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Isolation and characterization of an antifungal protein from *Bacillus licheniformis* HS10

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ABSTRACT

Bacillus licheniformis HS10 is a good biocontrol agent against *Pseudoperonospora cubensis* which caused cucumber downy disease. To identify and characterize the antifungal proteins produced by *B. licheniformis* HS10, the proteins from HS10 were isolated by using 30–60% ammonium sulfate precipitation, and purified with column chromatography on DEAE Sepharose Fast Flow, RESOURCE Q and Sephadex G-75. And the SDS–PAGE and MALDI-TOF/TOF-MS analysis results demonstrated that the antifungal protein was a monomer with molecular weight of about 55 kDa, identified as carboxypeptidase. Our experiments also showed that the antifungal protein from *B. licheniformis* HS10 had significantly inhibition on eight different kinds of plant pathogenic fungi, and it was stable with good biological activity at as high as 100 °C for 30 min and in pH value ranged from 6 to 10. The biological activity was negatively affected by protease K and 10 mM metal cations except Ca^{2+}.

1. Introduction

Cucumber downy mildew (CDM), is an air-borne disease caused by *Pseudoperonospora cubensis* (Berk.et Curt.) Rostov, which is one of the most important cucumber leaf diseases in cucumber cultivation areas of more than 70 countries around the world [1]. Currently, the control of cucumber downy is mainly depending on chemical pesticides, such as Metalaxyl, Propamocarbmetalaxyl, Fosetyl, Mancozeb, Chlorothalonil [2,3]. And these chemicals have limited disease control effects and serious environmental pollutions. Therefore, the biological control of cucumber downy is going into sights as a potential method due to its safety [4,5].

Bacillus is a common advantage of microbial populations, which is widely distributed in plants, soil and plant microflora. It has good capability of stress resistance, disease prevention and increase yield for crops [6]. *Bacillus licheniformis*, a potential *Bacillus* strain, has developed metabolic system and rich extracellular antifungal substances, including glucanase [7], chitinase [8], antifungal protein [9], peptide [10] and lipopeptide [11].

Our previous studies showed *B. licheniformis* HS10 had a good biocontrol effect against variety of pathogens, and also had good activity of enzymes such as protease, chitinase and cellulase (unpublished). This study reported that an extracellular protein could contributed to the stable and highly antifungal activity of *B. licheniformis* HS10.

2. Materials and methods

2.1. Preparation of the tested strains

Antagonistic strains: *B. licheniformis* HS10 was isolated from a healthy cucumber rhizosphere surrounded by cucumber plants that died of cucumber downy mildew in a cucumber field in Huai'an city, Jiangsu province.

Pathogenic strains: *Fusarium graminearum*, *Bipolaris sorokinianum*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia cerealis*, *Bipolaris maydis*, *Gaeumannomyces graminis* and *Pseudoperonospora cubensis* were preserved in our lab.

Bacteria was cultured in LB medium (peptone,10 g; yeast extracts, 5 g; NaCl, 10 g; agar, 18 g and distilled water, 1 L).

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P. cubensis was cultured with in vivo leaf blade, and the other eight kinds of pathogenic fungi were cultured in PDA medium (potato, 200 g; glucose, 20 g; agar, 18 g; and distilled water, 1 L).

2.2. Purification of the antifungal protein from B. licheniformis HS10

2.2.1. Extraction of crude protein from B. licheniformis HS10

B. licheniformis HS10 was cultured with shaking at 200 rpm in 500 ml LB medium for 48 h at 28 °C. The supernatant was collected with centrifugation (8000 *g* for 20 min). Then solid ammonium sulfate was slowly added to the supernatant to reach about 30–60% saturation. The precipitate was collected after standing at 4 °C overnight and centrifugated (12,000 *g* for 20 min), then was redissolved in 5 ml 20 mM PBS (pH7.2), and dialyzed extensively with ultrapurified water for 2 days to remove ammonium sulfate. Finally, crude proteins were yielded with freeze-drying method at 0 °C.

2.2.2. Isolation of antifungal protein

The crude extract was passed through a DEAE-Sepharose Fast Flow column (2.5 cm × 20 cm) on an AKTA Prime system (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with 20 mmol/L PBS (pH 7.2), and then the column was washed with the same buffer to remove unabsorbed proteins. The absorbed proteins were eluted with a linear gradient of 1 mol/L NaCl from 0% to 100% concentration in 20 mmol/L PBS (pH 7.2) at a flow rate of 2 mL/min. Antifungal activity of the proteins against *P. capsici* was monitored in each fraction. The antifungal fractions were pooled, and then applied to a RESOURCE Q column (1.6 cm × 3 cm) in the same methods. At last, active fraction respectively was injected to Sephadex G-75 column (2.6 × 50 cm) on an AKTA Prime system (Amersham Biosciences) pre-equilibrated with 20 mmol/L PBS (pH7.2), the column was eluted with buffer at a flow rate of 2.0 mL/min. Individual peak fractions were collected and condensed in a cut-off dialysis tubing. After dialysis with ultrapurified water, the samples were used for further analysis. All purification steps were performed at room temperature, and the column effluent was monitored by absorbance at 280 nm.

