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Identification and Characterization of an Anti-fungi *Fusarium oxysporum* f. sp. *cucumerium* Protease from the *Bacillus subtilis* Strain N7

Yi Luo, Lifei Sun, Zhen Zhu, Wei Ran*, and Qirong Shen

Jiangsu Key Lab for Organic Solid Waste Utilization, Nanjing Agricultural University, Nanjing 210095, P. R. China

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A newly discovered alkaline antifungal protease named P6 from *Bacillus subtilis* N7 was purified and partially characterized. *B. subtilis* N7 culture filtrates were purified by 30–60% $(NH_4)_2SO_4$ precipitation, anion-exchange chromatography and gel filtration chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single band of 41.38 kDa. Peptide sequence of protease P6 was determined using a 4800 Plus MALDI TOF/TOF™ Analyzer System. Self-Formed Adaptor PCR (SEFA-PCR) was used to amplify the 1,149 bp open read frame of P6. Dimensional structure prediction using Automatic Modeling Mode software showed that the protease P6 consisted of two β-barrel domains. Purified P6 strongly inhibited spore and mycelium growth of *Fusarium oxysporum* f. sp. *cucumerium* (FOC) by causing hypha lysis when the concentration was 25 μg/ml. Characterization of the purified protease indicated that it had substrate specificity for gelatin and was highly active at pH 8.0–10.6 and 70°C. The P6 protease was inhibited by EDTA (2 mmol/L), phenyl methyl sulfonyl fluoride (PMSF, 1 mmol/L), Na^+, Fe^{3+}, Cu^{2+}, Mg^{2+} (5 mmol/L each) and H_2O_2 (2%, v/v). However, protease activity was activated by Ca^{2+}, K^+, Mn^{2+} (5 mmol/L each), mercaptoethanol (2%, v/v) and Tween 80 (1%, v/v). In additon, activity was also affected by organic solvents such as acetone, normal butanol and ethanol, but not hexane (25%, v/v each).

Keywords: purification, characterization, *Bacillus subtilis*, anti-fungal protease, self-formed adaptor PCR

Introduction

Bacillus subtilis has been used as a model system for studying the ecology, biochemistry, genetics, and physiology of Gram-positive bacteria for more than 50 years (Harwood, 1992; Becher *et al.*, 2011). Extensive knowledge of *B. subtilis* molecular biology has led it to be used as a biological control agent in agricultural sector where it can produce more than two dozen antibiotics with an amazing variety of structures (Stein, 2005). Based on the capacity of growing within many environments (Earl *et al.*, 2008), several *B. subtilis* strains and their derivatives were found to have broad suppressive abilities over a variety of plant pathogens. For example, Chung *et al.* (2008) reported that the ME488 *B. subtilis* isolate suppressed the growth of 39 of 42 plant pathogens tested. Recently, with the special emphasis on antibiotic mechanism, three families of lipopeptides in the lower mass range (1–1.5 kDa), including surfactins, iturins, and fengycins have been isolated from the bacterium and studied for their surfactant and antibiotic properties (Stein, 2005). Additional attempts also have been made to produce these lipopeptides through solid state fermentation of agricultural by-products and waste products, because they confer low toxicity, low allergic effect on humans and animals (Mizumoto and Shoda, 2007). In the view of present papers, many other lower mass antimicrobial peptides including bacilysin (0.27 kDa) (Özcengiz and Alaeddinoglu, 1991), sublancin (3.88 kDa), (Oman *et al.*, 2011), and rhizocticin (0.45 kDa) (Rapp *et al.*, 1988; Kugler *et al.*, 1990; Borisova *et al.*, 2010) have also been extracted from strains of this bacterium. Moreover, *B. subtilis* also secretes an abundances of antifungal proteins with differences in molecular weight such as 50.8 kDa (Chen *et al.*, 2010), 42.3 kDa (Li *et al.*, 2009), 41.9 kDa (Liu *et al.*, 2007), 59 kDa (Xie *et al.*, 1998), and 67 kDa (Manjula *et al.*, 2004). Recently, approximately 90 extra-cellular proteins have been identified by proteomic analysis of proteins secreted by *Bacillus* spp.

In the view of these findings, the application of proteins, particularly proteases, isolated from *Bacillus* spp., has rapidly grown in the food, leather, pharmaceutical and bioremediation industries as well as in the textile industry for removal of protein-based stains (Oberoi *et al.*, 2001; Gupta *et al.*, 2002). However, few reports have investigated the correlation between the bio-control function and extra-cellular proteases for suppressing plant pathogens, partly because it is difficult to characterize proteins due to the diversity of their actions and structures. With the advent of novel molecular biological techniques, protein sequencing (Peptide Mass Finger) and Self-Formed Adaptor PCR (SEFA-PCR) techniques have been confirmed powerful techniques to identify unknown proteins, by determining their sequence information (Wang *et al.*, 2007; Liu *et al.*, 2010).

