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ORIGINAL PAPER

Purification and characterization of a potential antifungal protein from *Bacillus subtilis* E1R-J against *Valsa mali*

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Abstract In order to identify the antagonistic substances produced by *Bacillus subtilis* E1R-J as candidate of biocontrol agents for controlling Apple *Valsa* Canker, hydrochloric acid precipitation, reverse phase chromatography, gel filtration, and ion exchange chromatography were used. The purified fraction EP-2 showed a single band in native-polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fraction EP-2 was eluted from native-PAGE and showed a clear inhibition zone against *V. mali* 03-8. These results prove that EP-2 is one of the most important antifungal substances produced by *B. subtilis* E1R-J in fermentation broth. SDS-PAGE and Nano-LC–ESI–MS/MS analysis results demonstrated that EP-2 was likely an antifungal peptide (trA0A086WXP9), with a relative molecular mass of 12.44 kDa and isoelectric point of 9.94. The examination of antagonistic mechanism under SEM and TEM showed that EP-2 appeared to inhibit *Valsa mali* 03-8 by causing hyphal swelling, distortion, abnormality and protoplasts extravasation. Inhibition spectrum results showed that antifungal protein EP-2 had significantly inhibition on sixteen kinds of plant pathogenic fungi. The stability test results showed that protein EP-2 was stable with antifungal activity at temperatures as high as 100 °C for 30 min and in pH values ranging from 1.0 to 8.0, or incubated with each 5 mM Cu^{2+}, Zn^{2+}, Mg^{2+}, or K^{+}. However, the antifungal activity was negatively affected by Proteinase K treatment.

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Keywords Endophytic *Bacillus subtilis* · Apple *Valsa* Canker (AVC) · Biocontrol agents (BCAs) · Antifungal substances

Introduction

Apple *Valsa* Canker (AVC), caused by *Valsa mali* (*Vm*) has been an destructive disease of apple trees (*Malus* spp.) in eastern Asia (Abe et al. 2007), especially in China (Wang et al. 2011), seriously hinder apple production in all apple producing areas world-wide (Abe et al. 2007). AVC is difficult to control through chemical treatments including conventional fungicides (Zhang et al. 2015) because the pathogen penetrates extensively into the host phloem and xylem, thus, the pathogen is withdrawn from the activity of conventional fungicides. Biocontrol agents (BCAs) as a more efficient and environmentally friendly alternative control measure are attracting more and more attention. Some species of microorganisms have been studied as candidates of BCAs for controlling AVC disease. For example, there are several reports that strains of *Trichoderma harzianum* (Gao et al. 2002), *Chaetomium spirale* (Xin and Shang 2005), and *T. atroviride* (Deng et al. 2009) exhibiting antagonistic effects against *V. mali*. Li et al. (2015) found that endophytic actinomycetes strains showed biocontrol effect against AVC disease under laboratory and field conditions. Another example is reported by Zhan et al. (2008), who found that *Streptomyces aureus* strain Z-6 isolated from soil also showed antifungal activity against *V. mali*.

Bacillus species are often regarded as ideal candidates for commercial BCAs partly due to their ability to form heat- and desiccation-resistant endospores (Kumar et al. 2011), and can protect plants against pathogens via

multiple mechanisms, including induction of systemic resistance (Kloepper et al. 2004), competition for space and nutrients (Chen et al. 2012), and secretion of various antifungal substances, such as antibiotics (Stein 2005), volatile organic compounds (Fiddaman and Rossall 1993), secondary metabolites (Gao et al. 2014), cell wall degrading enzymes (Pane et al. 2012), lipopeptides (Berry et al. 2010), and antifungal proteins (Ren et al. 2013). Among these antagonistic factors, production of antimicrobial substances is considered as one of the most important biocontrol mechanisms. The majority of *Bacillus* strains have the capacity to produce antimicrobial substances (Yang et al. 2015). A series of novel antimicrobial substances have been purified from *Bacillus* species (Baindara et al. 2013; Yang et al. 2015; Slimene et al. 2015). Therefore, *Bacillus* species have become an important source for discovering novel antifungal substances.

Bacillus subtilis E1R-J was isolated from healthy wheat roots in China (Qiao et al. 2006). Preliminary experimental results indicated that E1R-J exhibited high antifungal activity to *Gaeumannomyces graminis* var. *tritici* (*Ggt*) in vitro and in vivo (Liu et al. 2009), and showed inhibitory effects on wheat stripe rust in greenhouse and field trials (Li et al. 2013). The activity of E1R-J is mainly related to the production of antifungal proteins (Liu et al. 2009). The purpose of this study was to purify antifungal proteins produced by E1R-J and to partially characterize the active proteins. These studies will broaden the knowledge on the mechanisms exerted by *B. subtilis* strain E1R-J against *V. mali*, causing the AVC disease.

