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RESEARCH ARTICLE

Separation and Purification of Antimicrobial Protein of *Paenibacillus kribbensis* CX -7 Strain against *Fusarium oxysporum*

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Abstract

Plants wilt is a worldwide fungus soil-borne disease infected by *Fusarium*, the main pathogen is *Fusarium oxysporum*. The serious harm to the plant highlights the importance ofsearching new antimicrobial substances against wilt.*Paenibacillus kribensis* CX-7 strain with the ability of solubilizing phosphate and potassium was isolatedin previous study, and it had widely antagonism against pathogenic microorganism.Therefore, the aim of this study was to investigatethe potential antagonistic activity of *Paenibacillus kribbensis* CX-7 strainagainst *Fusarium oxysporum.*The crude extract was subjected to ammonium sulfate(NH_2SO_4) gradient precipitation, keeping the 50-60% saturationfraction in which activity was detected. Fraction 6 which had the most antibacterial activity was gottenby DEAE-Sephadex chromatography.It was a singleband, and the molecular weight was approximately 50 KDa detected by SDS-PAGE.The characters of fraction 6 showed that the suitable pH was 7.0, and the optimum temperate was 25℃ when it worked. Mn^{2+} and Mg^{2+} can activate it, while Cu^{2+}, Zn^{2+} and Na^{+} can inhibit it. The antimicrobial substance produced by*P. Kribbensis* CX-7 could be applied as an alternative orsupplementary method for *Fusarium oxysporum* control.

Introduction

Plants wilt is a worldwide fungus-caused soil-borne disease infected by *Fusarium*, the main pathogen is *Fusarium oxysporum*(Hao, et al., 2005). The plant could be infected from the root, then vascular bundle disease is caused and the plant died. The disease can occur in the whole growth period. Now in the worldwide, cotton, sugar cane, banana, tomato and melon are seriously infected by *Fusarium oxysporum.* The melon can be decreased by 20-30% infected by *Fusarium oxysporum* (Gao, et al., 2006).

The serious harm to the plant highlighted the importance ofsearching new antimicrobial substances against wilt. Several control strategies such chemical treatments have been considered, but they remain inefficient and difficult to apply. Biological control methods such as those using microorganisms that can suppress plant diseases represent a promising disease control alternative. And several biocontrol agents were identified, including species of *Trichoderma*, *Penicillium*, *Sporidesmium*, *Gliocladium*, *Burkholderia*, *Bacillus*, *Serratia*, and others (Asha et al.,2011; Ghorbany et al., 2010; Dihazi et al., 2012).

In previews study, seven multifunctional strains with the ability of solubilizing phosphate and potassium were isolated, and the most efficient strain (CX-7) was identified as *Paenibacillus kribbensis* after a series of physiological and biochemical experiments, morphological observation and 16S rRNA gene sequence analysis. Meanwhile, we found that CX-7 strain had widely antagonism against pathogenic microorganism including cotton yellow wilt pathogen, cotton wilt pathogen, wheat root rot diseases pathogen and wheat scab pathogens (Zhang et al. 2013). *Paenibacillus* defined in 1993 after analyzing 51 species of the genus *Bacillus*16S rRNA

gene sequences (Ash et al., 1993)is widely distributed in the environment, such as soil, water, plant rhizospheres andinside plant tissues (Ross, et al., 2001; Garbeva, et al., 2003;von der Weid, et al., 2002; Lal and Tabacchioni, 2009). Alongwith the different properties presented by the genus of *Paenibacillus*, oneimportant characteristic is the ability of producing a wide variety of secondary metabolites, such asantimicrobial substances and/or hydrolytic enzymes withactivity against a broad spectrum of microorganisms,including different pathogenic fungi (Kurusu, et al., 1987;Kajimura and Kaneda, 1997; Alvarez, et al., 2006; Aktuganov, et al., 2008; Tupinambá et al., 2008; Lal and Tabacchioni, 2009).

Paenibacillus kribbensis CX-7 which could solubilize phosphate and potassium had an effective role on the controlling of *Fusarium oxysporum*. To analyze the mechanism of this antagonism, we investigated the potential antimicrobial substance secreted by*Paenibacillus kribbensis* CX-7 strainagainst*Fusarium oxysporum* in this study. Protein separation and purification of *P. Kribbensis* CX-7 strainwas reported, together with the partial characterization ofthe antimicrobial substance.The antimicrobial substance produced by the*P. Kribbensis* CX-7strain would be important as an alternative orsupplementary method for *Fusarium oxysporum* control.