This study aimed to purify and identify an antifungal Protease P6 produced by *B. subtilis* N7 and to test its effect on the growth of *Fusarium oxysporum* f. sp. *cucumerium* (FOC). Peptide mass fingerprinting, degenerate oligonucleotide primer PCR and SEFA-PCR were performed to identify the open reading frame (ORF) sequence of P6. The dimensional

*For correspondence. E-mail: savetheday@163.com; Tel./Fax: +86-025-84396212

Table 1. Degenerate primer PCR and SEAF-PCR primers

Primers	Sequence (5'→3')	Product size (bp)
Up-2(F)	AACAAAACCTTATGATGATAATGGACAYGGNACNCA	603
Do-5(R)	GCAAATCGGTGTTGCCATNSWNGT	
F10-Lsp1	ATGGAATGAAGGATTCGTCGTCTGC	
F10-Lsp2	GACGATACGGTCGCTTCTTTCTCAA	
F10-Lsp3	TCAAACCGTGTAGGCNNNNNNNNNTTCAGTC	
F10-Rsp1	GAGCGGGTCGTCCGTTTCTT	
F10-Rsp2	CAAGGTTCCTGATCCTGATT	
F10-Rsp3	CGGCCCCTGATATTTACCNNNNNNNNAAGCGC	

Note: F, forward primer; R, reverse primer; Lsp1-3, upstream SEFA-PCR primers; Rsp1-3, downstream SEFA-PCR primers

structure of the protease P6 was predicted using Automatic Modeling Mode software. Subsequently, characterization of the purified P6 protease was conducted with special emphasis on its activity in different environments.

Materials and Methods

Strain

B. subtilis N7 was isolated from the cucumber rhizosphere. NCBI database blast of 16S rRNA gene sequence showed 99% homology to *B. subtilis* DSM 10. This strain was stored on nutrient agar (NA) medium at 4°C. The 16S rRNA gene sequence of the strain N7 was deposited into GenBank with the accession no. JQ317780. FOC fungal plant pathogens were obtained from the Institute of Vegetables and Flowers, the Chinese Academy of Agricultural Sciences in Beijing, China.

Isolation of the antifungal protease

B. subtilis N7 was cultured in 200 ml of Luria-Bertani medium (LB) with shaking at 180 rpm for 60 h at 30°C. The supernatant was collected after centrifugation at 10,000×g for 15 min, and then relatively saturated with solid ammonium sulphate to 0–30%, 30–40%, 40–60%, 60–80%, and 80–100%. The precipitate was stored at 4°C overnight and then centrifuged at 10,000×g for 20 min. The fractionated precipitation was resolved in 5 ml of 25 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer for 2 days to remove the ammonium sulphate prior to the anti-fungi activity assay. The anti-fungi activity protein fraction was applied to a DEAE-Sepharose Fast Flow (Amersham Biosciences) column (2.5 cm×20 cm). After removal of the non-adsorbed fraction, adsorbed proteins were eluted with linear concentration gradients of 0–1 M NaCl in 25 mM Tris-HCl buffer (pH 8.0). Subsequently, the eluted fractions were dialyzed for 2 days and then subjected to an anti-fungi activity assay. The anti-fungi activity fraction was further isolated and applied to a Superdex^{TM} 200 HR 10/30 column (GE Healthcare) in Tris-HCl buffer. The final purified protein was confirmed by its anti-fungi activity on Petri dishes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified antifungal protein from *B. subtilis* N7 was subjected to SDS-PAGE (Laemmli, 1970) using 10% resolving and 5% stacking gels. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. The molecular mass of the proteins was determined by comparison of electrophoretic mobility with a molecular mass protein marker (Institute of Biochemistry, China).

Peptide Mass Finger sequences

After SDS-PAGE, the band of protein P6 was excised and sent to Company of Biomedical Analysis (Sangon Co., China) to determine the peptide fractions that formed the protein. P6 band was digested with trypsin (sequencing grade, Promega) and peptide samples generated from the trypsin digest were loaded into a 4800 Plus MALDI TOF/TOF^{TM} Analyzer system. Sequence homology was analyzed using the Mascot Program and the NCBI BLAST online search service.