2.2.3. Molecular mass determination and peptide blasting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12% T, 5% C) was performed according to the method of Laemmli [12]. Gels were stained in 0.1% (w/v) Coomassie blue R250 −30% (v/v) methanol −10% (v/v) acetic acid in water. The destaining solution was 30% (v/v) methanol −10% (v/v) acetic acid in water. After being electrophoresed by SDS–PAGE, the band of protein C1 was excised and sent to Nanjing Medical University to determine peptide fractions of the protein by MALDI-TOF/TOF-MS [13].

2.2.4. Protein content determination

The protein contents were determined by Bradford's procedure (Bradford, 1976) [14].

2.3. Inhibition spectrum of antifungal protein

Using eight kinds of pathogenic fungi as the indicator, the antifungal activity of protein were tested by the cylinder-plate method [15]. After the mycelial colony had developed, sterile blank Oxford-cups (0.5 cm in diameter) were placed at a distance of 3 cm away from the rim of the mycelial colony. Each 30 μg purified antifungal protein sample in 20 mmol/L PBS (pH 7.2, 200 μL) was added into Oxford-cups and buffer (without protein) was used as control. Then, the plates were incubated at 25 °C for 72 h until the mycelial growth in control plates had enveloped peripheral disks. At the same time, the treated samples with antifungal activity had produced crescents of inhibition around Oxford cups. On the other hand, *P. cubensis* (10 μL, 10^5 sporangium/mL) was cultured in each leaf blade (0.625 cm in diameter) after the leaf blades were immersing in protein solution for 5 min. The control treatment was only treated with *P. cubensis* or buffer (pH 7.2). All of the plates were incubated at 25 °C for 10 days. All treatments were conducted in triplicate. Each trial was repeated three times.

2.4. Effect of temperature, pH, metal ions and enzymes on stability and activity of the antifungal protein

The effect of enzymes, temperature and pH on stability and antifungal activity of the protein was determined as described by Bizani and Brandelli [16]. The protein was incubated with 1 mg/ml proteinase K for 60 min at 37 °C. The effect of pH was investigated when pH was 2–12 for 1 h. To analyze the thermal stability, the antifungal protein was exposed to temperatures ranging from 50 to 100 °C for 30 min. The effect of the ions was determined with the trearment of 10 mM Ca^{2+}, K^+, Na^+, Li^+, Ag^+, Zn^{2+} with the protein. The antifungal activities of the protein was determined after its incubation with different enzymes, different temperature and different pH. Three repetitions were included in each treatment, and each trial was repeated three times.

3. Results

3.1. Purification and identification of antifungal protein

The fermentation supernatant of *B. licheniformis* HS10 was subjected with relative 30–60% saturation of ammonium sulfate for precipitation. After dissoluted, dialysised and filtrated, the crude protein was applied to DEAE-52, RESOURCE Q anion exchange chromatography and G-75 gel column chromatography. Only one main peak of protein was recovered individually from crude protein by those three chromatography. As Fig. 1A showed, the peak C1 was an obvious protein peck, and it was also tested to have the antifungal activity. Then the C1 was dialyzed, concentrated and saved in freezer for SDS–PAGE analysis. The concentrated C1 was analyzed by SDS–PAGE. Only single main band with molecular mass about 55 kDa was found (Fig. 1B). The 55 kDa band on SDS–PAGE gel was cut off and recovered for identification using MALDI-TOF/TOF-MS in Nanjing Medical University testing center. The sequence was analyzed by blast in NCBI database. Five highest matching rate proteins were carboxylase, α-amylase, two kinds of similar catalases and phage-like protein (Table 1). The relative molecular mass of C1 appeared to be 55 kDa. Then we effectively isolated and identified this protein for the subsequent antifungal bioassay.

3.2. Antifungal bioassay of protein towards different pathogenic fungi

The antifungal spectrum of the secreted protein (30 μg) from *B. licheniformis* HS10 was shown in Table 2 and Fig. 1C. The results showed that the protein had a broad spectrum antifungal activity against seven kinds of plant pathogenic fungi except *Rhizoctonia cereal*, and the inhibition zone diameter ranged from 8 mm to 16 mm. Moreover, the protein could also significantly suppress *P. cubensis* on cucumber leaves (Fig. 2).