Materials and methods

Microorganisms, origins, culture conditions, and reagents

All Fungal strains (*V. mali* strain 03-8, *Botryosphaeria dothidea*, *Sclerotinia sclerotiorum*, *Bipolaris sorokiniana*, *Exserohilum turcicum*, *Valsa pyri*, *Botrytis cinerea*, *Gibberella zeae*, *Fusarium solani*, *Rhizoctonia cerealis*, *Valsa leucostoma*, *Mycosphaerella musicola*, *Gloeosporium fructigenum*, *Gloeosporium musarum Cooke et Mass*, *Glomerella cingulata* and *Botytis cinerea Pers*) were isolated from different hosts. *B. subtilis* strain E1R-J was isolated from healthy wheat plants (Qiao et al. 2006). All strains were identified and preserved by the Plant Pathology Laboratory at Northwest A & F University, Yangling, PRC.

The fungal species were routinely grown on potato dextrose agar (PDA) at 25 °C for 48 h. *B. subtilis* strain E1R-J was grown in LB liquid medium for seed liquid culture and in Landy medium for antifungal proteins production (Yang et al. 2012).

All reagents and solvents used in this study were analytical grade.

Purification of antifungal proteins from *B. subtilis* strain E1R-J

Supernatant preparation

A single colony of *B. subtilis* strain E1R-J was cultured in LB liquid medium at 30 °C and 150 rpm for 24 h as seed liquid. 1.5 mL seed liquid was inoculated into 50 mL Landy medium and cultured at 30 °C, 200 rpm for 72 h (Yang et al. 2012). The supernatant was collected by centrifugation at 4 °C, 13,000×*g* for 30 min.

Crude extract from the supernatant

The culture supernatant was standing at 4 °C for 24 h after adjusting the pH to 4.0 using 6 M HCl. The precipitation collected by centrifugation at 4 °C, 10,621×*g* for 20 min, was dissolved in deionized water, the pH adjusted to 7.0 using 0.1 M NaOH, and then freeze-dried. The crude extract was extracted four times with analytical grade methanol. The methanol extracts were combined, the solvent evaporated, and the residues dissolved in a small amount of deionized water. Crude extracts were stored at 4 °C.

Further purification of the antifungal protein

The crude extract was applied to a RESOURCE RPC^{TM} column (1.6 cm × 3 cm), on an ÄKTA Prime system (Amersham Biosciences, Shanghai, China), pre-equilibrated with 0.09 % TFA. The column was washed with a linear gradient of 0.1 % TFA from 0 to 100 % concentration in ultrapure water at a flow rate of 1 mL/min to remove unabsorbed proteins. Fractions containing antifungal activity were pooled, and applied to a Superdex^{TM}75 10/300 GL column (1.0 × 30 cm) on an AKTA Prime system. The column was eluted with phosphate buffer (0.02 M, pH 7.0) at a flow rate of 0.5 mL/min. Fractions with antifungal activity were pooled, and injected to a DEAE-Sepharose Fast Flow column (0.7 × 2.5 cm) on an AKTA Prime system pre-equilibrated with 20 mM Tris–HCl pH 7.0. The column was washed and absorbed proteins eluted with a linear gradient from 0 to 1 M NaCl in 20 mM Tris–HCl pH 7.0 at a flow rate of 1 mL/min. Finally, active fractions were injected to a Superdex^{TM}75 10/300 GL column (1.0 × 30 cm) on an AKTA Prime system (the operation process was followed as described above) in order to remove salt ions.

Individual peak fractions were collected and concentrated by dialysis. After dialysis with PBS buffer, 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. Antifungal activity against *V. mali* strain 03-8 of the individual proteins was monitored using the agar plate method (Liu et al. 2010).

Polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide separation gels (15 %) and the Mini-Protean®3 system (Bio-Rad Laboratories, Hercules, CA, USA) were used to detect proteins. Gels were stained with Coomassie Brilliant blue R-250 (Fluka), or used directly for electro-elution of individual protein bands as follows: gel pieces containing the interest protein were put into a dialysis bag (MWCO 8000–14,000 Da) with an appropriate amount of native-PAGE gel electrophoresis buffer. Then the dialysis bag was placed in a horizons electrophoresis tank containing the same native-PAGE gel electrophoresis buffer. Proteins were dialyzed overnight in PBS buffer and freeze-dried after electrophoresis at 100 V for 3.5 h. Freeze-dried samples were dissolved in a small amount ddH_2O.

SDS-PAGE for determination of molecular weights

Purified protein was subjected to a mass determination of the subunits by SDS-PAGE following the method of Laemmli (1970) on 15 % separation gels. Gels were also stained with Coomassie Brilliant blue R-250 (Fluka).

Sequencing of protein EP-2 by Nano-LC–ESI–MS/MS

Identification of the protein EP-2 was accomplished by Nano-LC–ESI–MS/MS in Beijing Genomics Institute (BGI, Beijing, China). LC–ESI–MS/MS analysis was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150.

Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo), (5600 msconverter) and the MGF file were searched. Proteins identification were performed by using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against uniprot_*B. subtilis* (61,370 seqs) database (http://www.uniprot.org/uniprot/?query=taxonomy:1423).