Amplification of the open reading frame sequence of P6

High levels of conserved blocks from serine proteases were found by 6 amino acid sequence alignment against *B. amyloliquefaciens* FZB42 AprX GenBank no. ABS74070.1; *B. amyloliquefaciens* DSM 7 alkaline serine protease reference sequence no. YP_003920391.1; *B. subtilis* subsp. *spizizenii* ATCC6633 alkaline serine protease reference sequence no. ZP_06873415.1; *Clostridium* sp. D5 alkaline serine protease reference sequence no. ZP_08129737.1; *Thermoanaerobacter wiegelii* Rt8. B1 peptidase S8 and S53 subtilisin kexin sedolisin reference sequence no. YP_004819622.1; and *Clostridium* sp. D5 alkaline serine protease reference sequence no. ZP_8129737.1, which were downloaded from NCBI. Degenerate oligonucleotide primers Up-2(F) and Do-5(R) (listed in

Table 2. Thermal cycling conditions for SEFA-PCR

Primer addition	No. of cycles	Cycling conditions
F10-Lsp3 or F10-Rsp3	1	94°C 3 min, 94°C 90 sec, 35°C 3 min, then ramping to 58°C at 0.2°C/sec, 70°C 5 min
F10-Lsp1 or F10-Rsp1	35	94°C 30 sec, 56°C 5 min, 70°C 5 min 25 cycles; 10 cycles of 94°C 30 sec, 56°C 5 min, 70°C 5 min, 2 cycles and 94°C 30 sec, 50°C 30 sec, 70°C 5 min, 1 cycle
F10-Lsp2 or F10-Rsp2	25	94°C 3 min, 94°C 30 sec, 56°C 5 min, 70°C 5 min

Table 1) were designed by CODEHOP designer (http://blocks.Fhcrc.rg/codehop.html). These primers were derived from conserved sequence blocks and were used in PCR to amplify the region between them.

SEFA-PCR was used to clone the flanking DNA sequences of the known region amplified by the degenerate primers. The two sets of primers located sequentially on the known DNA sequences are listed in Table 1. The reaction conditions are listed in Table 2. The final DNA bands were recovered and sequenced by cloning into T-vector (pMD18-T; TaKaRa Co.).

Dimensional structure prediction of P6

The completed DNA sequence coding for P6 was interpreted using ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). After ORF obtained, analysis of dimensional structure for P6 was conducted by Automatic Modeling Mode Program (http://www.expasy.org/swissmod).

Assay of anti-fungi activity

Anti-fungi activity against FOC assays were carried out in 90×15-mm Petri dishes containing 20 ml of potato dextrose agar, under sterile conditions. After mycelial colonies developed, sterile Oxford cups (0.6 cm in diameter) were placed 0.5 cm away from the rim of the mycelial colony. An aliquot of protein solutions filtered through a 0.22-μm filter was added to the Oxford cup. The dishes were then incubated at 30°C for 48 h until mycelial growth had enveloped the Oxford cup containing Tris-HCl as a control.

Evaluation of spore growth inhibition by P6 in a quantitative 96-well plate format was performed as described by Pillay *et al.* (2011) with minor modifications. Spores of FOC were isolated from the Potato Dextrose Agar (PDA) by adding 10 ml of sterile water to the petri dish and shaking it gently. The spore suspension was then decanted and centrifuged at 1,000×g for 15 min at room temperature. The pelleted spores were re-suspended in 0.5 ml of sterile 10% glycerol. The spore concentration was determined by Hemocytometer counts before use. The growth assay was carried out in sterile 96-well microtiter plates fitted with lids. Spores in 80 μl aliquots of half strength Potato Dextrose Broth (PDB) at the density of 50,000 spores/well. 20 μl of filter sterilised P6 (at final concentrations of 12.5, 25, and 50 μg/ml) was added to an 80 μl suspension of FOC spores. Sterile Milli-Q water was added to the appropriate wells as a control and a PDB blank was also included. Three replicate wells were set up for each treatment. Plates were incubated in the dark at 30±0.5°C and absorbance at 595 nm was measured every 24 h for 6 days using a Spectra Max M5 microplate reader (Molecular Devices, USA). IC_{50} value of anti-fungi FOC activity of P6 was determined by three doses of the protein (at the concentrations of 10, 30, and 60 μg/ml) added separately to three aliquots each containing 15 ml of PDA medium at 50°C. A small amount of mycelia was added to the center of each solidified agar plate. An equal volume of 25 mM Tris-HCl buffer (pH 8.0) without P6 was poured in the Petri dishes as a control. Plates were incubated at 30°C for 72 h until mycelial growth of the control enveloped the entire plate. For comparison, the IC_{50} value of the anti-fungi FOC activity of a known antifungal inhibitor, iturin A (Sigma-Aldrich, USA), was determined by the method described above for three different concentrations (12.5, 25, and 50 μg/ml). The area of the mycelial colony was measured, and inhibition of fungi growth in the plates was determined by calculating the % reduction in the area of the mycelia colony (Wang and Ng, 2005). The IC_{50} value was the concentration that caused a 50% reduction in the area of the mycelial colony.

