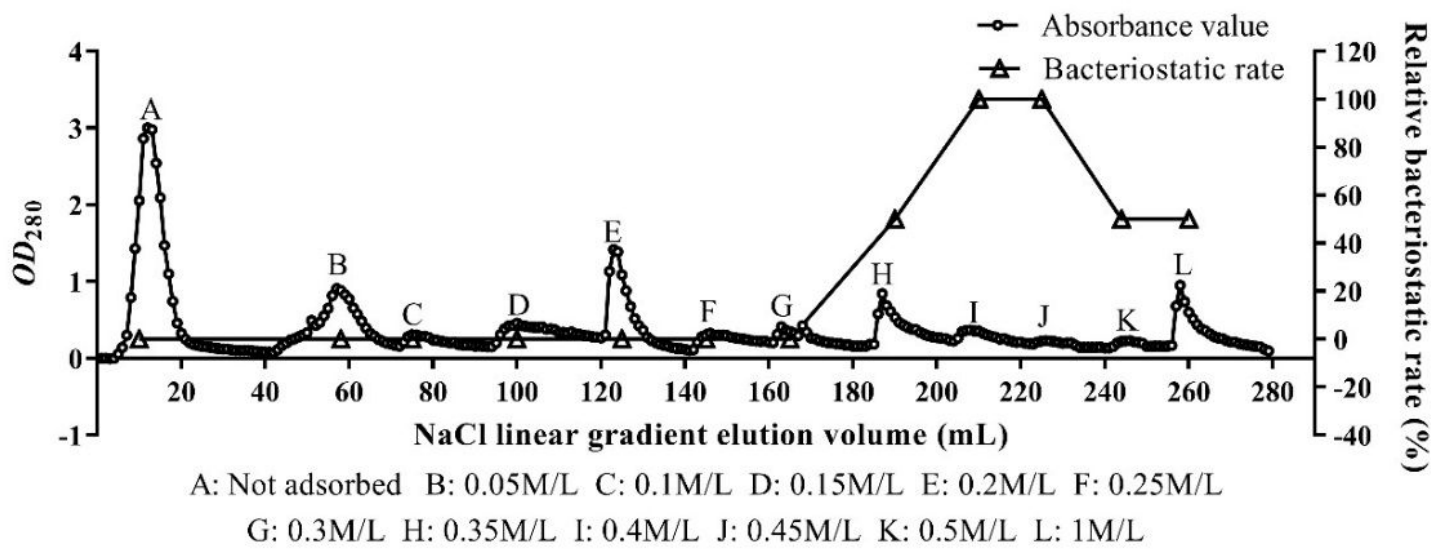


Figure 5

Ion exchange chromatography of crude extract from *Bacillus licheniformis* TG116

Note: A: Not adsorbed; B: 0.05 mol·L⁻¹; C: 0.1 mol·L⁻¹; D: 0.15 mol·L⁻¹; E: 0.2 mol·L⁻¹; F: 0.25 mol·L⁻¹; G: 0.3 mol·L⁻¹; H: 0.35 mol·L⁻¹; I: 0.4 mol·L⁻¹; J: 0.45 mol·L⁻¹; K: 0.5 mol·L⁻¹; L: 1 mol·L⁻¹

Figure 6

The bacteriostatic effect of components H, I, J, K and L separated by DEAE Sepharose Fast Flow, aseptic water as a control

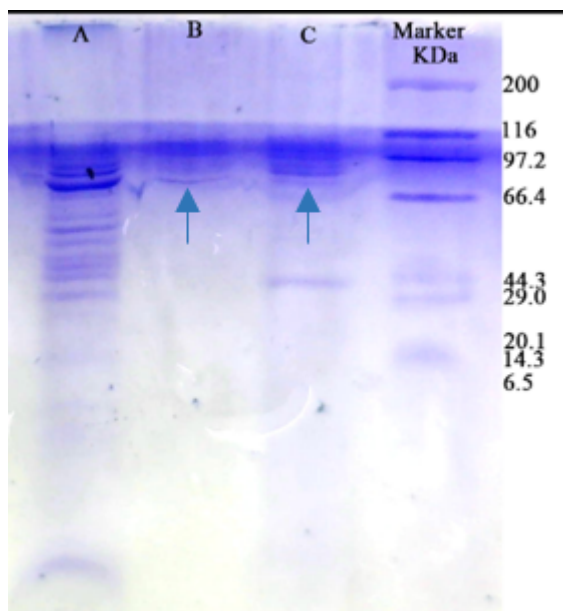


Figure 7

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of TG116 protease

Note: Lane Marker molecular weight markers; Lanes A Sterile fermentation broth total protein; Lanes BM fraction eluted from Sephadex G-50; Lane C I fraction eluted from DEAE Sepharose Fast Flow; respectively. The separated bands were visualized after Coomassie brilliant blue R-250 staining