Materials and methods

Microorganisms and culture conditions

Paenibacillus kribbensis CX-7 strainwas preserved in the laboratory of Pharmaceutical Engineering of Hebei Agricultue University. It was grownin the media (sucrose 10.0 g, soybean powder 3.0 g, $MgSO_4$ 0.5 g, K_2HPO_4 1.0 g, yeast extract 0.5 g, $(NH_4)_2SO_4$ 0.5 g, distilled water 1000 mL) at 32℃for24–48 h. It was stored aerobically at room temperaturein NA (Nutrient Agar) slants supplemented with 1.2% agar and 1% $CaCO_3$(w/v). Fungal strain belonging to *Fusarium oxysporum*(provided by Plant Protection Institute of Hebei province) was grown in PDB (Potato DextroseBroth, Difco) at 25 ℃ from 2 to 7 days.

Extraction of antimicrobial protein of CX-7 strain

The CX-7 broth supernatant was collected by centrifuge (10 min, 8000 r/min). After a linear gradient 10%、20%、30%、40%、50%、60%、70% and 80% $(NH_4)_2SO_4$ was added in the supernatant, the sediments were collected each by centrifugation (10 min, 8000 r/min). Then the sediments were dialyzed using 10,000 MW dialysis tubing cellulose membrane. Antagonism experiment was done by using the protein sediments of different $(NH_4)_2SO_4$ concentration. The optimal $(NH_4)_2SO_4$ concentration can be determined (Karossi, 2012).

Antimicrobial substance (AMS) activity assay

The overlay method described by Rosado and Seldin(1993) was used to detect antimicrobial activity. The culture plate was made containing 5 mL *Fusarium oxysporum*spore suspension (about 1.0×10^8 CFU/mL) and 20 mL PDA media. 2.0 mm hole was perforate at the plate. Then 50 μL CX-7 protein solution was added into the hole. Antimicrobial substance activity was indicatedby a clear zone of inhibition around the hole after incubation for 72 h at 25 ℃.

Purification of antimicrobial protein of CX-7 strain

The DEAE-Sephadex chromatograph column was balance using 0.01 mol/L Tris-HCl buffer (pH 7.5) from AKTA explorer high pressure liquid chromatography system (HPLC). The injection volume was 200 μL and detection wavelength was 280 nm. The absorption peak fractions were collected in a flow rate of 0.4 mL/min with 0.05~2 mol/L NaCl gradient elution (0.05~2 mol/L). The fraction was concentrated by Alpha-6 vacuum freeze-drying machine and the antimicrobial activity assay was done as above(Zhao et al., 2008).

Fractions purity and molecular weight

Antimicrobial protein fractions purity and molecular weight were determined by SDS-PAGE method(Jian,2012).Theconcentrated gel concentration was 5%, and the separating gel concentration was 12%, the gel was 1mm thick and the dye was coomassie blue R250 (Zhang et al., 1997).

Optimum temperature and thermal stability of antimicrobial protein

Antimicrobial protein lyophilized powder (0.5 g) were dissolved into 100 mL 0.02 mol/L pH 7.5 Tris-HCl buffer, then 50 mg/mL protein solution can be gotten. The antimicrobial activity was determined after heat preservation for 18 h at the temperature 4℃, 25℃, 30℃, 37℃, 42℃, 50℃ and 60℃ condition or for 30 min at the temperature 80℃, 100℃ and 115 ℃, or for 20 min at the temperature 121℃. The antimicrobial activity was 100% in the process at 37℃.

Appropriate pH and pH stability of antimicrobial protein

The antimicrobial protein lyophilized powder were dissolved into 0.05 mol/L pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0 and pH 8.0 citric acid - Na_2HPO_3 buffer

or pH 9.0, pH 10.0 and pH 11.0 glycine-NaOH buffer,50 mg/mL protein solution can be gotten. The antimicrobial activity was respectively determined after heat preservation for 2 h at appropriate temperature. The antimicrobial activity was 100% in pH 7.2 buffer.

Effect of metal ion on antimicrobial protein activity

$CaCl_2$, $CuSO_4$, $MgCl_2$, $MnCl_2$, $FeCl_3$, $ZnCl_2$, KCl and NaCl were dissloved separately to 0.02 mol/L pH 9.0 Tris-HCl buffer, then 5 mmol/L metal ion solution can be get. Then CX-7 antimicrobial protein lyophilized powder was dissolved in the different metal ion solution, the terminal concentration was 50 mg/mL. The antimicrobial activity was respectively determined after heat preservation for 30 min at 37℃. The contrast was the activity of antimicrobial protein with no metal ion.

Results and Discussion

Extraction of antimicrobial protein of CX-7 strain

The CX-7 broth sediments were collected by centrifuge (10 min, 8000 r/min) treated by different concentrations $(NH_4)_2SO_4$ salting. Then they were dissolved to pH 6.5 K_2HPO_4-KH_2PO_4 buffer respectively and the antimicrobial activities (inhibiting zone) were measured with *Fusarium oxysporum* pathogen as indicator. The inhibiting zone was 20 mm (Fig1), when $(NH_4)_2SO_4$ salting concentration was 55%. With the $(NH_4)_2SO_4$ salting concentration increasing, there were no inhibiting zones. From above, we can draw a conclusion that the most antimicrobial substance can be gotten at 55% $(NH_4)_2SO_4$ salting concentration from CX-7 broth.