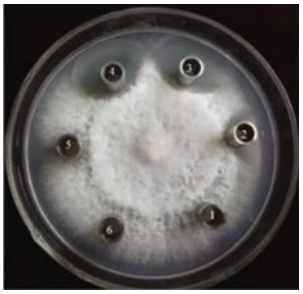

Fig. 1. FOC inhibition by precipitated proteins from different ammonia sulfate saturation conditions (1, CK; 2, 0–30% ammonia sulfate saturation; 3, 30–40% ammonia sulfate saturation; 4, 40–60% ammonia sulfate saturation; 5, 60–80% ammonia sulfate saturation; 6, 80–100% ammonia sulfate saturation).

Protease assays and protein measurements

Protease activity was determined by the method of Oberoi *et al.* (2001) using casein as a substrate. A volume of 50 μl of the protease was mixed with 2 ml of 50 mmol/L glycine-NaOH buffer (pH 9.0) containing 1% (w/v) casein, incubated at 60°C for 5 min along with the respective controls. The reaction was stopped by addition of 2 ml of 10% (w/v) trichloroacetic acid. The mixture was allowed to stand at 40°C for 15 min, centrifuged at 13,000×g for 10 min to remove the precipitate. The acid-soluble material was estimated by

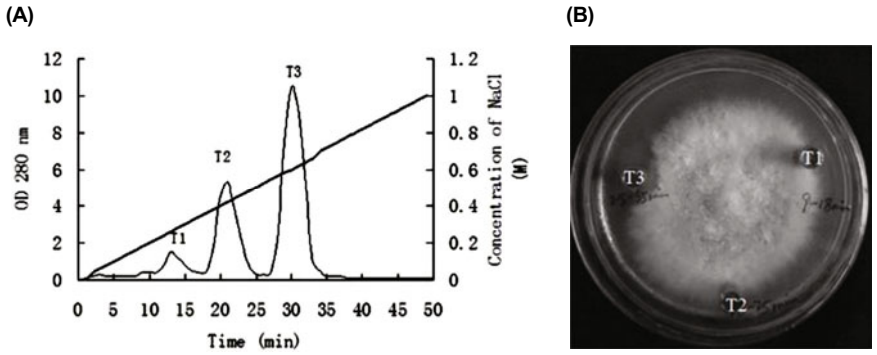

Fig. 2. Purification results from the DEAE Fast flow column [—, concentration of NaCl (A) and inhibition efficiency of separated peaks on FOC (B)].

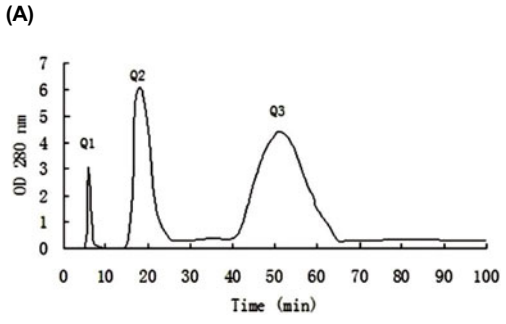

Fig. 3. Purification results from the Superdex^{TM} 200 HR 10/30 column (A) and inhibition efficiency of separated peaks on FOC (B).

spectrophotometric absorbance at 275 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg tyrosine/ml/min, under the standard assay conditions. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Effects of pH and temperature on protease activity

The optimum pH was determined at 60°C for 30 min with 1% (w/v) casein as a substrate. The pH of the reaction mixture was adjusted using 0.1 mol/L of following buffers: citrate buffer (pH 5.0–6.0), Tris-HCl buffer (pH 7.0–9.0), and glycine-NaOH buffer (pH 10.0–10.6). The pH stability was also determined at the same pH range by pre-incubation of 50 μl of the enzyme solutions and 2 ml of the above mentioned buffers at 60°C for 30 min. The relative activities were quantified under standard assay conditions. To study the thermal stability of the enzyme, P6 was pre-incubated in glycine-NaOH buffer at temperatures rang from 30 to 90°C for 30 min. The relative activities were quantified under standard assay conditions.

Effects of inhibitors, metal ions, detergents, oxidants, and organic solvents on protease activity

Protease aliquots were pre-incubated with metal ions (e.g., Ca^{2+}, Na^{+}, K^{+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, and Mg^{2+}), inhibitors (PMSF, EDTA, and mercaptoethanol), detergents (SDS and Tween 80) and oxidizing agents (H_2O_2) at 60°C for 30 min. Enzyme stability in the presence of organic solvents was investigated by incubating the purified protease with acetone, n-butanol, hexane, and ethanol at 60°C for 30 min. The relative activities were quantified under standard assay conditions.

Effects of different substrates on protease activity

The substrate specificity of the protease for BSA, casein, and gelatin was assessed as described above.

