Appl Microbiol Biotechnol (2006) 69: 722–730
DOI 10.1007/s00253-005-0019-5

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Bacillus sp. B16 kills nematodes with a serine protease identified as a pathogenic factor

Received: 22 March 2005 / Revised: 17 April 2005 / Accepted: 2 May 2005 / Published online: 15 July 2005

Abstract An endospore-forming bacterium, strain B16, was isolated from a soil sample and identified as a *Bacillus* sp. The strain presented remarkable nematotoxic activity against nematode *Panagrellus redivivus*. The crude extracellular protein extract from culture supernatant of the bacteria killed about 80% of the tested nematodes within 24 h, suggesting the involvement of extracellular proteases. A homogeneous extracellular protease was purified by chromatography, and the hypothesis of proteinaceous pathogeny in the infection of B16 strain was confirmed by the experiments of killing living nematodes and by the degradation of purified nematode cuticle when treated with the homogenous protease. The gene for the virulence protease was cloned, and the nucleotide sequence was determined. The deduced amino acid sequence showed significant similarity with subtilisin BPN' but low homology with the other cuticle-degrading proteases previously reported in fungi. Characterization of the purified protease revealed the molecular mass of 28 kDa and the optimum activity at pH 10, 50°C. The purified protease can hydrolyze several native proteinaceous substrates, including collagen and nematode cuticle. To our knowledge, this is the first report of a serine protease from a *Bacillus* genus of bacteria that serves as a pathogenic factor against nematodes, an important step in understanding the relationship between bacterial pathogen and host and in improving the nematocidal activity in biological control.

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Introduction

Plant-parasitic nematodes cause serious losses to a variety of agricultural crops worldwide. Since the traditional methods based on the use of nematocides and antihelminthic drugs are associated with major environmental and health concerns, the development of biocontrol agents to control nematodes is of major importance (Duncon 1991). At present, several successful biocontrol agents against nematodes have been put into use, which mainly come from nematophagous fungi (Åhman 2000; Tikhonov et al. 2002). While it has also been reported that the bacterial pathogens could infect a variety of evolutionary distinct hosts including plant-parasitic nematodes, nematophagous bacteria have, because of their rapid culturing and production compared with fungi, been used extensively as bioinsecticides against nematodes in soil, and levels of control equivalent to those of chemical pesticides have been established (Zhou et al. 2002). *Pasteuria* spp. could parasitize most of plant-parasitic nematodes, but their potential applications in the field are hampered because the bacteria have not been successfully cultured axenically (Atibalentja et al. 2000). Some parasporal crystal toxins from *Bacillus thuringiensis* have been manifested to infect larvae and eggs of plant-parasitic nematodes (Wei et al. 2003). However, very little new information about nematophagous bacteria has been added since bacterial biocontrol agents from *Pasteuria*, *Pseudomonas*, and *B. thuringiensis* were reported.

In the molecular mechanisms of nematophagous fungi infecting their hosts, it was suggested that hydrolytic enzymes participate in several steps of host infection (Huang et al. 2004). Moreover, ultrastructural and histochemical studies have revealed that penetration of the nematode cuticle involves the activity of hydrolytic enzymes (Jansson and Nordbring-Hertz 1988). Some extracellular proteases have been detected and partly characterized from a few nematode-trapping fungi, as well as from endoparasites of cyst nematodes (Tunlid et al. 1994; Leger et al. 1999). As for the nematophagous bacteria, no extracellular proteases

have been identified to serve as the pathogenic factor in the infection against the nematodes till now.

In this study, we described the isolation of a *Bacillus* sp. with remarkable ability to kill nematode *Panagrellus redivivus* from soil. The bioassay experiments of the bacteria showed that the extracellular cuticle-degrading proteases were involved in the processes to penetrate the cuticle and eventually digest them. The focal points in this study were the purification and characterization of an extracellular serine alkaline protease from the bacterium and the cloning of the corresponding gene. Our results suggested that this serine protease belonging to subtilisin BPN' could play an important role in the infection of the nematode *P. redivivus*.