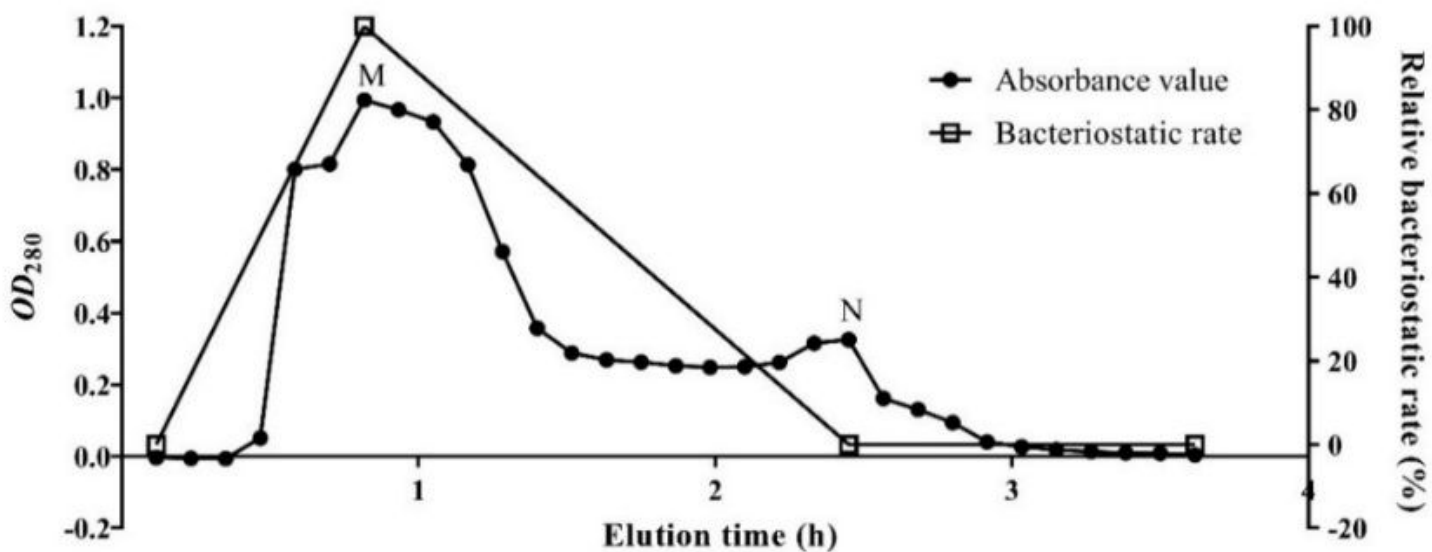


Figure 8

Sephadex G-50 molecular sieve chromatography of M

Note: The column was eluted with distilled water, at a rate of 2 mL·min⁻¹

Figure 9

The bacteriostatic effect diagram of component M was obtained by Sephadex G-50 molecular sieve chromatography