For protein identification, a mass tolerance of 0.05 Da was permitted for intact peptide masses and 0.1 Da for fragmented ions, with allowance for one missed cleavages in the trypsin digests. Gln->pyro-Glu (N-term Q), oxidation (M), deamidated (NQ) as the potential variable modifications, and carbamidomethyl (C), as fixed modifications. The charge states of peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of database is generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides with significance scores (≥20) at the 99 % confidence interval by a Mascot probability analysis greater than "identity" were counted as identified. And each confident protein identification involve at least one unique peptide.

Effect of antifungal protein on hyphal morphology of *V. mali*

A hole was punched at the distance of 25 mm from the center of a PDA plate after *V. mali* was inoculated into the center of the plate and cultured at 25 °C for 24 h. Then 20 μL of electro-eluted protein from native-PAGE gels was added into the hole and culture continued at 25 °C. SEM samples were prepared by taking mycelium blocks (5–7 mm) from the edge of the inhibition zone from 24 to 72 h cultures. Carefully processed as described by Kang (1995) and the morphological alterations of the hyphae observed using a JSH 6360 scanning electron microscope (SEM, JEOL Ltd, Tokyo, Japan) at 15 kV. TEM samples from 72 h culture were prepared in the same way as described by Kang (1995), and the ultrastructure of hyphae was observed using a HT7700 transmission electron microscope (HITACHI Company, Tokyo, Japan).

Inhibition spectrum of antifungal protein

Antifungal activity of protein EP-2 was detected using Oxford Cup method, using the sixteen fugal species as target organism, Each 30 μg protein EP-2 in sterile water (100 μL) was added into Oxford-cups and sterile water (without protein) was used as control. Each test was repeated three times.

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

In order to determine the antifungal protein stability, protein EP-2 was exposed to pH ranging from 1 to 12 for 1 h, temperatures ranging from 40 to 100 °C for 30 min, or incubated with 5 mM Cu^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, and 1 mg/ml protease K

respectively, the antifungal activities of the protein (0.1 μg/μL) was determined after treated with different conditions as described above, and the protein without treated as control. Each treatment was repeated three times.

Results

Purification and identification of antifungal protein

The summary of the purification of the antifungal protein from *B. subtilis* E1R-J is presented in Table 1. The purification processes were showed from Fig. 1a: I to d: I. The antifungal activity of fractions eluted from each columns were showed from Fig. 1a: II to d: II. As shown in Fig. 1d: I, one main protein peak was recovered from those four columns. This peak is an obvious protein peak and shows antifungal activity against *V. mali* (Figs. 1d: II, 2c). This main peak was showed only one main band on 15 % native-PAGE (Fig. 2a). Analysis by SDS-PAGE after electro-elution from native-PAGE gels confirmed the presence of a single polypeptide with an apparent molecular mass of about 12 kDa (Fig. 2b).

The single band on SDS-PAGE gel was cut off for identification using Nano-LC–MS/MS. Proteins identification were performed by using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against database containing 61,370 sequences. Four highest matching rate proteins were blasted (Table 2). Among them, antifungal polypeptide (Protein ID: tr|A0A086WXP9) are with the highest protein score and coverage.

Examining the antifungal activity of protein EP-2 against *V. mali* by SEM and TEM

SEM studies revealed that severe morphological alterations in hyphae of *V. mali* were caused after cultured for 72 h in the presence of protein EP-2. Compared to untreated hyphae (Fig. 3a), the protein evoked hyphal swellings (Fig. 3b), hyphal collapse, shriveling and protoplasm extrusion (Fig. 3c), all hyphal cells showed severe distortions (Fig. 3d).

TEM results show that EP-2 had a severe impact on mycelial ultrastructure of *V. mali* after cultured for 72 h in the presence of protein EP-2, compared to untreated hyphae (Fig. 4a), including mycelium cell and membrane abnormalities, cytoplasmic condensation, plasmolysis and vesicle swellings (Fig. 4b–d).

Antifungal activity of protein against different pathogenic fungi

The antifungal spectrum of the protein EP-2 was shown in Fig. 5. The results showed that protein EP-2 had a broad spectrum antifungal activity against sixteen kinds of plant pathogenic fungi and the inhibition zone diameter ranged from 6 to 35 mm. Among them, the antagonist activity towards *V. mali* was the strongest inhibition effect.

Stability of the antifungal protein EP-2

The stability of antifungal protein EP-2 is shown in Fig. 6. EP-2 was incubated at 40, 50, 60, 70, 80, 90, and 100 °C for 30 min. Antifungal activity could be detected at all temperatures. Comparison of treatment at 100 and 40 °C indicated that the antifungal activity decreased only by 14.9 %. Thus antifungal protein EP-2 showed good thermal stability (Fig. 6a).

EP-2 exhibited its strongest antifungal activity under conditions of pH 5.0 (Fig. 6b). With a decrease or increase in pH, antifungal activity of the protein significantly decreased. Especially above a pH of 11.0, the protein completely lost its antifungal activity. These results indicate that antifungal protein EP-2 is active over a wide pH-range, but it is sensitive to extreme alkaline conditions.

