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Isolation and partial characterization of an antifungal protein from the endophytic *Bacillus subtilis* strain EDR4

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ABSTRACT

An antifungal protein E2, from the culture filtrate of the endophytic *Bacillus subtilis* strain EDR4 of wheat with a high activity against numerous fungal species *in vitro* and take-all in wheat caused by *Gaeumannomyces graminis* var. *tritici in vivo*, was purified by $(NH_4)_2SO_4$ precipitation, hydrophobic-interaction chromatography, anion-exchange chromatography and polyacrylamide gel electrophoresis (PAGE). The molecular mass of the protein was about 377.0 kDa determined by gel permeation chromatography (GPC) using a Superdex 200 10/300 GL pre-packed column and the pI value of the protein detected by isoelectric focusing PAGE was 6.59. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) the antifungal protein showed a band with a molecular mass of 39.1 kDa, which suggest that the native protein consists of multi-subunits. The amino acid sequences of three peptides from the antifungal protein were obtained by using a nano-ESI-MS/MS (Q-TOF2) System. The protein isolated may be regarded as a new protein according to amino acid sequences of three peptides. The purified protein exhibited inhibitory activity on mycelium growth of e.g. *Fusarium graminearum*, *Macrophoma kuwatsukai*, *Rhizoctonia cerealis*, *Fusarium oxysporum* f.sp. *vasinfectum*, *Botrytis cinerea* and *G. graminis* var. *tritici* (*Ggt*). Scanning electron microscopy showed that hyphae of *Ggt* treated with the antifungal protein were severely deformed. The antifungal protein E2 exhibited ribonuclease and hemagglutinating activities as well as a trifle protease activity. However, no β-1,3-glucanase, β-1,4-glucanase, chitinase or protease inhibitory activities were detected.

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1. Introduction

In recent years endophytes as biocontrol agents became of interest because of their abilities to colonize internal tissues of plants without causing detrimental effects in their hosts [1,2]. Endophytes may excrete antimicrobial compounds, which may be involved in a symbiotic association with a host plant [3,4]. Many biologically active substances excreted by endophytes may be different from compounds identified [5]. In addition, the antibiotics produced by endophytes may reduce cell toxicity towards higher organisms because the plant itself serves as a natural selection system [3].

Bacillus spp. can produce structurally diverse secondary metabolites with a wide spectrum of antibiotic activity [3,6]. Many of these antifungal substances have been characterized and identified as peptide antibiotics [7]. The antifungal peptides produced by *Bacillus* species, included mycobacillins [8,9], iturins [10], bacillomycins [11,12], surfactins [13], mycosubtilins [14,15], fungistatins [16], subsporins [17] and rhizocticins [18]. *Bacillus* sp. produces also a range of other metabolites including chitinases, other cell wall-degrading enzymes [19,20] and some antifungal proteins [21–23].

Therefore, to screen novel, highly active antimicrobial substances from endophytes seems to be of high potential. In our laboratory the endophytic *B. subtilis* strain EDR4 was identified which displayed a high antifungal activity and controlled take-all in wheat evoked by *Gaeumannomyces graminis var. tritici (Ggt)* in greenhouse and field experiments [24] and unpublished results.

The purpose of this study was to purify an antifungal substance produced by the EDR4 strain and to partially characterize this protein in order to broaden the knowledge on the mechanisms exerted by the endophytic strain EDR4 of *Bacillus subtilis* against the take-all pathogen *Ggt*.

2. Materials and methods

2.1. Microorganisms, cultivation, substrates and reagents

The endophytic *B. subtilis* EDR4 strain was isolated from wheat roots [25] and stored at −80 °C in 25% glycerol-containing nutrient broth-yeast extract (NBY) [26]. For activation, the bacterial strain taken from storage was grown on nutrient agar (NA) for 48 h.

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The plant pathogens (*Fusarium graminearum*, *Macrophoma kuwatsukai*, *Rhizoctonia cerealis*, *Fusarium oxysporum* f.sp. *vasinfectum*, *Botrytis cinerea* and *Ggt*) (Collection of Plant Pathology Laboratory of Northwest A&F University) were routinely grown on potato dextrose agar (PDA) at 25 °C for 7 days and thereafter stored at 4 °C.

Laminarin from *Laminaria digitata*, chitin, carboxymethyl cellulose (CMC), casein, trypsin, ribonuclease and yeast tRNA were purchased from Sigma Chemical Co. The standard protein markers for molecular weight estimation by gel electrophoresis and gel permeation chromatography (GPC) were obtained from Hov-bio (Hong Kong) Tech. Co. Ltd. and Amersham Biosciences, respectively. The other chemicals and reagents used were of the highest grade commercially available.