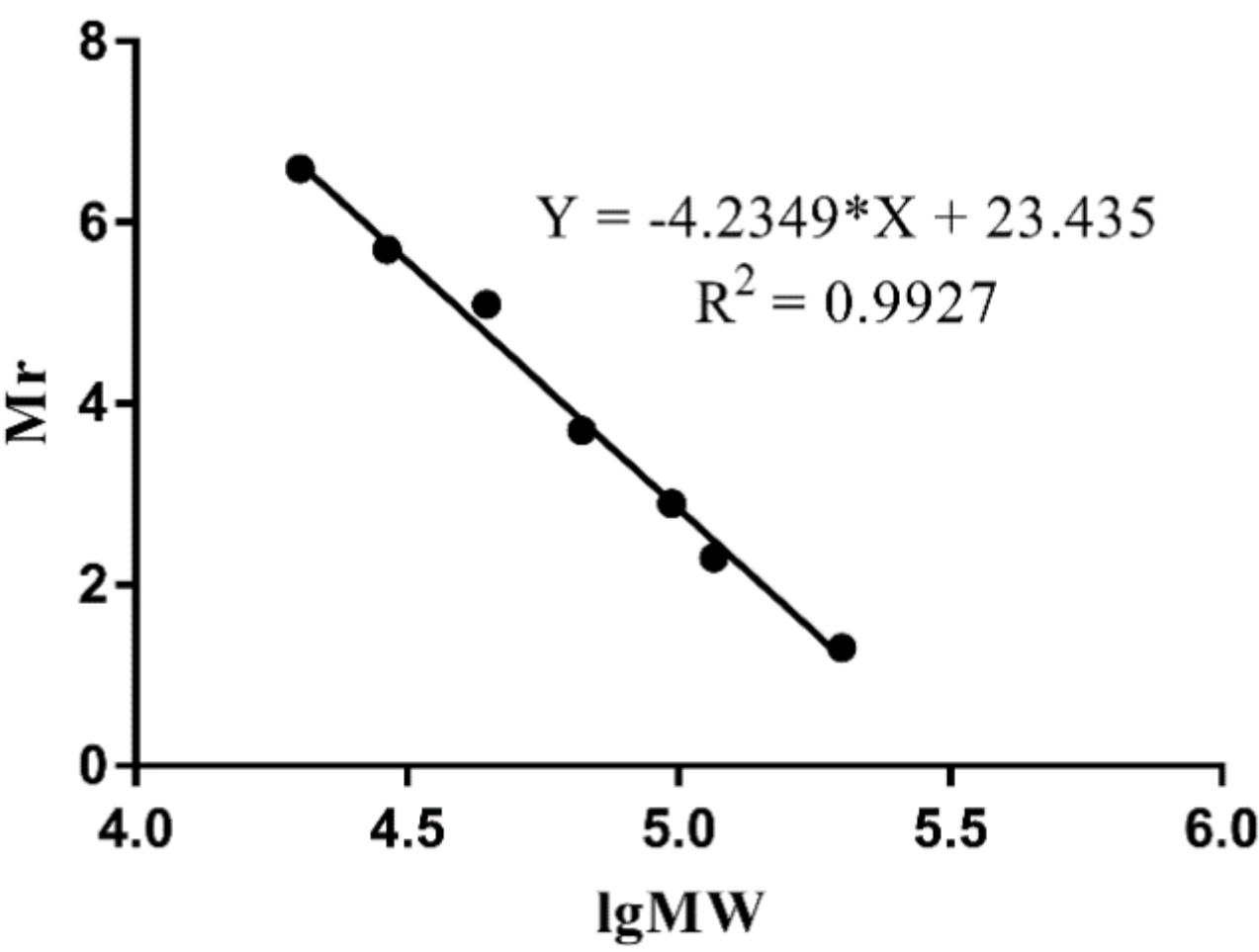


Figure 10

Standard protein migration rate standard curve

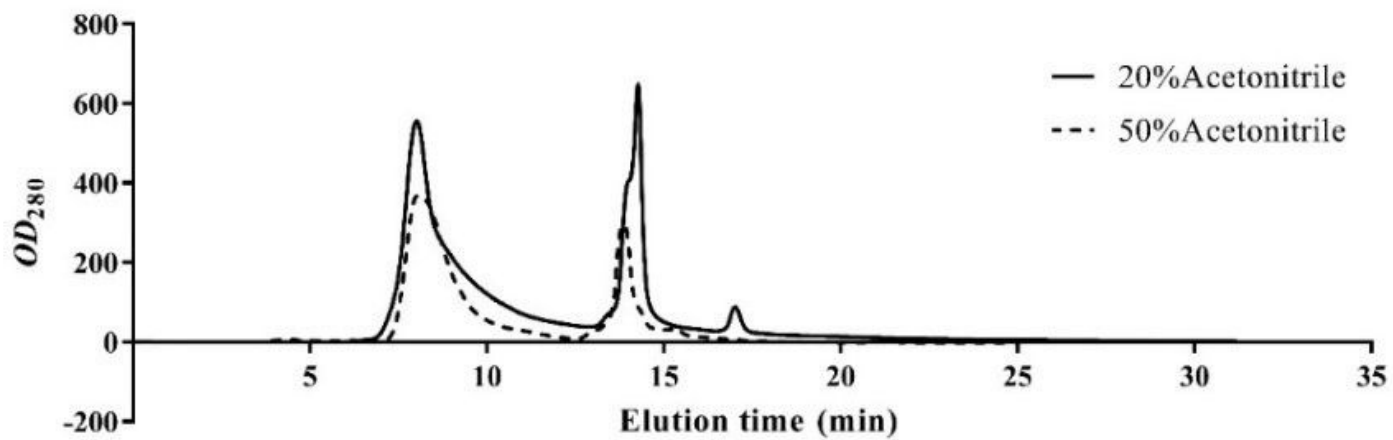


Figure 11

HPLC chromatogram of I fraction

Figure 12

Conserved domain prediction of Pase protein