3.3. Stability of the antifungal protein

3.3.1. Effect of pH and temperature on the antifungal activity of the protein

The results shown in Fig. 3A, different pH conditions had a great effect on the antifungal activity of protein (30 μg). The antifungal

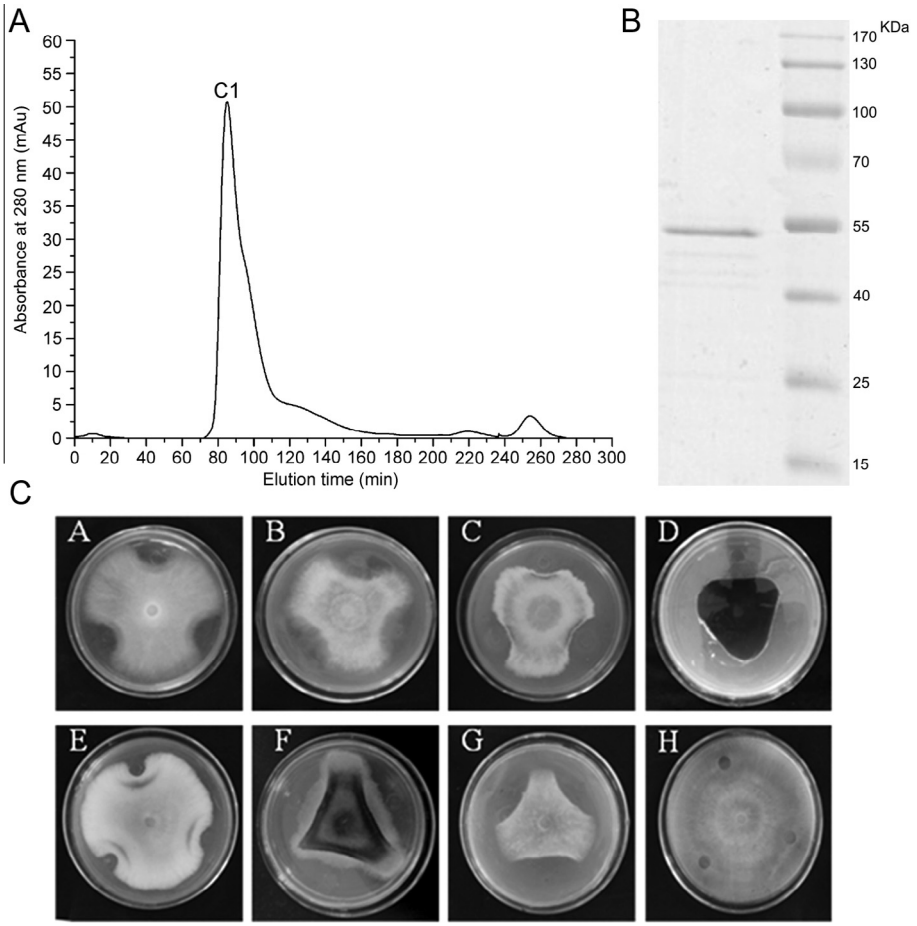

Fig. 1. Purification and identification of antifungal protein from *B. licheniformis* HS10 by gel filtration (A) and SDS–PAGE (B), and the antifungal activity test (C). *Note:* (A) The fraction was pretreatment by 30–60% (v/v) ammonium sulfate, DEAE-Sepharose Fast Flow column (2.5 cm × 20 cm), RESOURCE Q column (1.6 cm × 3 cm) and Sephadex G-75 column (2.6 × 50 cm) on an AKTA Prime system (Amersham Biosciences). (B) Left lane: peck C1 (purified antifungal protein) separated by Sephadex G-75; Right lane: protein molecular mass marker. (C) A: *Phytophthora capsici*; B: *Botrytis cinerea*; C: *Sclerotinia sclerotiorum*; D: *Bipolaris maydis*; E: *Fusarium graminearum*; F: *Bipolaris sorokinianum*; G: *Gaeumannomyces graminis*; H: *Rhizoctonia cerealis.* Each sample was tested by 30 μg protein in 20 mM PBS buffer (pH 7.2).

Table 1
The blast result of the protein sequence in NCBI.