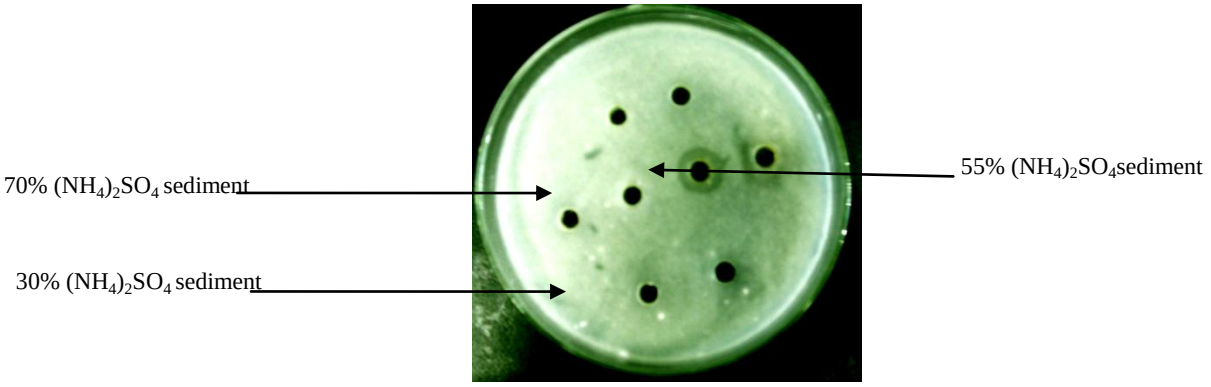
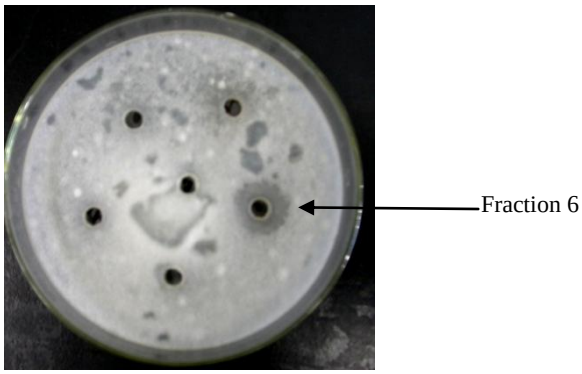


Fig.1 Antimicrobial activities of CX-7 at different $(NH_4)_2SO_4$ salting concentrations

Purification of antimicrobial protein of CX-7 strain

High pressure liquid chromatography system(HPLC) was used to purify the antimicrobial protein. For detectingthe wavelength at 280 nm,the absorption peak fractions were collected in a flow rate of 0.4 mL/min with 0.05~2 mol/L NaCl gradient elution. Nine fraction samples can be gotten, of which 6 fractions were measured having antimicrobial activities. But the activitieswere different. Fraction 6 was the best one of these. The inhibiting zone can be reached 25mm with *Fusarium oxysporum* pathogen as indicator(Fig. 2).

Fig.2 Antimicrobial activities of the different fractions of CX-7strain



Purity and molecular weight of fractions

Antimicrobial protein fraction purity and molecular weight were determined by SDS-PAGE methods. Of the 9 fractions, only fraction 6 had single band in the electrophoresis, and the molecular weight was about 50KD (Fig. 3). So in the bio-control field, it will be produced as a kind of fungicide, and be used in many plants.

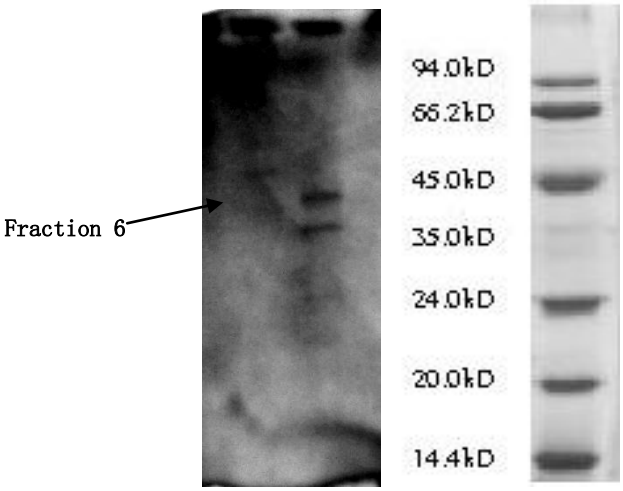
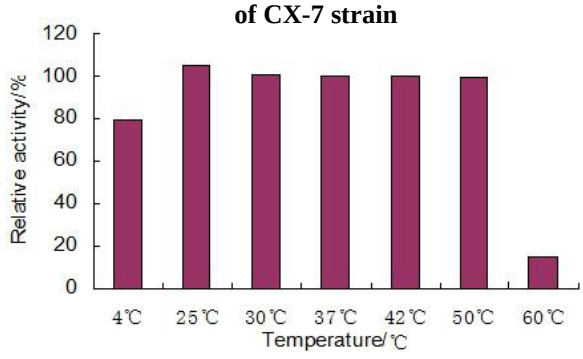