2.2. Preparation of the crude protein extract and testing for its antifungal activity

After activation, strain EDR4 was grown in 100 ml LB medium (5 g yeast extract, 10 g tryptone, and 10 g sodium chloride per 1000 ml water) on a shaker at 150 rpm for 48 h at 28 °C, and the supernatant was collected after centrifugation (8000 rpm, 20 min). Solid ammonium sulphate was gently added to the supernatant to 70% relative saturation. The precipitate (from 100 ml cultural filtrate) was collected by centrifugation (12,000 rpm, 30 min), redissolved in 10 ml 25 mM phosphate buffer (pH 7.0) and dialyzed extensively against distilled water to remove $(NH_4)_2SO_4$ before freeze drying. One millilitre of the crude protein was dissolved in 10 ml 25 mM phosphate buffer (pH 7.0) to test the antifungal activity against *Ggt*. Two *Ggt*-grown 4-mm-diameter agar disks were transferred into a flask containing 50 ml Potato Dextrose Broth (PDB) medium and to each flask 1 ml sterile distilled water (the control) or 1 ml of the crude protein extract concentrated after aseptic filtration (0.22 μm) or 1 ml of the residual supernatant (from which proteins had been extracted) or 1 ml of the original supernatant was added. The flasks were incubated in the dark on a shaker at 100 rpm at 25 °C for 7 days, and mycelia were harvested, washed with distilled water and dry weights determined. Following the above, the antifungal activity of the crude protein precipitated by $(NH_4)_2SO_4$ at 30% and 30–70% relative saturation were determined, respectively.

2.3. Further purification of the antifungal protein

The antifungal protein was purified further by a Fast-performance Liquid Chromatography system (FPLC system, Amersham Biosciences) and electrophoresis. A portion of the crude protein extract prepared by ammonium sulfate fractionation was redissolved in 20 mM phosphate buffer (pH 7.0, containing 1 M $(NH_4)_2SO_4$), applied to a phenyl-Sepharose 6 fast flow (high sub) pre-packed column (0.7 × 2.5 cm, Amersham Biosciences, Uppsala, Sweden) and eluted with a stepwise $(NH_4)_2SO_4$ (0.5 M and deionized water) at the flow rate of 0.5 ml/min. Fractions showing antifungal activity were pooled and passed over a DEAE-Sepharose fast flow prepacked column (0.7 × 2.5 cm, Amersham Biosciences) and eluted with a linear sodium chloride (0–0.5 M) after washing the column with 6 ml of 20 mM Tris–HCl buffer (pH 7.0) at 0.5 ml/min. Fractions of bound parts were pooled, dialyzed against deionized water and freeze-dried.

2.4. Polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide separation gels (7.5%) and stacking gels (5%) prepared in Mini-Protean® 3 system (Bio-Rad Laboratories, Alfred, Hercules, CA, USA) were applied to detect the proteins following ammonium sulfate precipitation and ion-exchange chromatography. Portions of the pooled fractions from ion-exchange chromatography were redissolved in phosphate buffer (pH 7.0) and subjected to native PAGE for 2 h at 4 °C and 10 mA. A gel was stained with Coomassie Brilliant blue R-250 (CBB R-250, Fluka) and other used to elute individual interested protein bands in 500 ml 1× Tris–glycine buffer (25 mM Tris, 192 mM Gly, pH 8.3) at 4 °C and 30 mA for 48 h. Portions of the protein residues were dissolved in phosphate buffer and tested for their antifungal activity against *Ggt*.

The isoelectric point was determined by the procedure of Stahmann et al. [27] using an 8 × 7 × 0.75 cm gel (Bio-Rad mini-protein® 3 cell electrophoresis system). The pH gradient was generated with a 0.8% ampholine (3.5–10.0), 1.2% ampholine (4.0–6.0) and 1.2% ampholine (5.0–7.0) (all from LKB, Bromma, Sweden) solution, using a broad range pI marker standard (pH 3.0–10.0, Pharmcia). The gel was also stained with CBB R-250. The image of gel stained was analyzed by the GeneTools program version 3.06.04 (Syngene, USA) and the isoelectric point of protein E2 was determined precisely.

2.5. SDS–PAGE and GPC for determination of molecular weight

The purified antifungal protein from strain EDR4 was subjected to the relative molecular mass determination of the subunits by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli [28]. The low range protein marker comprising rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa) and egg white lysozyme (14.4 kDa) was used. The gel was run for 2 h at 10 mA, and then stained with CBB R-250. The image of gel stained was analyzed by the GeneTools program version 3.06.04 (Syngene, USA) and the molecular weight of protein E2 was determined precisely.