Materials and methods

Isolation, screening, and identification of *Bacillus* sp.

Bacteria were isolated from soil samples by the dilution plating technique on Luria Bertani agar medium. The bacteria with significant nematotoxic activities were screened according to the modified method described in Åhman et al. (2002). In brief, the tested bacteria were inoculated onto pieces of axenic cellophane papers and incubated on agar plates containing a low-nutrient mineral salt medium at 37°C for 7 days. Then the infection experiments were started by adding a block of oat medium containing free-living nematodes to the middle of the plate. The numbers of mobile and immobile (i.e., with arrested movements) nematodes were determined every 12 h under a dissecting microscope. *Escherichia coli* and the plates without bacteria were used as controls.

The strain named B16 with significant nematotoxic activity from screening was identified as a *Bacillus* sp. in the China Center for Type Culture Collection (CCTCC) using a combination of phenotypic and biochemistric properties and phylogenetic analysis based on 16S rDNA sequences amplified by polymerase chain reaction (PCR) using two universal primers (Yoon et al. 2003). At the same time, this train was collected as No. CGMCC1128 by the China General Microbiological Culture Collection Center.

Culturing of *Bacillus* sp. and *P. redivivus*

Yeast Extract/Peptone/Dextrose (YPD) medium composed of 1% yeast extract, 2% peptone, and 2% glucose was adjusted to pH 7.0 and sterilized by autoclaving at 120°C for 20 min. Two typical colonies of strain B16 were inoculated into a 250-ml YPD medium in 500-ml flasks and cultured at 37°C for 3 days under shaking (200 rpm).

The nematode *P. redivivus* was axenically cultured on an oatmeal medium (oatmeal 20 g; water 80 ml) at 25°C for 4–7 days and separated from the culture medium using the Baerman funnel technique (Gray 1984), and an aqueous suspension of nematode was prepared for use as a working stock.

Protease assays

Protease activity was measured by adding a 125-μl aliquot of samples (or diluted as needed) to the protein substrate of 2% casein solution (125 μl) in a 1.5-ml eppendorf tube. Incubations were performed at 37°C for 10 min. The reaction was stopped by adding 250 μl 10% (w/v) trichloroacetic acid, followed by the incubation and centrifuge at 12,000 rpm for 5 min. The supernatant was transferred to tubes containing 2.5 ml sodium carbonate (0.55 M), and then 0.5 ml Folin phenol reagent was added and incubated at 37°C for 20 min. Protease activity was measured by the absorbance value of the mixture at 680 nm. A calibration curve was obtained using L-tyrosine as a standard to survey quantity of L-tyrosine in the reactive solution. Proteolytic unit was defined as increased quantity of L-tyrosine in one unit at 37°C.

Protein content was measured with a BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

Purification of the extracellular protease

The 500-ml culture filtrates were centrifuged at 8,000 rpm for 10 min at 4°C, and the supernatant collected was subjected to ammonium sulfate saturation (40–70%) by slow continuous stirring at 4°C. The solution was left overnight at 4°C, followed by centrifuging at 8,500 rpm for 30 min at 4°C. Then the precipitate was dissolved in a minimum amount of 50 mM sodium phosphate buffer (PBS) (pH 7.5). Following dialyses, this resultant sample was designated as the crude protease extract and was tested for nematocidal activity with the bioassay method described below.

The crude protease extract was supplemented with 1 M ammonium sulfate, adjusted to pH 7.5 and applied to a HiPrep 16/10 column (Pharmacia Biotechnology, Amersham, Switzerland). Buffers used were (1) 50 mM PBS (pH 7.5) and 1 M $(NH_4)_2SO_4$ and (2) 50 mM PBS (pH 7.5). The gradient was 0–100% B for 25 min with a flow rate of 2 ml/min. Elution of proteins followed at 280 nm. Each fraction was collected, dialyzed, and assayed for protease and nematocidal activity.