Protein ID	Molecular functions	Sequence length	Sequence coverage [%]	Mol. weight [kDa]	Organism
gi|510141549	Putative carboxypeptidase	547	65.4	60.61	*B. licheniformis*
gi|429844636	Alpha-amylase	512	70.3	58.529	*B. licheniformis*
gi|510144704	Vegetative catalase KatA	485	71.3	54.87	*B. licheniformis*
gi|383441191	Vegetative catalase 1	485	67.2	54.82	*B. licheniformis*
gi|383438495	Phage-like protein	448	41.3	48.632	*B. licheniformis*

Table 2
Analysis of antifungal activity of the protein from *B. licheniformis*.

Indicator strains	Inhibition zone/mm
Phytophthora capsici	11.3
Botrytis cinerea	11.0
Sclerotinia sclerotiorum	8.5
Bipolaris maydis	14.1
Fusarium graminearum	7.5
Bipolaris sorokinianum	15.6
Gaeumannomyces graminis	14.8
Rhizoctonia cerealis	0

activity of the protein was highest with pH = 7. With the increase or decrease of pH, the antifungal activity was significantly decreased, especially in lower pH, it even lost in pH = 2. This result indicated that the acidic condition was not conducive to the antifungal activity of the protein.

The protein still had high antifungal activity after incubation in 25, 50, 60, 70, 80, 90 and 100 °C for 30 min. The antifungal activity decreased by 21.2% after incubation in 100 °C for 30 min (Fig. 3B). This result showed that the antifungal protein from *B. licheniformis* HS10 had good thermal stability.

As shown in Fig. 4A–H, the antifungal protein from *B. licheniformis* HS10 was only affected by 10 mM Ca^{2+}, but not K^+, Na^+, Li^+, Ag^+, Zn^{2+}. Meanwhile, the antifungal activity decreased 14.5% when the protein incubated with proteinase K at 37 °C for 1 h (Fig. 4I). The result showed that the antifungal activity of the protein was less affected by metal cations and proteinase K.

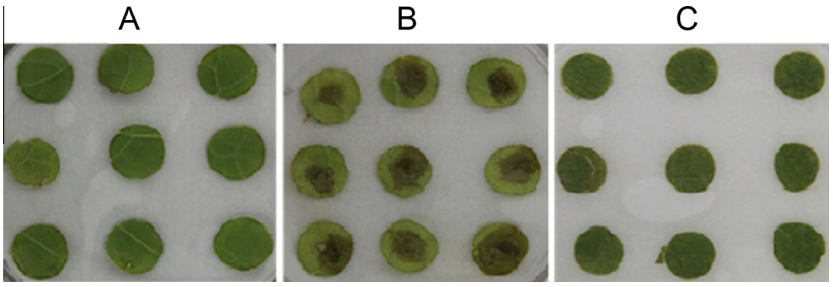

Fig. 2. The antifungal activities of protein against *P. cubensis*. *Note:* (A) CK; (B) *P. cubensis*; (C) *P. cubensis* and protein.

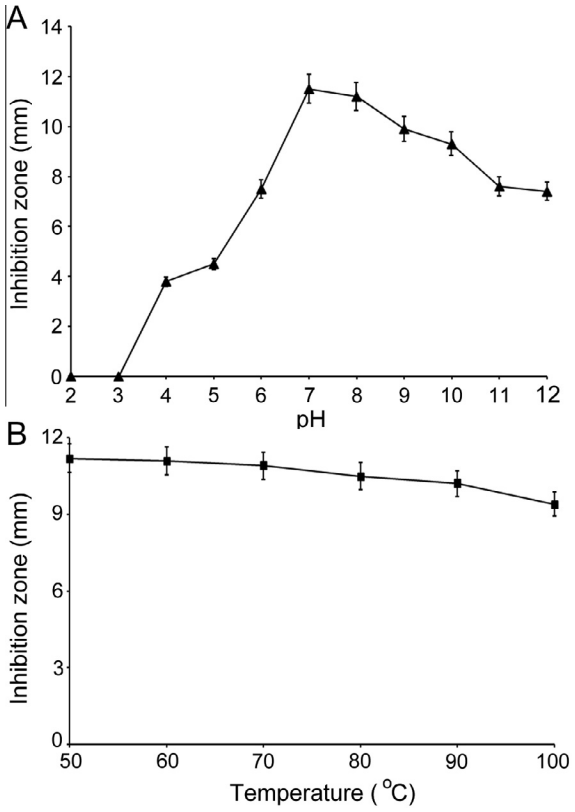

Fig. 3. Effect of pH (A) and temperature (B) on antifungal activity of protein.