The native molecular mass of the antifungal protein was estimated by GPC (FPLC system, Amersham Biosciences) using a Superdex 200 10/300 GL (1.0 × 30 cm,) pre-packed column at a flow rate of 0.5 ml/min. The column was calibrated with reference substances such as dextran blue 2000 (2000 kDa), aldolase (158.0 kDa), bovine serum albumin (66.7 kDa), ovalbumin (43.0 kDa), and cytochrome *c* (13.6 kDa) (each 2 mg/ml, 100 μl) for molecular weight estimation under identical conditions. Protein concentration of each step was determined by the method of Bradford [29] with bovine serum albumin (BSA, Sigma) as standard.

2.6. De novo sequencing of protein E2 by a nano-ESI-MS/MS (Q-TOF2)

After being electrophoresed by SDS–PAGE, the band of protein E2 was excised and sent to the National Center of Biomedical Analysis, China (NCBA, China), to determine peptide fractions of the protein. The band was digested with trypsin (sequencing grade, Promega) and peptide samples generated from the tryptic digest were loaded into palladium-coated borosilicate nanospray needles. Nano-ESI-MS for peptide sequencing were performed on a Q-TOF2 System (Micromass, UK). The complex CID-MS/MS spectra containing fragment ions in multiple charge states were replotted into a simple spectrum of monovalent ions using MaxEnt-3 software. Sequences were interpreted from the resulting fragment ion spectra using the PepSeq *de novo* sequencing tool (Micromass). Sequence homology was analyzed using mascot program and the NCBI BLAST online search service.

2.7. Assay of antifungal activity

The assay of the purified protein for antifungal activity toward *F. graminearumm*, *M. kuwatsukai*, *R. cerealis*, *F. oxysporum*, *B. cinerea* and *Ggt* was carried out using the Oxford plate assay system. Two

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sterile Oxford cups were placed symmetrically at a distance of 2.5 cm from the rim of the mycelium grown agar disk (4-mm-diameter taken from the edge of actively growing colonies of each species) in the center of a PDA plate. One hundred and fifty microliters of a 1.04 μg/ml protein solution filtered through a 0.22-μm filter was added to each Oxford cup. The plates were incubated at 25 °C until mycelia growth had enveloped the control Oxford cups containing distilled water. The diameter of inhibition zones around the Oxford cups containing the samples were measured (mm) [30,31].

To determine the IC50 value (the concentration of the antifungal protein required to reduce the area of the mycelial colony to 50%) for the antifungal activity of the protein E2 against *Ggt*, five doses (0.0204, 0.0407, 0.0813, 0.1626 and 0.3252 mg) of the protein dissolved in phosphate buffer (pH 7.0) were mixed with each 10 ml PDA at 45 °C, respectively. The Petri dishes (9 cm diameter) were inoculated with *Ggt*-grown agar disks and incubated at 25 °C. Buffer only (without protein) served as control. For comparison a dilution series of the culture filtrate was included in this experiment. When the mycelium of the control had reached the edge of the plate, the areas of the mycelium colonies of the different treatments were measured, and the IC50 values were determined [31].

2.8. Effect of antifungal protein on hyphal morphology of Ggt

Samples (1 cm^2) of the different treatments from the PDA culture dishes around the Oxford cups, containing the different concentrations of the antifungal protein, were taken, carefully processed as described by Huang et al. [32] and the morphological alterations of the hyphae observed using a JSH 6360 scanning electron microscope (SEM) at 15 kV.

2.9. Partial characterization of the protein

2.9.1. Assay for ribonuclease activity

The activity of protein toward yeast tRNA was assayed according to the method of Liu et al. [23]. Protein was incubated with 200 μg tRNA in 150 μl 100 mM MES buffer (pH 6.0) at 37 °C for 1 h then, the reaction was terminated by addition of 350 μl of ice-cold 3.4% (v/v) perchloric acid. The mixture was placed on ice for 15 min thereafter centrifuged (15,000 rpm, 15 min) at 4 °C. The absorbance at 260 nm of the supernatant was measured after appropriate dilution. RNase activity was defined as the amount of enzyme which brings about an increase in absorbance at 260 nm of one unit per min in the acid soluble fraction per ml of reaction mixture under the specified conditions. All spectrophotometric assays were performed with a Nicolet Evolution 300 spectrophotometer (USA).