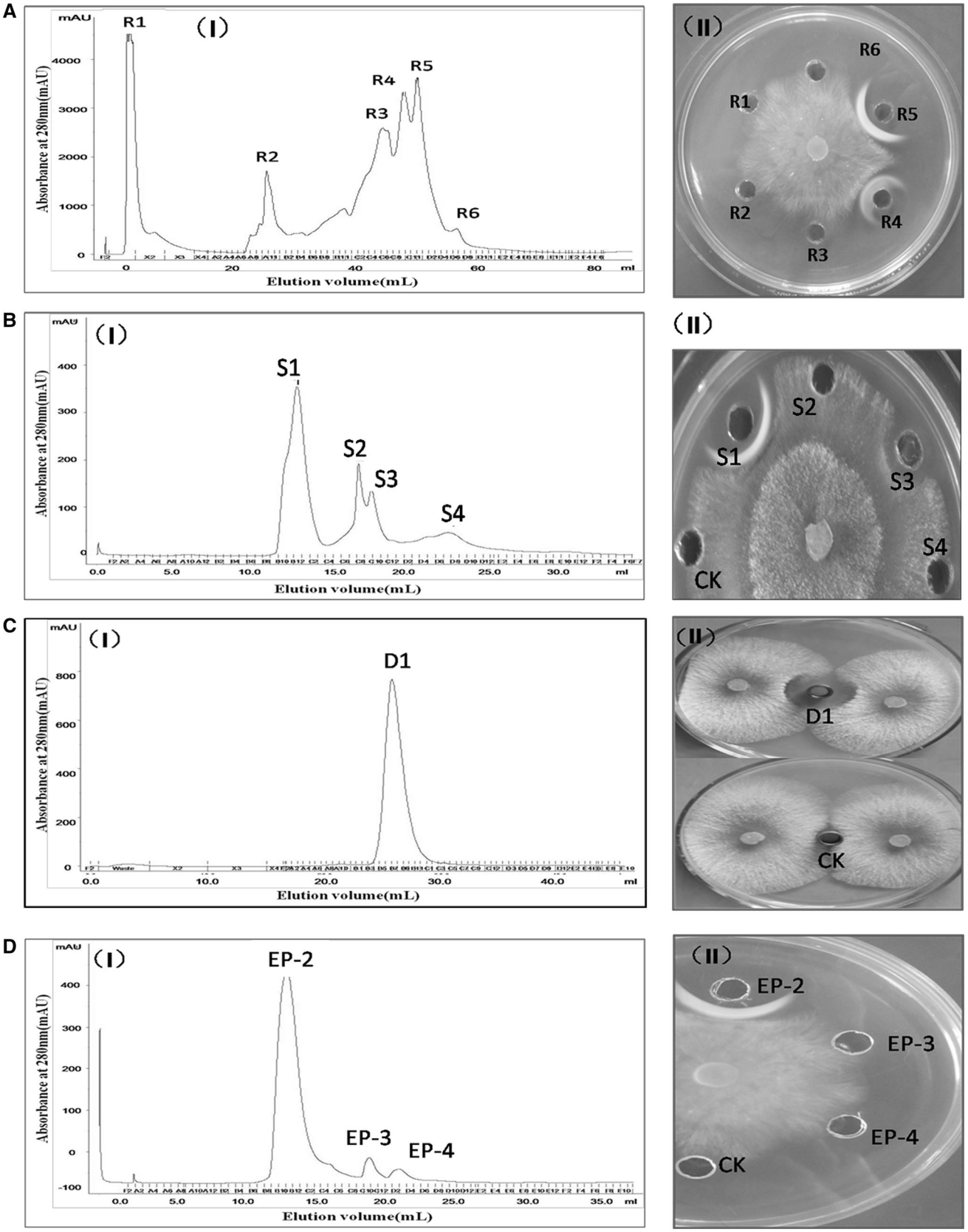
Antifungal protein EP-2 was not sensitive to treatment with different cations at the indicated concentrations. However, antifungal activity of EP-2 was severely affected by proteinase K treatment. After incubation with proteinase K at 37 °C for 1 h antifungal activity decreased by 71.9 % (Fig. 7). These results show that antifungal activity of the protein EP-2 was less affected by metal cations, but seriously affected by proteinase K treatment.

Discussion

Antifungal proteins are extremely attractive candidates for use as BCAs owing to their wide spectrum of antifungal activities and mechanisms of action, which differ from

Table 1 Protein yield of each purification step

Purification step	Volume (mL)	Total protein (mg)	Recovery (%)
Crude protein	10,000	3870	100
Reverse phase chromatography	80	425.7	11
Gel filtration	36	154.8	4
Anion exchange desalting	60	58.05	1.5
Desalting	36	19.5	0.5


A
(I)
R1
R2
R3
R4
R5
R6
mAU
Absorbance at 280nm(mAU)
Elution volume(mL)
ml
(II)
R1
R2
R3
R4
R5
R6
B
(I)
S1
S2
S3
S4
Absorbance at 280nm(mAU)
Elution volume(mL)
ml
(II)
S1
S2
S3
S4
CK
C
(I)
D1
Absorbance at 280nm(mAU)
Elution volume(mL)
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(II)
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CK
D
(I)
EP-2
EP-3
EP-4
Absorbance at 280nm(mAU)
Elution volume(mL)
ml
(II)
EP-2
EP-3
EP-4
CK