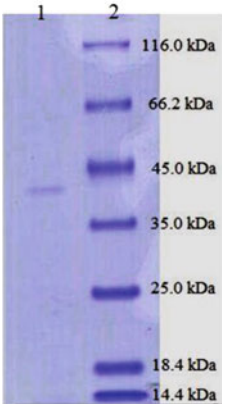

Fig. 4. SDS-PAGE for anti-fungal proteins. Lane 1 shows anti-fungi protein after Sepharose Fast Flow column and Superdex^{TM} 200 HR 10/30 column chromatography. Lane 2 shows the protein marker.

Data analysis

The data were analyzed in SPSS 16.0. The data were subjected to Duncan's analysis of variance (ANOVA), and the means were separated by Duncan's multiple range tests at $P \leq 0.05$.

Results

Isolation of antifungal protein

Antifungal protein was isolated using saturated ammonium sulfate, DEAE-Sepharose Fast Flow column chromatography and Superdex^{TM} 200 HR 10/30 column chromatography. The crude protein precipitated by $(NH_4)_2SO_4$ at 30–60% relative saturation showed stronger antifungal activity than those at the other relative saturations (Fig. 1) and was purified in next steps. The saturated ammonium sulfate purified crude protein extracted on the DEAE-Sepharose Fast Flow column

Table 3. Sequence homology of protein P6 with other proteins using Mascot Search Tool

The name of bacterium and homologous protein (NCBI no.)	Matched peptides sequence	Matching position	Ions score
Bacillus amyloliquefaciens FZB42 AprX (gi|154686140)	GVTVAVIDTGIYQHPDLEGR	148–167	135
	VIGFADFVNQK	168–178	64
	YQGPAPEADLIGVK	206–219	75
	IISMSLGGDALR	253–264	28
	VITVGAYDDNDTAGNEDDTVASFSSR	310–335	145
	EKPDILAPGVDIVSLR	343–358	136

Note, Individual ions scores > 56 indicate identity or extensive homology ($P<0.05$).

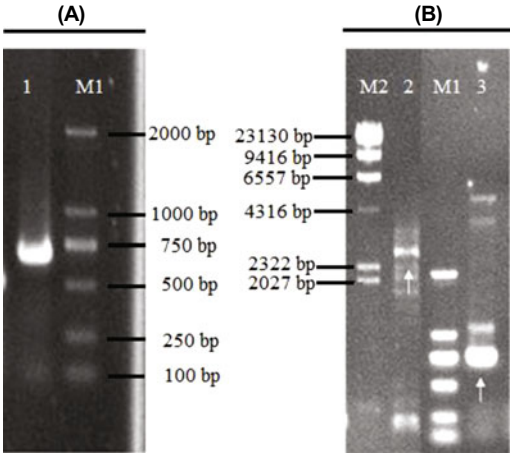

Fig. 5. Results from degenerate primer PCR (A) and SEFA-PCR (B). Lane 1 shows the partial sequence of P6 (approximately 640 bp in in length); Lane M1 shows the DL 2000 maker; Lane M2 shows the *Hin*dIII Marker; Lane 2 shows the downstream sequence of Fig. 5A (white arrow indicates the target band for sequencing, approximately 2,730 bp in length); and Lane 3 shows the upstream sequence of Fig. 5A (white arrow indicates the target band for sequencing, approximately 820 bp in length).

produced three adsorbed fractions (9–18 min, 18–25 min, 25–35 min) (Fig. 2A). Of these fractions, only the 25–35 min fraction (T3) showed high antifungal activity (Fig. 2B). Fraction T3 was further loaded on a Superdex™ 200 HR 10⁄30 column (GE Healthcare) and yielded three fractions: Q1, Q2, and Q3 (Fig. 3A). Only fraction Q3 exhibited antifungal activity (Fig. 3B). SDS-PAGE showed that fraction Q3 was a single band with a molecular mass of 41.38 kDa (Fig. 4) as calculated by the quantity one program (Bio-Rad, USA). This protein was designated as P6.

Peptide sequences from Protease P6

Six peptides from the purified anti-fungi protein were analyzed using a 4800 Plus MALDI TOF/TOF™ Analyzer. The protein was classified as a novel serine protease after a blast of the peptide sequences against the NCBI databank (Table 3). The amino acid sequences of the five peptides showed homology with AprX from *Bacillus amyloliquefaciens* FZB42, which is a serine protease with a mass of 48.69 kDa. However, partial sequences of the fourth peptide exhibited low similarity to protease from *Bacillus* sp., suggesting its hypothetical traits form other species.

Fig. 6. Dimensional structure of P6 of *B. subtilis* N7. The black arrow indicates the typical β-barrel conformation.