2.9.2. Assay of hemagglutinating activity

In the assay for hemagglutinating activity, a 2% suspension of rabbit red blood cells was prepared following the method of Ji et al. [33]. The blood from a healthy rabbit, added an anticoagulant, was washed with phosphate-buffered saline (pH 7.2) before centrifugation at 3500 rpm for 10 min. The red blood cells deposited were diluted to 2% suspension with the above buffer for detection the hemagglutinating activity of the antifungal protein E2 following the method of Liu et al. [23]. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity was the number of hemagglutination units per mg protein.

2.9.3. Assay of protease activity

In the assay for protease activity, a solution of casein as substrate was freshly prepared using the method of Liu et al. [23]. To 2 g casein, 10 ml distilled water and 10 ml of 0.2 M NaOH were added. After addition of 60 ml distilled water, the mixture was stirred to make a solution. The pH of the solution was adjusted to pH 7.5 with HCl, and the solution was heated at 90 °C for 15 min before cooling down to 25 °C and diluted with 100 ml of 100 mM Tris–HCl buffer (pH 8.0) containing 40 mM $CaCl_2$. The precipitate was removed and the resulting solution was used. The antifungal protein or trypsin solution (positive control) (50 μl) preheated for 10 min to 37 °C was mixed with 350 μl of the above prepared preheated casein solution. After 25 min, 1 ml of 4% (w/v) trichloroacetic acid was added to end the reaction. The mixture was placed at room temperature for 30 min before centrifugation at 15,000 rpm for 15 min. The absorbance of the supernatant, which reflects the amount of casein fragments produced by proteolytic action, was read at 280 nm against water as blank.

2.9.4. Assay of protease inhibitory activity

The assay was conducted following the method of Liu et al. [23], using trypsin as enzyme. The purified antifungal protein was tested for the ability to inhibit trypsin in 50 mM Tris–HCl buffer (pH 8.0) after incubation at 25 °C for 30 min. The samples were mixed with trypsin prior to preheating.

2.9.5. Assay of glucanase and chitinase activities

Following the method of Abeles and Forrence [34], the activity of β-1,3-glucanase and β-1,4-glucanase of the protein was also clarified. Protein solution was incubated with laminarin or CMC in 50 mM sodium acetate (pH 5.5) for 30 min at 37 °C before adding the dinitrosalicylic acid reagent to the reaction mixture. The samples were heated at 100 °C for 5 min and cooled to room temperature and the absorbance read at 540 nm. One unit of activity of β-1,3-glucanase and β-1,4-glucanase is defined as the amount of enzyme that catalyses the release of reducing groups equivalent to 1 μmol glucose, under standard conditions (min^{-1}, 37 °C). Similarly, chitinase activity of the protein was tested. Colloidal chitin as substrate was prepared according to the method of Berger and Reynolds [35]. Glucose was used as standard in the assay.

3. Results

3.1. Determination of the antifungal activity

The antifungal activities of the original supernatant, the crude protein extract and the residual supernatant (from which proteins had been extracted) were tested against mycelium growth of *Ggt* in PDB. When 1 ml of the original supernatant was added to 50 ml PDB, dry weight of mycelium was reduced by 67.5% compared to the control (1 ml sterile distilled water). However, when 1 ml of the residual supernatant was used, mycelium dry weight of *Ggt* was not affected compared to the control treatment. In the presence of 1 ml supernatant of the crude protein extract, growth of *Ggt* was markedly suppressed and dry weight of mycelium was reduced by 61.5% compared to the control. In addition, the antifungal activity of the crude protein precipitated by $(NH_4)_2SO_4$ at 30–70% relative saturation was better than at 30% relative saturation, and was purified in next steps (data not shown).

3.2. Purification of the antifungal protein

The protein was isolated and purified by ammonium sulfate fractionation, phenyl-Sepharose 6 FF column chromatography, DEAE-Sepharose FF column chromatography and PAGE. The yield of each purification step is shown in Table 1. Precipitation using 30–70% saturation of $(NH_4)_2SO_4$ removed 83.3% of the protein. The IC50 of protein E2 on *Ggt* was only 0.02 mg/ml and the yield of E2 obtained was only 0.5% of the total protein in the culture filtrate after a series of purification steps.

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Table 1
Protein yields after each purification step.

Purification steps	Total volume (ml)	Total protein (mg)	%	IC50 (on *Ggt*) (mg/ml)[a]
Culture filtrate	2000	491.9	100.0	0.82
$(NH_4)_2SO_4$ 30%-70%	17.3	82.2	16.7	–
Phenyl-Sepharose	94.5	28.1	5.7	–
DEAE-Sepharose	13.9	6.1	1.2	–
PAGE (E2)	9.3	1.5	0.5	0.02

[a] IC_{50} value is the concentration of the antifungal protein required to reduce the area of the mycelial colony to 50%.