◀**Fig. 1** Purification of antifungal protein EP-2 from *Bacillus subtilis* E1R-J by reverse phase chromatography (**a**), gel filtration (**b**), anion exchange chromatography (**c**) and desalting (**d**). *I* Absorbance peaks of crude proteins purified process, *II* inhibition of *Valsa mali* 03-8 growth with the fractions collected from the absorption peaks, *CK* sterile water

those of small-molecule antibiotics. In this study, antifungal protein EP-2 was purified from fermentation broth (FB) of *B. subtilis* E1R-J by using acid precipitation, combined with various chromatographic steps; this approach is considerably different from the widely used ammonium sulfate precipitation method (Senol et al. 2014; Sathishkumar et al. 2015). This protein shows excellent tolerance to acid and heat, while still maintaining its antifungal activity at pH 1.0. Some reports on the heat resistance of antifungal proteins have been published (Skouri Gargouri and Gargouri 2008; Huang et al. 2009; Rao et al. 2015); however, very few reports discuss their acid stability (Xie et al. 1998). The results of this study provide a new resource for obtaining purified acid-resistant antimicrobial protein. During the acid precipitation process, the broth supernatant from *B. subtilis* E1R-J showed no antifungal activity following its treatment with hydrochloric acid, which proves that the active substances in the broth supernatant were completely precipitated. Meanwhile, the precipitate obtained showed strong antifungal activity, which is indicative of the acid resistance exhibited by the antifungal proteins. Moreover, extraction by hydrochloric acid precipitation should separate the acid-resistant antifungal proteins from other proteins, thereby simplifying the subsequent purification process, and the antifungal protein obtained by this method must be stable under acidic conditions.

In this study, protein EP-2 exhibited broad-spectrum antifungal activity against many pathogenic fungi with a strong inhibitory effect, especially against *V. mali*, wherein the diameter of the inhibition zone was up to 35 mm when 30 μg protein dissolved in 100 μL sterile water was applied for evaluation (Fig. 5). Thus, protein EP-2 is a very promising candidate for use as a BCA against AVC.

During the protein purification process, six absorbance peaks of fractions R1, R2, PR3, R4, R5 and R6 were recorded after the crude extract was subjected to separation by reverse phase chromatography using a RESOURCE RPC^{TM} column (Fig. 1a: I). According to the results of the evaluation of antifungal activity, fractions R4 and R5 showed antagonistic activity against *V. mali* (Fig. 1a: II). Protein EP-2 was obtained from fraction R5. These results suggest that protein EP-2 is just one of the several antifungal substances present in FB of *B. subtilis* E1R-J. Other

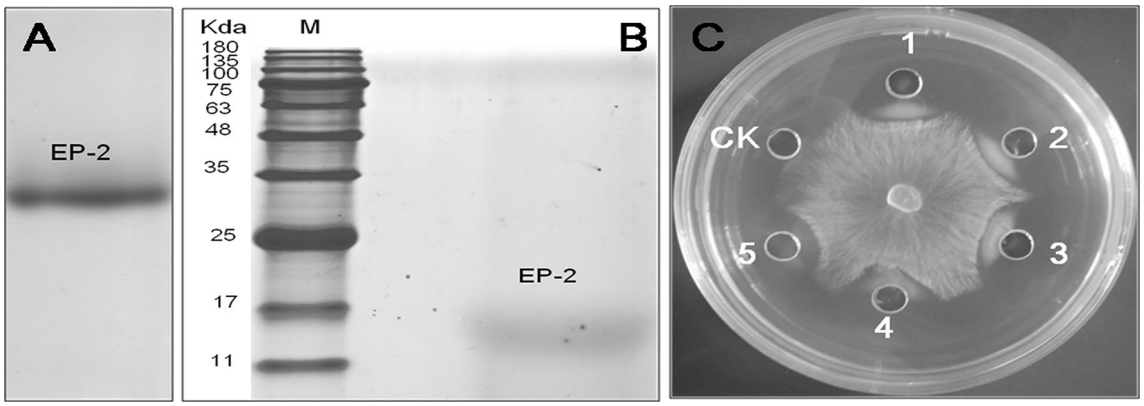

Fig. 2 Protein EP-2 identification and antifungal activity test. **a** EP-2 detected by native-PAGE, **b** EP-2 detected by SDS-PAGE, *left lane* protein molecular mass marker, *right lane* SDS-PAGE spectrum of antifungal protein EP-2 recovered by electric elution from a native-PAGE gel. **c** Antifungal activity of EP-2 against *Valsa mali* 03-8, *1* protein from the main peak EP-2 (such as D), *2–5* protein recovered from native-PAGE, *CK* sterile water

Table 2 The blast result of the protein sequence in NCBI

Protein ID	Molecular functions	Protein score	Coverage (%)	Molecular mass (kDa)	Isoelectric point	Organism
tr|A0A086WXP9	Antifungal polypeptide	109.63	28.21	12.4	9.94	*Bacillus subtilis*
tr|A0A080UHX6	Thioredoxin	77.98	22.12	11.5	4.24	*Bacillus subtilis*
sp|C0SP85	Uncharacterized protein YukE	43.9	7.22	11.0	4.14	*Bacillus subtilis*
tr|A0A080UC24	HTH-type transcriptional regulator tnrA	32.48	11.82	13.1	10.42	*Bacillus subtilis*