Fractions containing protease and nematocidal activity from hydrophobic interaction chromatography were pooled, desalted by dialyzing against sterilized water at 4°C, adjusted to pH 6.0, and applied to a Source 15Q PE4.6/100 column (Pharmacia Biotechnology). Buffers used were (1) 10 mM PBS (pH 6.0) and (2) 10 mM PBS (pH 6.0) and 1 M NaCl. The gradient was 0–30% B for 2 min, 30–60% B for 1 min, and 60–100% B for 1 min, with a flow rate of 2 ml/min. Elution of proteins followed at 280 nm. Fractions collected were dialyzed and assayed for protease and nematocidal activity.

The purified enzyme was checked on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Laemmli (1970) using 12% run-

ning gels, and the gels were stained with Coomassie brilliant blue R-250. The molecular mass standard includes rabbit phosphorylase *b* (97 kDa), BSA (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), and hen egg-white lysozyme (14.4 kDa).

N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified protease sample was determined on an ABI Procise 491 (ABI, Perkin Elmer, MA, USA) protein sequencer. The protein solution was loaded onto a Prosorb (ABI) cartridge and washed thrice with 200 μl of 0.1% triflouroacetic acid. Then the polyvinylidene fluoride was cut and placed in the reaction cartridge of the ABI Procise 491. The N-terminal sequence of the first ten amino acids was blasted in the National Center for Biotechnology Information (NCBI) GenBank.

Cloning of the gene encoding the purified enzyme

Genomic DNA of *Bacillus* strain B16 extraction was conducted with the Promega wizard genomic DNA purification kit (Promega, Madison, WI, USA) for Gram-positive bacteria and used as a template for PCR with the following oligonucleotide primers, which were designed based on the gene sequence of subtilisin BPN': 5′-GGAGAGG ATAAAGAGTGAGAGGCAAAA-3′ and 5′-CTGAGCT GCCGCCTGTACGT-3′. Cycling conditions were 4 min at 95°C for the first denaturation; 35 cycles of 40 s at 95°C, 40 s at 55°C, and 1.5 min at 72°C for amplification; then 10 min at 72°C for extension.

The PCR product of 1163 bp was inserted into pGEM-T vector according to the manual (Promega) and sequenced. The deduced amino acid sequence was aligned with other cuticle-degrading proteases that have been reported using the program of multiple alignment in DNAMAN software.

Bioassays

P. redivivus nematodes were washed thoroughly with a 10-mM PBS (pH 7.0, sterile), and suspension containing 40–50 nematodes (20 μl) was transferred to a sterile 1.5-ml eppendorf tube. The tested protease sample was added to the nematodes. Protease was boiled for 10 min or treated with 1.0 mM phenylmethylsulfonyl fluoride (PMSF) before being added to the nematode cuticle as control. After incubating the mixture at 25°C for 24–48 h, the numbers of living and dead nematodes were counted under a dissecting microscope.

Fragments of cuticle of the nematode *P. redivivus* was purified according to the method described by Cox et al. (1981). Two hundred microliters of purified enzyme was mixed with nematode cuticle, the result was observed under a dissecting microscope every 12 h. As controls, 0.1 M BSA and protease K added to the nematode cuticle were used.

Characterization of the purified enzyme

The optimum pH of the purified protease was determined by incubating the enzyme with substrates of casein using the Britton–Robinson universal buffer system at pH value between 3 and 12; pH stability of the purified enzyme was investigated by adding it to buffers with different pH, incubating at 4°C for 2 h, and adjusting pH to 7, followed by the protease assay of the proteolytic activity as described above.

To determine the optimum temperature for proteolytic reaction, the activity of the purified enzyme was measured by incubating the reaction mixture at different temperatures, ranging from 4 to 90°C (4, 20, 30, 40, 50, 60, 70, 80, 90); to determine the enzyme stability at different temperatures, the activities of purified enzyme were assayed in the samples after they were incubated at temperatures ranging from 4 to 90°C for 30 min.