Hydrophobic-interaction chromatography (HIC) of the partially purified protein, precipitated by $(NH_4)_2SO_4$ at 30–70% saturation, on phenyl-Sepharose 6 Fast Flow produced an unadsorbed fraction (A) and three adsorbed fractions (B, C and D) (Fig. 1I). Of these fractions, only fraction B showed high antifungal activity. Ion-exchange chromatography of fraction B on a DEAE-Sepharose Fast Flow column yielded an unadsorbed fraction E and three adsorbed fractions F, G and H. Anti-fungal activity was detected only in fraction F (Fig. 1II). Fraction F was electrophoresed using PAGE and after staining the gels with CBB R-250 two bands (E2 and E3) were observed (Fig. 1III). Then, the bands E2 and E3 were electro-eluted following excising the gel bands separately. The antifungal activations of the protein fraction E2, E3 and the culture filtrate of strain EDR4 were tested in PDA medium against *Ggt*. The protein E3 exhibited a trifle of antifungal activity. The IC50 value of the antifungal protein E2 on mycelium growth of *Ggt* was 0.02 mg/ml (Table 1) and was significantly lower than the value of the culture filtrate (0.82 mg/ml). E2 showed a single band with a molecular mass of about 39.1 kDa in SDS–PAGE (Fig. 1IV).

3.3. Peptide sequences from the antifungal protein

Amino acid sequences of three peptides of protein E2 were obtained by *de novo* sequencing using a Q-TOF2: VYGVGTVKEERLR (E2a), FCLEPFADDVMWR (E2b) and AATADRPYDR (E2c) which were assigned to the precursor 753.46 *m*/*z* (a), 814.94 *m*/*z* (b) and 568.33 *m*/*z* (c), respectively. The protein E2 was identified as a new protein by the National Center of Biomedical Analysis, China. In order to validate this conclusion, we blasted these sequences obtained in the NCBI databank and the results indicated that amino acid sequences of the three peptides did not accord with any of the known proteins. However, partial sequences of the peptide E2a and E2b exhibited similarities to peptidase, glucanase, cellulase, aminopeptidase and protease from *Bacillus* sp., but peptide E2c showed some hypothetical protein sequences from other species.

3.4. Activity of the purified antifungal protein E2 and scanning electron microscopy (SEM)

The IC50 value of the antifungal protein on mycelium growth of *Ggt* was 0.02 mg/ml, determined on PDA medium after incubation for 7 days. Protein E2 showed also antifungal activity toward the fungal activities tested using the Oxford cup assay. At a concentration of each 150 μl of 1.04 μg/ml protein per cup, E2 produced the different inhibition zones toward *F. graminearum*, *F. oxysporum*, *M. kuwatsukai*, *R. cerealis*, *B. cinerea* and *Ggt*, respectively, and the diameter of inhibition zones toward *Ggt* get up to 225 mm (Table 2).

SEM studies revealed that severe morphological alterations in hyphae of *Ggt* were caused in the presence of 1.0381 μg/ml of E2. Compared to the untreated hyphae (Fig. 2A), the protein evoked irregular hyphal growth, such as abnormal thickenings at the branching sites and hyphal tips became swollen (Fig. 2B). Hyphal cells showed severe distortions, shriveling or swellings (Fig. 2C). Protein E2 cause leakage in hyphal of *Ggt* (Fig. 2B).

3.5. Isoelectric point (pI) and molecular mass of the purified protein

The pI of the purified protein determined by isoelectric focusing PAGE was 6.59 (Fig. 3).

In SDS–PAGE, the protein appeared as a single protein band after staining the gel with CBB R-250, and the molecular mass was estimated of about 39.1 kDa (Fig. 1IV).

Using GPC, a single symmetrical peak was observed for E2, a molecular mass of about 377.0 kDa was estimated after calibration the column with known molecular weight standards. This result suggests that the native antifungal protein E2 consists of multi-subunits.

3.6. Other activities of the antifungal protein

The antifungal protein E2 displayed ribonuclease specific activity which was 1.285 U/mg, hemagglutinating specific activity which was 649.35 U/mg, and protease specific activity which was 0.422 U/mg. The protein, however, showed no β-1,3-glucanase, cellulase or chitinase activities as well as no protease inhibitory activity.