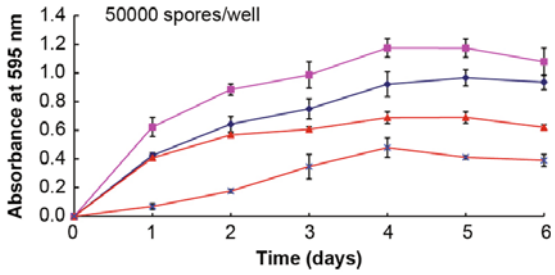

Fig. 7. The FOC growth inhibition assay. Fungal spores were immersed in half strength PDB to a concentration of 50,000 spores/well and the assay plates were incubated in the dark at 30±0.5°C. Varying concentrations of protease P6 (■, 12.5 μg/ml; ▲, 25 μg/ml; ×, 50 μg/ml) were tested against the fungal spores. Assays without P6 served as negative controls (◆). Data represent the means of 3 independent measurements.

Open reading frame sequence of Protease P6

Degenerate oligonucleotide primer PCR protocol was used to clone a portion of sequences from the protease P6 synthesis gene, approximately 640 bp in length (Fig. 5A). Further, this DNA sequence was used as a template for chromosomal walking primer design. After two orientated SEFA-PCR tests, target sequences through three rounds of PCR for each amplification orientation were obtained (Fig. 5B). The complete ORF of P6 was estimated 1,149 bp in length after sequencing and assembling analysis (GenBank accession no. KC422672).

Dimensional structure prediction

The dimensional structure of P6 was predicted using its ORF sequence and showed in Fig. 6. P6 was mainly consisted of two typical β-barrel domains and four α-helix domains. In addition, irregular folds were also found.

Anti-fungi activity assay

The purified antifungal protease P6 was tested for FOC spore growth inhibition using a quantitative 96-well plate assay (Broekaert *et al.*, 1990). Figure 7 shows that FOC spores germinated more rapidly in the presence of low concentration of P6 (12.5 μg/ml) than treatment in the absence of P6. Spores germinated slowly at P6 concentrations of 25 and 50 μg/ml. The optical densities achieved after 6 days of incubation indicate that P6 could be a potent inhibitor of FOC growth, exerting strong inhibition of fungal growth at concentrations greater than 25 μg/ml. According to the inhibition standard curves (Table 4), the IC_{50} values of P6 and iturin A on FOC mycelium were 32.2 and 23.6 μg/ml, respectively.

Table 4. IC_{50} values of iturin A and Protease P6 inhibitory activity towards FOC

Antagonist	Y=ax+b	R^2	IC_{50} (μg/ml)
Protease P6	Y=1.261x+9.3539	R^2 = 0.9451	32.2
iturin A	Y=1.881x+5.6	R^2 = 0.9804	23.6

Note: Y and x represent percentage of inhibition area of mycelial and concentration of antagonist in plates respectively.

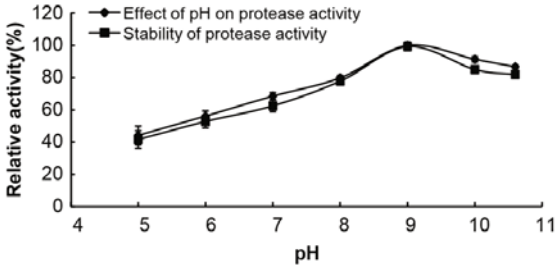

Fig. 8. Effect of pH on activity and stability of P6. The activity of the protease at pH 9.0, corresponding to 665 U/ml, was considered to be 100% activity. Results represent the mean values of three independent replicates and error bars indicate standard deviation.

Effects of pH and temperature on protease activity

The effect of pH on protease activity is shown in Fig. 8. The optimum pH for P6 was 9.0 within the pH range of 5.0–10.6. P6 was stable in a pH range between pH 8.0 and 10.0 maintaining over 70% of its original activity. P6 showed the high activity at temperatures between 60 and 80°C, with an optimal temperature of 70°C (Fig. 9). However, the enzyme only retained 54% of its activity after incubation for 30 min at 70°C.

Effects of inhibitors, metal ions, detergents, oxidants, and organic solvents on protease activity

P6 was severely inhibited by the serine protease inhibitor PMSF (1 mmol/L). However, the metalloprotease inhibitor EDTA (2 mmol/L) only caused slight (8%) inactivation (Table 5). Most of the metal ions tested (Ca^{2+}, K^{+}, and Mn^{2+}) had stimulatory effects, although Fe^{3+} was slightly inhibitory. Among these, the activity of purified P6 was enhanced by 22% by 5 mmol/L of $MnCl_2$, because of activation by Mn^{2+}. Two detergents were assayed for their effects on protease activity. Tween 80 increased protease activity; whereas, SDS decreased protease activity by 20%. The activity of purified P6 seemed to be stimulated by reducing environments (Table 5), as compared with exposure to oxidizing agents (2% H_2O_2).

As most proteins are inactivated in the presence of organic solvents, purified P6 was also found to be unstable in organic solvents. More than 20% inactivation was observed in acetone, n-butanol and ethanol after 30 min of pre-incubation. Among the organic solvents studied, the enzyme was only stable in the presence of hexane (Table 5).