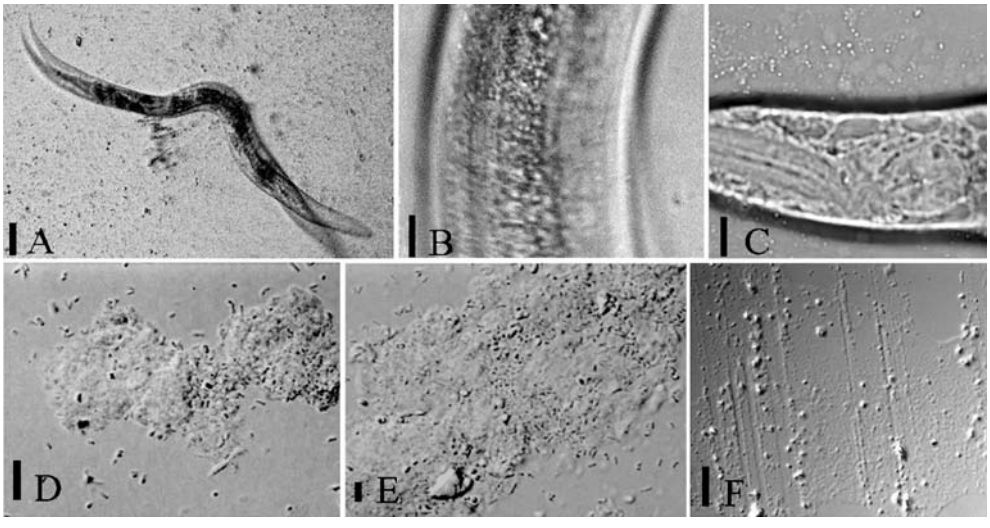

Fig. 1 The action of bacterium strain B16 against nematode *P. redivivus* observed under a dissecting microscope. **a** The nematode was alive on the plates incubated with *E. coli* after 3 days. **b** and **c** The cuticle of the nematode was intact on the control plate without bacterium and on the control plate with *E. coli* during the same time. **d** The bacteria were attracted to nematodes and could grow well on them. **e** The bacteria reproduced in large quantities on the nematodes. **f** The cuticle of nematodes was destroyed by the bacteria

The effect of metal ions was assayed by adding different metals (0.1 M Ca^{2+}, 0.1 M Mg^{2+}, 1.0 M Cu^{2+}, 0.1 M Zn^{3+}, 0.1 M Al^{3+}, 1.0 M Hg^{2+}) into the reaction mixture. Protease activity was measured.

The effects of various inhibitors on the protease activity were examined by incubating them with the purified enzyme for 5 min at room temperature. The proteolytic activity was then measured in the protease assay above.

Proteolytic activity vs protein substrates was carried out by using the methods previously described by Tunlid et al. (1994).

The GenBank accession number for our obtained serine protease is AY708655.

Results

Identification and characterization of strain B16

The strain B16 showing significant nematocidal activity was sent to CCTCC and proposed as a new species of *Bacillus*. The sequence for 16S rRNA presented 99% similarity with the 16S rDNA sequences of *Bacillus subtilis*, *Bacillus vallismortis*, *Bacillus* sp. *Bch1*, and *Bacillus mojavensis*. The phenotypic and genotypic characteristics and DNA–DNA relatedness data indicated that strain B16 should be distinguished from all the relative species of genus *Bacillus*. Therefore, on the basis of the polyphasic taxonomic data presented, *Bacillus nematocida*, a new species of the genus *Bacillus*, is proposed (Huang et al. 2005).