4. Discussion

Bacillus and *Streptomyces* species have been used since many years for insect biocontrol, industrial enzyme production and antibiotic production. In recent years, *Bacillus* species have received much attention for the development of biological agents to control soil borne diseases. The Gram-positive microorganisms *Bacillus* and *Streptomyces* offer the advantage to produce heat and desiccation resistant spores which in contrast to fluorescent pseudomonads can be readily formulated and stored [36]. *Bacillus* spp. are also known to produce a wide variety of antifungal compounds, including volatiles [37], lipopeptides [38,39] and several modified small peptides as well as proteins [21,23]. Our study revealed that proteins represented the main antifungal substances in the culture filtrate of the endophytic *B. subtilis* strain EDR4 which reduced the growth of mycelium of *Ggt* by 61.5%. These antifungal proteins consist of two parts: one is precipitated by 30% relative saturation ammonium sulphate, another precipitated between 30% and 70%. Proteins in the 30–70% precipitation part were further purified using phenyl-Sepharose and DEAE-Sepharose chromatography. One antifungal protein was purified to homogeneity and identified by native PAGE, it was designated E2. Protein E2 yielded 0.5% of the protein recovery.

The protein E2 strongly inhibited mycelial growth of different plant pathogenic fungi including *F. graminearum*, *M. kuwatsukai*, *R. cerealis*, *F. oxysporum*, *B. cinerea* and *G. graminis* var. *tritici* which belong to Ascomycotina and Deuteromycotina. The IC50 value of the antifungal protein E2 on mycelium growth of *Ggt* was 0.02 mg/ml. The result obtained so far revealed that the antifungal activity of protein E2 comprised a relatively wide spectrum against plant pathogenic fungi. However, other species, especially members of the Oomycetes, should also be tested. From these structural alterations of hyphae, the primary mode of action of protein E2 in *Ggt* cannot be deduced. Detailed biochemical and ultrastructural studies are required to determine the primary target of protein E2.

The antifungal protein E2 represents a novel protein. Experimental results indicate that the antifungal protein E2, with a native molecular mass of about 377.0 kDa, is made up of multi-subunits of each 39.1 kDa. The molecular weight of protein E2, however, did not much differ from the molecular weight of bacisubin from

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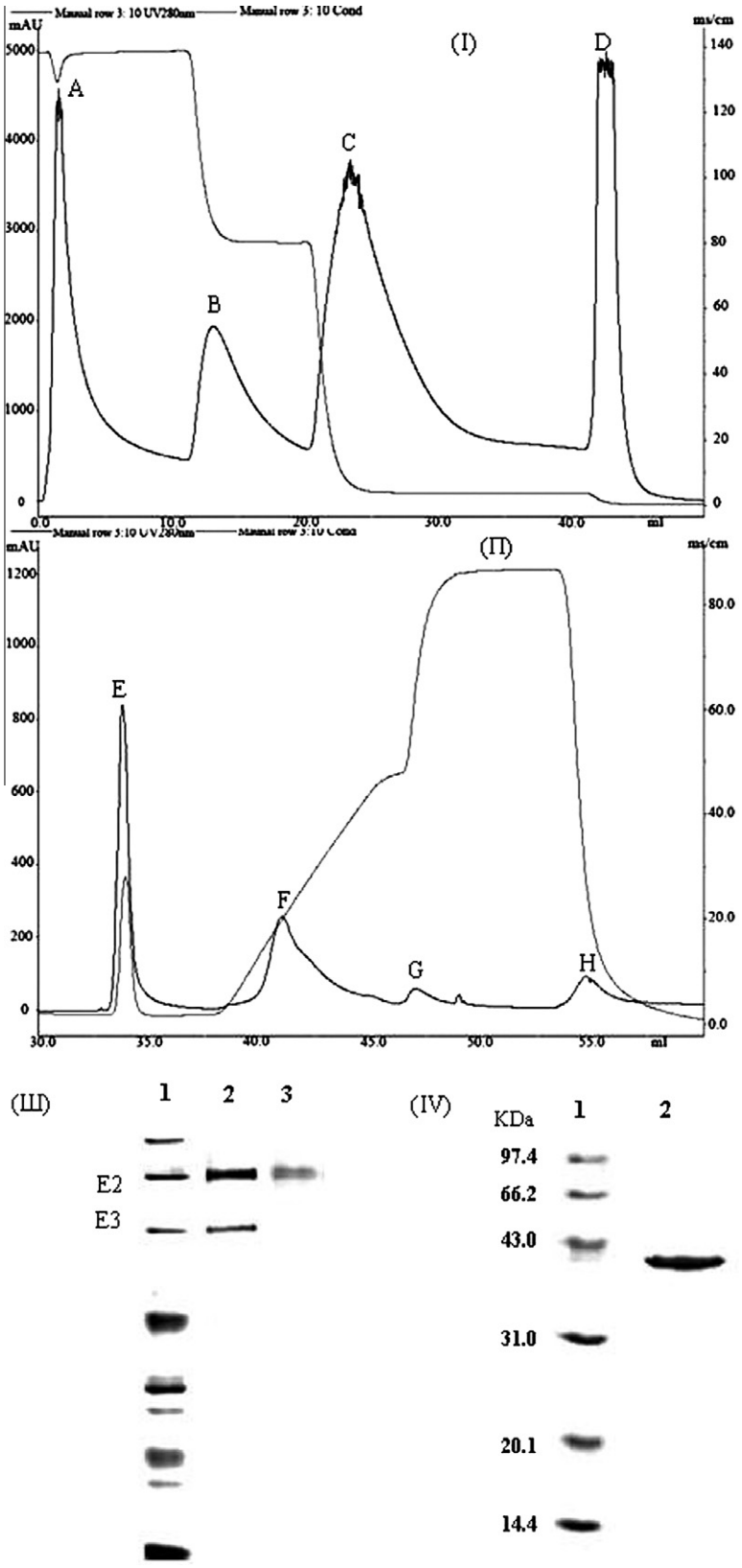