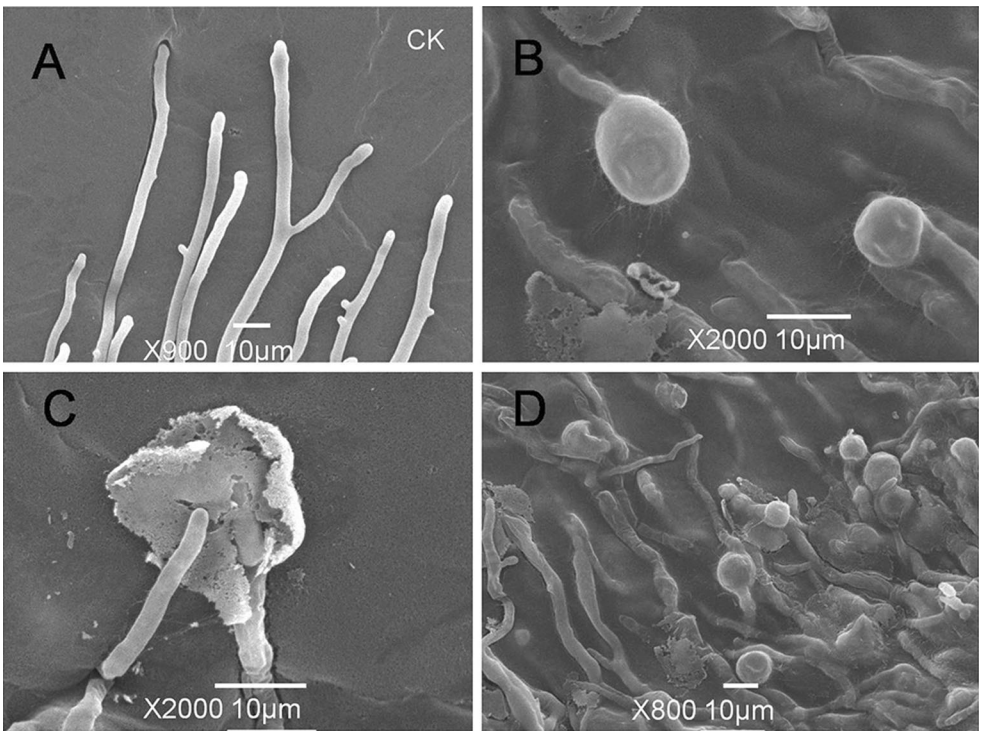

Fig. 3 Effect of the antifungal protein EP-2 on hyphal growth of *Valsa mali*. **a** Hyphae of *V. mali* 03-8 untreated. **b–d** Hyphae of *V. mali* 03-8 treated with antifungal protein EP-2 for 72 h

antifungal substances will need to be purified in the next study.

Identification of protein EP-2 was performed by using the Mascot search engine (Matrix Science, London, UK; version 2.3.02) against a database containing 61,370 sequences. Four proteins with the highest matching rates were antifungal polypeptide, thioredoxin, uncharacterized protein YukE, and the HTH-type transcriptional regulator TnrA. Among them, the protein with the highest protein score and coverage was antifungal polypeptide (Protein ID: tr|A0A086WXP9) (Table 2). The function of the antifungal polypeptide (Protein ID: tr|A0A086WXP9) is likely predicted from the genome of *B. subtilis*, and our results confirmed its antagonistic activity. Moreover, based on the amino acid sequences of EP-2, prediction of the signal peptide and transmembrane region was accomplished at http://www.cbs.dut.dk/services/SignalP and http://www.cbs.dut.dk/services/TMHMM/, respectively. The predicted results show that protein EP-2 has a strong hydrophobic region at the N terminus, with a typical signal peptide structure spanning residues 1–27 of the amino acid sequence. There are two typical protein transmembrane regions spanning residues 7–29 and residues 97–116 of the amino acid sequence. Based on the results of the prediction of the signal peptide and transmembrane structures, antifungal polypeptide is likely to be an extracellular protein. The predicted results were consistent with the origin of protein EP-2 from the FB of *B. subtilis* E1R-J. Moreover, we analyzed the function of the other three proteins with high matching rates. Thioredoxin is a catalyst and is usually involved in biochemical reactions; uncharacterized protein YukE is a type of carrier protein associated with secretion system VII, which is responsible for the transport of proteins associated with the secretion system to the extracellular environment; and HTH-type transcriptional regulator TnrA is a transcription factor that controls gene expression. The three proteins show no antimicrobial activity according to their functions. Therefore, owing to these reasons, we believe that the purified antifungal protein EP-2 is likely antifungal polypeptide. In our further studies, we will attempt the molecular cloning and sequence analysis of EP-2 to gain more information.