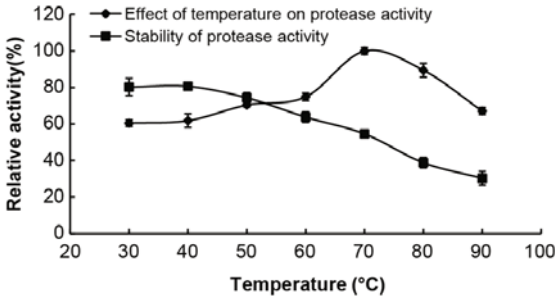

Fig. 9. Effect of temperature on activity and stability of P6. The activity of the protease at 70°C, corresponding to 778 U/ml, was considered to be 100% activity. Results represent the mean values of three independent replicates and error bars indicate standard deviation.

Table 5. Effect of various metal ions, surfactants, inhibitors and organic solvents on protease activity

Reagents	Concentration	Relative activity (%)
Ca^{2+} ($CaCl_2$)	5 mmol/L	110 ± 2.7
Na^{+} (NaCl)	5 mmol/L	77 ± 9.2
K^{+} (KCl)	5 mmol/L	112 ± 8.4
Mn^{2+} ($MnSO_4$)	5 mmol/L	122 ± 2.9
Fe^{3+} ($FeCl_3$)	5 mmol/L	90 ± 3.2
Cu^{2+} ($CuCl_2$)	5 mmol/L	87 ± 6.3
Mg^{2+} ($MgSO_4$)	5 mmol/L	86 ± 4.7
H_2O_2	2% (v/v)	82 ± 8.1
EDTA	2 mmol/L	92 ± 4.1
Mercaptoethanol	2% (v/v)	104 ± 3.4
SDS	1% (w/v)	80 ± 7.7
Tween 80	1% (v/v)	103 ± 2.2
PMSF	1 mmol/L	0
Acetone	25% (v/v)	57 ± 3.2
N-Butanol	25% (v/v)	71 ± 3.9
Ethanol	25% (v/v)	76 ± 7.8
Hexane	25% (v/v)	102 ± 5.6

Note: PMSF, phenymethylsulfonyl fluoride; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate. Data represent the mean values of three independent replicates, and ± means standard deviation (n=3). Protease activity without any chemical was taken as 100% which corresponded to 502 U/ml.

Substrate specificity

P6 substrate specificity was studied using different protein substrates at 1% w/v at 60°C. The results showed gelatin was the most preferred substrate for P6 with high activity (100%), followed by BSA (52%) and casein (43%) (Table 6).

Discussion

The Gram-positive microorganism *Bacillus* offers the advantage that it can be used in the development of a wide variety of antifungal agents for control of spore formation and soil born pathogens (Kim and Chung, 2004; Toure *et al.*, 2004). In this paper, we isolated a novel serine protease from *B. subtilis* N7, which can inhibit the growth of the pathogen FOC. The antifungal protein can be precipitated with 30% and 60% relative saturation of ammonium sulphate. Protein in the 30–60% precipitation fraction was further purified and isolated using DEAE-Sepharose and gel filtration chromatography. SDS-PAGE showed a single clear protein band of

Table 6. Substrate specificity of the protease P6

Substrate	Concentration	Relative activity (%)
BSA	1% (w/v)	52 ± 2.6
Casein	1% (w/v)	43 ± 2.1
Gelatin	1% (w/v)	100

Note, Data represent the mean values of three independent replicates, and ± denotes standard deviation (n=3). Protease activity measured in the presence of gelatin was taken as 100%,which corresponded to 3072 U/ml.

41.38 kDa after three steps of purification. This band was distinct from several other antifungal proteins from *B. subtilis* strains with molecular masses of 50.8 kDa (Chen *et al*., 2010), 59 kDa (Xie *et al*., 1998), and 67 kDa (Manjula *et al*., 2004), and however it was similar to proteins of 42.3 kDa (Li *et al*., 2009) and 41.9 kDa (Liu *et al*., 2007).

To confirm the identity of P6, Peptide Mass Finger technology was conducted using a MALDI TOF/TOF Analyzer System, which can identify a protein by sequencing several peptides of the protein after enzymatic digestion. Unlike Edman degradation chemistry, which is the most widely used protein sequence technology (Liu *et al*., 2007), Peptide Mass Finger technology is not restricted to N-terminal modifications and also has high sensitivity, high mass accuracy and high speed (Liu *et al*., 2010). By blasting against the NCBI databank, the antifungal protein was confirmed as a serine protease. Five of the six sequenced peptides matched the AprX protease from *B. amyloliquefaciens* FZB42, which belongs to the serine protease family. However, SDS-PAGE demonstrated that the molecular mass of P6 was different from other reported serine proteases of 30.9 kDa (Guangrong *et al*., 2010); 15 kDa (Adinarayana *et al*., 2003) and 29.0 kDa (Bhaskar *et al*., 2007). The results obtained indicate that P6 may be identified as a novel protein.