Fig. 1. Chromatographic methods as well as PAGE and native PAGE after electro-elution for purification and SDS–PAGE of the antifungal protein. (I) HIC of crude protein on phenyl-Sepharose 6 FF column using an AKTA Explorer system. (A) An unadsorbed fraction; (B), (C) and (D) the adsorbed fractions. (B) Showed high antifungal activity. (II) Ion-exchange chromatography of fraction B (low HIC) on a DEAE-Sepharose Fast Flow column using an AKTA Explorer system. (E) An unadsorbed fraction; (F), (G) and (H) three adsorbed fractions. Anti-fungal activity was detected only in fraction F. (III) PAGE of the crude protein and fraction F (low DEAE-sepharose FF column) and the purified antifungal protein. Lane1: Crude protein following ammonium sulfate precipitation; lane 2: fraction F pooled by ion-exchange chromatography; lane 3: protein obtained by excising the gel band E2 from bands in lane 2 and electro-elution. (IV) SDS–PAGE (15% gel) of the antifungal protein. Lane1: Lower molecular protein marker; lane 2: the subunit of the antifungal protein. All gels were stained with CBB R-250.

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Table 2
The effect of the antifungal protein E2 from *Bacillus subtilis* EDR4 tested on PDA plate *in vitro* on different pathogens.

Pathogens	*Fusarium graminearum*	*Rhizoctonia cerealis*	*Fusarium oxysporum* f.sp. *vasinfectum*	*Botrytis cinerea*	*Macrophoma kuwatsukai*	*Gaeumannomyces graminis* var. *tritici*
Inhibition zones (mm)	125	125	145	155	185	225

Note: The diameter of inhibition zones were measured after incubating the plates in the dark at 25 °C for 3 days as well as *Gaeumannomyces graminis* var. *tritici* for 5 days; 0.52 μg of protein E2 is used in each Oxford Cup.