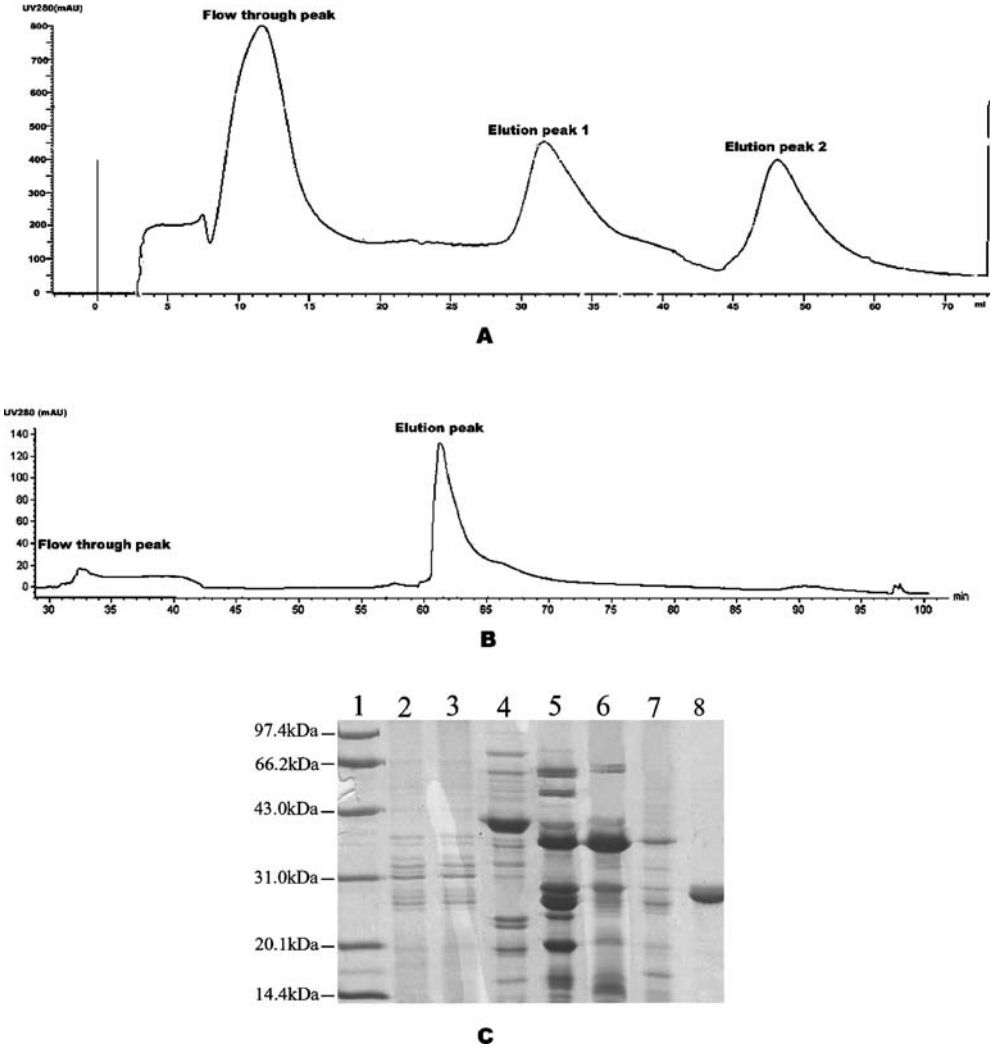

Fig. 2 **a** Result of HIC. Only fractions of elution peak 1 have protease and bioassay activity. **b** Result of cation-exchange chromatography. The purified enzyme with protease and bioassay activity was obtained by collecting elution peak fraction. **c** SDS-PAGE. Based on the electrophoretic mobility, the molecular mass of this purified protease was approximately 28 kDa. *Lane1*, the molecular mass standard; *Lanes 2* and *3*, fractions of flow through peak in HIC; *Lane 4*, fractions of elution peak 2 in HIC; *Lane 5*, fractions of elution peak 1 in HIC; *Lane 6*, fractions of elution peak 1 after salting out; *Lane 7*, fractions of flow through peak in cation-exchange chromatography; *Lane 8*, fractions of elution peak in cation-exchange chromatography