Development of a novel antifungal substance is of great interest for devising plant protection strategies (van der Weerden et al. 2013). To develop effective biocontrol approaches based on the biological effect of these antifungal substances, their modes of action must be understood. These anti-fungal substances exert their antifungal action through three main modes: disrupting the fungal cell membrane (Emrick et al. 2013), damaging the integrity of the fungal cell wall (Onishi et al. 2000), and causing the death of fungal cells by inhibiting DNA synthesis (Gopinathan 2013). In recent years, the biological roles of many purified antifungal peptides via different mechanisms of

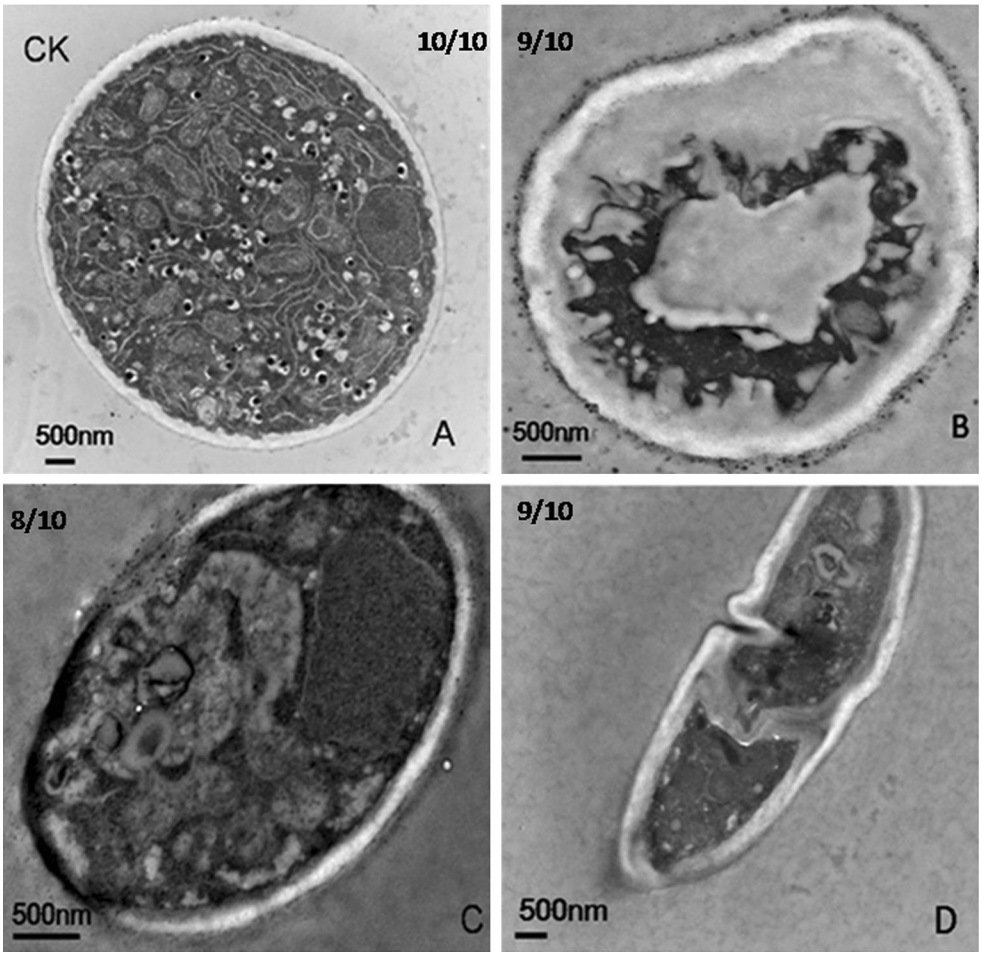

Fig. 4 Effects of antifungal protein EP-2 on ultrastructure of *Valsa mali* observed by transmission electron microscopy. **a** Hyphae of 03-8 *V. mali* untreated, **b–d** abnormal cells of *V. mali* 03-8 treated with antifungal protein EP-2 for 72 h; *a/b* "a" means times of similar phenomena observed, "b" means the total times of experiments conducted