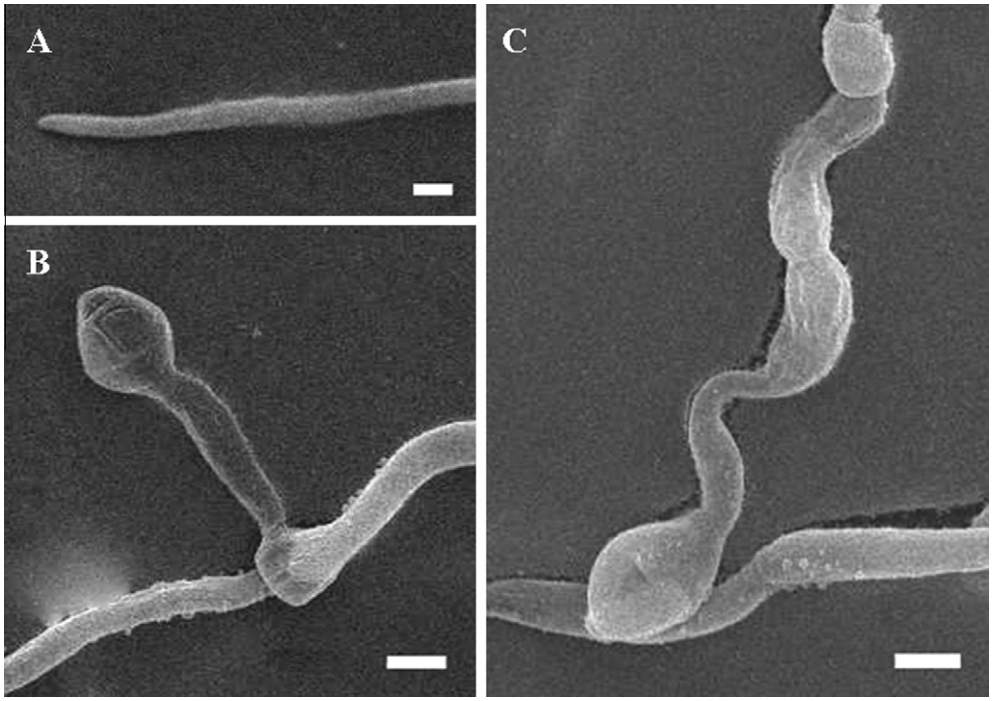

Fig. 2. Effects of the antifungal protein E2 from endophytic *Bacillus subtilis* strain EDR4 on hyphal growth of *Gaeumannomyces graminis* var. *tritici*. (*Ggt*) observed by the scanning electron microscopy. (A) Hypha from untreated *Ggt* colony. Bar = 2.5 μm; (B) and (C) abnormal hyphal growth of *Ggt* treated with the antifungal protein. Bar = 5 μm; (B) tip of hyphal branch was swollen; (C) hyphal branch was swollen and severely distorted.

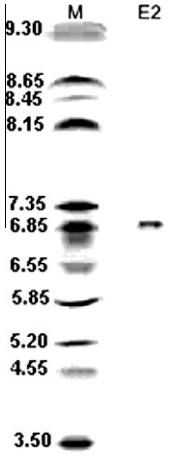

Fig. 3. IEF of the antifungal protein from the endophytic *Bacillus subtilis* strain EDR4. Left lane: Broad range pI marker; right lane: purified antifungal protein E2. The gel was stained with CBB R-250.

B. subtilis B-916, but differed clearly from other antifungal agents (enzymes, peptides and proteins) produced by *B. subtilis* such as bacillomycin D synthetase A (448.2 kDa), bacillomycin D synthetase B (607.2 kDa), bacillomycin D synthetase C (309.0 kDa), putative sensor kinase (53.4 kDa), BamD (44.9 kDa), endo-1,4-β-glucanase (46.6 kDa), YnfF (45.4 kDa), putative response regulator (27.7 kDa), endo-1,4-β-xylanase (54.3 kDa), and YxjF (fragment) (12.2 kDa) [40]. Ribonuclease, protease, hemagglutinating, and protease inhibitory activities have been reported for some antifungal proteins [30,41–43]. The antifungal protein bacisubin, from *B. subtilis* strain B-916, manifested ribonuclease and hemagglutinating activities but no protease and protease inhibitory activity [23]. The results obtained for E2 did not accord with characteristics of the antifungal protein bacisubin and other antifungal proteins reported so far from *Bacillus* spp. Thus, these findings most likely suggest that protein E2 may be a novel protein.

Among existing methods for sequence determination, the Edman degradation chemistry is the notably the most used one. However, some proteins cannot be directly sequenced by the Edman degradation because they have a blocked N-terminal residue. Nano-ESI-Q-TOF-MS/MS is the best complementary method to Edman degradation method in peptide and protein sequencing because this method has no restriction to N-terminal modification and has high sensitivity, high mass accuracy and high speed. To identify the antifungal protein E2, we had used automatic Edman degradation analyzes for N-terminal amino acid sequencing, but no peak emerged in the sample. Possibly, the protein was blocked at the N-terminal. To make an affirmative identity of protein E2, *de novo* sequencing which can identify a protein by sequencing 2–3 peptides for certain was carried out using nano-ESI-MS/MS by NCBA, China, and the protein was identified as a novel protein. By blasting in NCBI databank, the assumption was confirmed that protein E2 is likely a novel antifungal protein: the peptides which

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had been sequenced did not collectively match, in whole or in part, to a single protein from the *B. subtilis*. The findings accord with the conclusion of the protein identification from NCBA, China. Our present work is focusing on amplifying a portion of the E2 open reading frames using degenerated oligonucleotide primers in order to obtain the full gene sequence. Further studies will concentrate on the involvement of the antifungal protein in control of take-all and other plant diseases by the strain EDR4 of *B. subtilis*.

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