Fig.3 Electrophoresis of the antagonistic protein by SDS-PAGE （M：marker）

Optimum temperature and thermal stability of antimicrobial protein of CX-7

The antimicrobial activitiesof antimicrobial protein were determined by using 50mg/mL solution after heat preservation for 18h at 4℃, 25℃, 30℃, 37℃, 42℃, 50℃ and 60℃, for 30min at 80℃, 100℃ and 115℃, and for 20min at 121℃. The antimicrobial activity was 100% in the process at 37℃. In the experiment, the antimicrobial activity had no activity in the treatment of 80℃ for 30min, and the optimal temperature was 25℃(Fig. 4).

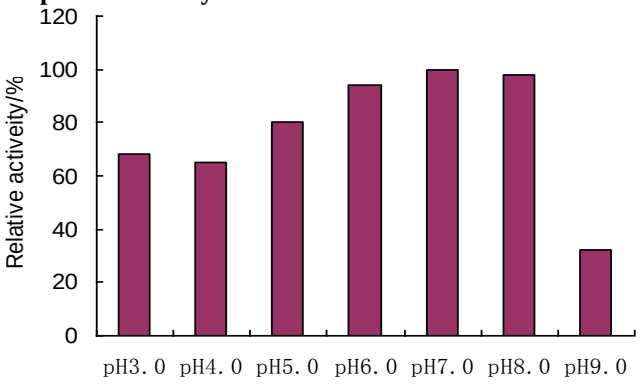
Fig.4 Thermal stability of antagonistic protein of CX-7 strain



Appropriate pH and pH stability of antimicrobial protein of CX-7

The CX-7 strain antimicrobial activity can be determined at different pH buffer with *Fusarium oxysporum* as pathogenindicator. From the experiment, we know that the antimicrobial protein activity was stable during pH 5.0-8.0 and the optimum is pH7.0 (Fig. 5).

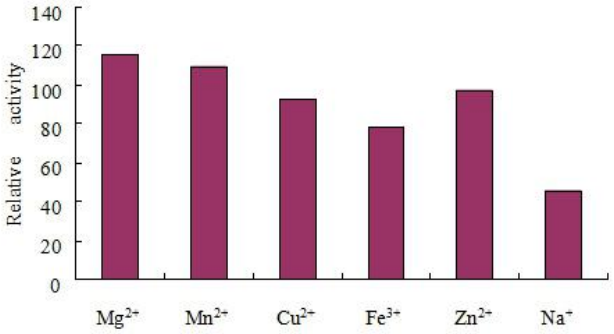
Fig.5 The optimal pH and stability of antagonistic protein activity of CX-7 strain



Effect of metal ion on antimicrobial protein activity

The CX-7 strain antimicrobial activity can be determined at different metal ion buffer with *Fusarium oxysporum* as pathogenindicator. From the experiment, we know that the antimicrobial protein activity can be activated by Mg^{2+} and Mn^{2+} and it would be inhibited by Cu^{2+}, Fe^{2+}, $Zn^{2+}$and Na^{+} (Fig 6).

Fig.6 Effect of different metal ion on antagonistic protein activity of CX-7 strain



Conclusion

The preliminary studies on the antimicrobial substance of CX-7 strain showed that CX-7 can produce a molecular weight of 50KD protein substance. The antimicrobial activity had been measured at different condition with *Fusarium oxysporum* as indicator pathogen. It showed that the antimicrobial protein had high activity during pH6.0-8.0. So it had the ability of acid and alkali resistance and the optimum was pH7.0. It maintain high antimicrobial activity during 25-50℃, but it was inhibited at 60℃. So the CX-7 antimicrobial protein can not resist to high temperature and the optimal temperature was 25℃. In the experiment of effect on the antimicrobial activity by different metal ions, it showed Mg^{2+} and Mn^{2+} can activated it, but it will be inhibited by Cu^{2+}, Fe^{2+}, Zn^{2+} and Na^{+}.

In this study, the purification and character of the antimicrobial protein had been studied. Although the fraction 6 had been separated and purified, but the amino acid sequence has not finished and the mechanism of antimicrobial protein would be studied further.

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