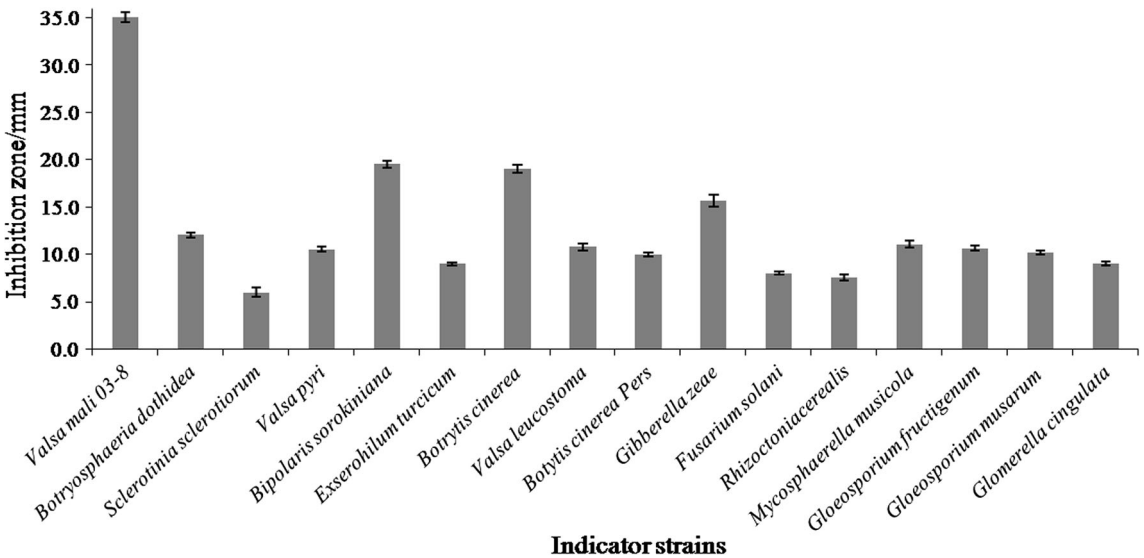

Fig. 5 Analysis of antifungal activity of protein EP-2. Each 30 μg protein EP-2 in sterile water (100 μL) was added into Oxford-cups. Each test was repeated three times

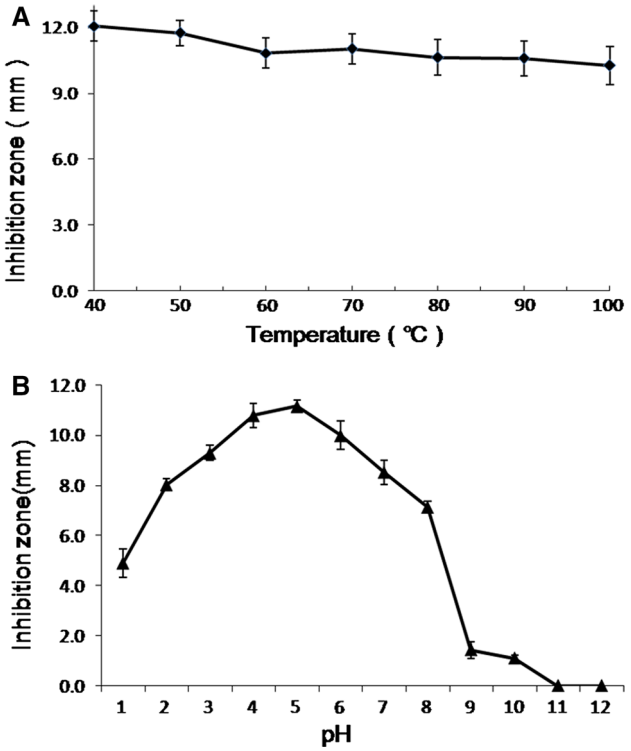

Fig. 6 Effect of temperature (**a**) and pH (**b**) on the antifungal activity of protein EP-2. Each 10 μg protein EP-2 in sterile water (100 μL) treated with different pH ranging from 1 to 12 for 1 h or temperatures ranging from 40 to 100 °C for 30 min was added into Oxford-cups and the protein EP-2 without treated was used as control. Each test was repeated three times

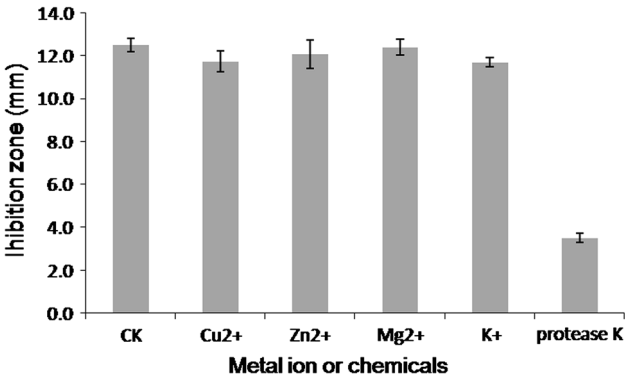

Fig. 7 Effect of ions and proteinase K on the antifungal activity of protein EP-2. Each 10 μg protein EP-2 in sterile water (100 μL) treated with 5 mM Cu^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, or 1 mg/mL proteinase K was added into Oxford-cups and the protein EP-2 without treated was used as control. Each test was repeated three times

action have been established. Moreover, some studies have shown that a single peptide is often capable of possessing more than one mode of action, depending on the type of target cell (van der Weerden et al. 2013). According to the results of SEM and TEM examinations, severe morphological alterations in the mycelial ultrastructure of *V. mali* hyphae were caused after *V. mali* was cultured for 72 h in the presence of protein EP-2. Further research is needed to understand the mechanism by which this component inhibits *V. mali*.

Conclusion

We purified and characterized an antifungal protein, EP-2, from *B. subtilis* E1R-J. Our results indicate that protein EP-2 is a promising BCA for use against AVC. In our further research, we will attempt to optimize the fermentation conditions during culture to increase the yield of the antifungal metabolites. Moreover, we can determine the gene sequences corresponding to the antifungal protein amino acid sequences, synthesize probes specific to resistance genes, and introduce gene probes encoding the antifungal protein into plants to obtain resistant plants. We could also generate an overexpression vector and facilitate industrial production of the antifungal protein. The findings of this study will provide new ideas and highlight the potential possibilities of developing new BCAs against *V. mali*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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