SEFA-PCR was used to amplify 1,149 bp of the full gene sequence of P6. Amino acid alignment showed that the amino acid sequence of P6 had high similarity (64.79%) with that of *B. amyloliquefaciens* FZB42 AprX. Furthermore, P6 had two β-barrel domains, which is the typical conformation for serine proteases (Hedstrom, 2002; Perona and Craik, 2008).

Automated microtiter plate broth assay was used to study fungi growth inhibition. FOC spores germinated more rapidly in the presence of low concentrations of P6 as compared to control (in absence of P6). Few of the relevant literature shows low concentrations of antifungal protein can enhance fungi growth, this may because most fungi growth inhibition assays were performed using solid medium plates (Liu *et al*., 2010; Das *et al*., 2011) where slight changes in spore growth were hard to observe. The results also showed both of 25 and 50 μg/ml of protease P6 strongly inhibited FOC growth, indicating that P6 may be considered as an effective fungi inhibitor when used at appropriate concentrations.

P6 was also effective on inhibiting FOC mycelium growth (IC_{50} of 32.2 μg/ml), compare to the IC_{50} values of iturin A (23.6 μg/ml). A few reports are available on IC_{50} values of antifual inhibitors. Cho *et al.* (2009) suggested 52.0 μg/ml of iturin A could reduce the growth of *Aspergillus flavus* by half and Liu *et al.* (2010) found that the IC_{50} value of the antifungal protein E2 on mycelium growth of *Gaeumannomyces graminis* var. *tritici* was 20 μg/ml. The primary mode of action of P6 on FOC inhibition could be related to hydrolysis and loss of integrity of the FOC cell wall. A similar morphological change in pathogenic bacterial cells has been reported at enzyme concentrations of 65.7 μg/ml (Bhaskar *et al*., 2007).

Further more, purified P6 revealed good stability over broad ranges of pH and temperature. P6 retained 54.6% of its relative activity after 30 min at 70°C and pH 9.0. This pH and temperature stability is very important for commercial protease applications. Oberoi *et al.* (2001) reported an industrially promising protease that had the highest activity at 70°C when the pH ranged from 8–11. Thus, the present protease P6 is also promising for industry, as it is active over a broad temperature and pH range.

In terms of the effect of metal ions, the literature showed that certain bacterial serine proteases were repressed by Zn^{2+}, Hg^{2+}, and Cu^{2+}, while others were not (Nilegaonkar *et al*., 2007). Our results confirmed that Cu^{2+} inhibited the activity of P6. Whereas, the presence of Ca^{2+}, Mn^{2+}, and K^{+} activated P6, This may due to the effects of cations on maintaining or diminishing active confirmation of the enzyme (Nilegaonkar *et al*., 2007). In addition, activity of purified P6 was completely inhibited by PMSF and slightly inhibited by EDTA (8%), which confirms that PMSF and EDTA are inhibitors for proteases (Riffel *et al*., 2003). Importantly, in the presence of anionic surfactants (SDS), oxidizing agents (H_2O_2), and reducing agents (mercaptoethanol), P6 showed high stability and compatibility. However, the results did not accord well with the earlier reports. For example, Reddy *et al.* (2008) reported that an alkaline protease produced by *Bacillus* sp. RKY3 retained approximately 77% of its original activity after treatment with 1% (w/v) SDS for 30 min and earlier report on the stability of alkaline proteases in the presence of oxidants showed that an alkaline protease from *Bacillus* sp. RGR-14 lost 40% of its activity in 1% (v/v) H_2O_2 (Oberoi *et al*., 2001). Thus, these findings most likely suggest that protease activity may vary with demension differences. Proteins are easily inactivated or denatured in organic solvents. In aqueous environments, proteins possess the conformational mobility necessary for optimal catalysis. In contrast, organic solvents cannot engage multiple hydrogen bonds and have lower dielectric constants, leading to stronger intra-protein electrostatic interactions (Reddy *et al*., 2008). Therefore, proteases that are naturally stable in the presence of organic solvents are less. P6 was not stable after 30 min of pre-incubation in the organic solvents we tested, except for hexane. Although casein is usually chosen as the preferred substrate for protease reactions (Oberoi *et al*., 2001; Reddy *et al*., 2008), P6 showed greater activity on BSA and gelatin.

In present study, we reported a new protease from *B. subtilis* N7 obtained by combining protein purification and gene amplification methods. Protease P6 has the potential for use in industry for bioremediation in addition to its antifungal agricultural uses. However, further studies on the pilot-scale production of Protease P6 need to be conducted before it can be used as for production of protein hydrolysates or for bio-control of fungi.

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