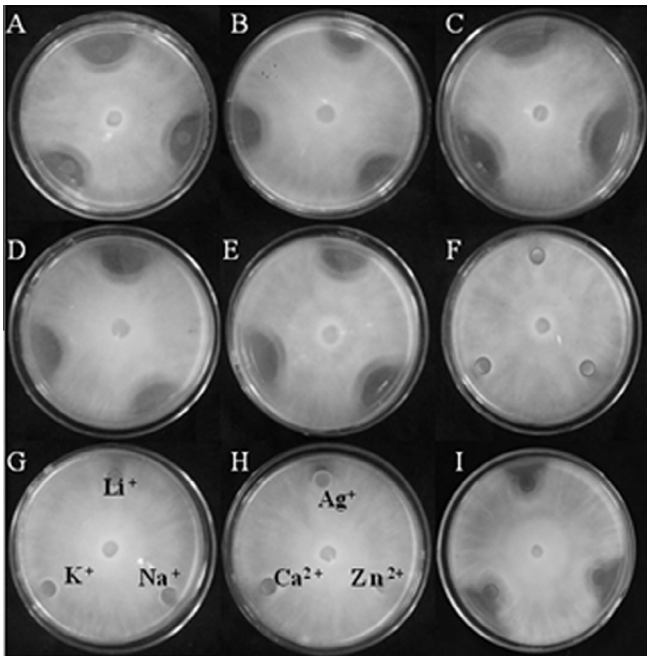

Fig. 4. Effect of ions and proteinase K on antifungal activity of protein. *Note:* A–F: antifungal protein treated with 10 mM Ag^+, K^+, Li^+, Na^+, Zn^{2+}, Ca^{2+}; G and H:10 mM metal cations without protein as control; I: antifungal protein treated with 1 mg/mL protease K.

4. Discussion

Many *Bacillus* strains including *B. licheniformis* strains were used for controlling plant disease, especially for fungal diseases such as *Botrytis cinerea*, *Phytophthora capsici* [17–19]. *Bacillus* offered the advantage to sporulate so that they had strong abilities of heat-resistant, desiccation-tolerance and colonization in plant micro-environment to prevent pathogen infection or induce plant resistance. *Bacillus* also could produce a variety of antibiotic compounds, including volatiles, lipopeptides [20], peptides [21] and proteins [22,23]. *Bacillus* species had already been widely used for agricultural biocontrol [24], industrial enzymes [25] and antibiotics production [26].

B. licheniformis HS10 had a good control effect on cucumber downy mildew in preliminary experiments. In this study, the extracellular protein had a significant suppressive activity on *F. graminearum*, *Bipolaris sorokinianum*, *S. sclerotiorum*, *Botrytis cinerea*, *Phytophthora capsici*, *R. cerealis*, *Bipolaris maydis* and *G. graminis* (Figs 1C and 2).The protein was still stable at 100 °C for 30 min and also remain stable in pH 6–10 (Fig. 3), and this suggested that its antifungal protein was heat-resistant and neutral. Besides, it kept a good fungal resistance by 10 mM metal cations treatment excepted for Ca^{2+} (Fig. 4A–H). And it was negatively affected by proteinase K (Fig. 4I). The antifungal protein was likely to be a cyclic structure [27]. The protein with good stability and broad-spectrum antibacterial had a biocontrol potential. The results had similarity with characteristics of other antifungal proteins reported so far from *B. licheniformis* [28], which is retaining more than 97% activity at temperature below 70 °C and above 90% in pH 6–10 conditions, but it was moderately sensitive to proteinase K. Those proteins with good stability and broad antifungal -spectrum showed good biocontrol potential.

B. licheniformis produces various of antifungal substances with molecular masses mainly ranging from 1.4 to 32 kDa [28]. Only chitinase, which has similar molecular weight of 55 kDa from *B. licheniformis* strain MY75, was found in previous studies [29]. It showed that some significant characteristics including high secretions of chitinase and antifungal activity toward *Aspergil lusniger* and *Gibberella saubinetii* without any other above mentioned fungi. Our protein C1 from *B. licheniformis* HS10 was identified as carboxylase, a-amylase, catalasesorphage-like protein instead of chitinase

by using MALDI-TOF/TOF-MS and blasting in NCBI. Among them, the highest coverage of putative carboxy peptidase (Protein ID:gi|510141549), was dissociated 39 peptides [30]. This protein function is likely predicted from the genome of *B. licheniformis*. The antifungal activity of carboxy peptidase was not previously reported in *Bacillus* species, so that it was unclear whether this enzyme had antifungal activity [31]. And the theory molecular mass of protein C1 was higher than the actual measurement due to shearing the signal peptides or specific modification along with the process of protein expression [32–33].The assumption was confirmed that protein C1 is likely a novel antifungal protein. A further study would aim at the molecular cloning and sequence analysis of C1 to gain more information.

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