Table 1 Summary of the purification steps of the protease

Step	Total protein (mg)	Total enzyme activity (PU)	Specific activity (PU mg^{-1})	Purification fold
Culture filtrate	60.30	300.10	5.10	0.00
Crude extract	17.40	227.90	13.11	2.60
HIC	8.20	164.00	20.00	3.90
Purified enzyme	5.06	155.80	30.80	6.00

For definitions, see Materials and methods. Protease activity was assayed using casein as substrate. The purification scheme and calculation were repeated three times with similar results
PU Proteolytic units

Nematocidal activity of strain B16

The infection experiments showed that about 85% of the tested nematodes were killed within 84 h in the plates containing strain B16. However, on the control plates with non-pathogenetic *E. coli* and without any bacterium, more than 90% of the nematodes were still mobile after 100 h. Observation under a dissecting microscope recorded that the tested B16 strain first attached to the epidermis of their host, penetrated and degraded the cuticle, then propagated and formed spores on the nematodes until, eventually, only white traces were visible under the microscope (Fig. 1). In the experiments of infection against *P. redivivus*, the bacteria strain B16 demonstrated apparent infectious activity and the observed degradation of nematode cuticle.

Purification of proteases in culture filtrates

The activities of extracellular proteases were assayed during the growth of *Bacillus* sp. B16, and maximum enzyme production was observed after 70 h in the late logarithmic growth phase. After the strain was cultured for 80 h, production of protease began to decrease. Therefore, it was determined that the bacterium culture of 70 h would be used to purify the extracellular protease with virulence to nematodes.

The bacterial extracellular cuticle-degrading protease was purified to homogeneity by using 40–70% salting out of ammonium sulfate, hydrophobic interaction chromatography (Fig. 2a), and cation-exchange chromatography (Fig. 2b), and then one elution peak with both nematocidal and protease activity was pooled. The purity of the resulting elution was confirmed by single band after Coomassie brilliant blue staining on 12% SDS-PAGE (Fig. 2c). The molecular weight of the enzyme was found to be 28 kDa. After each purification step, proteolytic activities were assayed, and the concentrate fold was shown in Table 1.

Bioassay

Bioassays of the fractions with positive proteolytic activity were also performed after each step of purification. Mortalities of nematodes in eppendorf tubes with crude protease extracts were 60, 80, and 95% within 12, 24, and 48 h, respectively, whereas in the negative controls with sterilized water and PBS (pH 7.0), it was shown that mortalities were below 20%. After hydrophobic interaction chromatography (HIC), only a fraction of elution peak 1 had the ability of nematocide, and within 24 h, 90% of the tested nematodes were killed. However, after being boiled or treated with 1.0 mM PMSF, the elution lost the ability to kill nematodes. Following cation-exchange chromatography, the resultant purified protease was obtained, and bioassay showed 90% of the nematodes could be killed within 24 h at the concentration of 1.79 μg/ml; after 48 h, all of the tested nematodes were almost killed and degraded (Table 2). However, in the controls of BSA and protease K, less than 10% of the nematodes were killed. Under the microscope, we noticed that the cuticles of nematodes had been degraded and destroyed by the proteolytic activities. However, in the negative controls, most of the nematodes were mobile and the cuticles were complete when treated for the same time (Fig. 3). Furthermore, we purified the cuticle from nematode *P. redivivus* and treated it again with the purified protease to verify the hydrolysis of nematode cuticle in vitro. When treated with the protease, many incomplete and degraded fragments were observed, whereas the cuticles were intact and the striae were clear in the negative controls (Fig. 4).

Table 2 Killing of the nematode *P. redivivus* by protease extracts from *Bacillus* sp.

Extract	Protein (μg/ml)	PU ($\times 10^{-3}$)	Mortalities of nematodes (%) (SD)			
			12 h	24 h	36 h	48 h
Filtrate	2.13	5.10	40(2.3)	50(1.8)	70(3.2)	80(2.8)
Crude	8.33	13.11	60(4.5)	80(2.4)	90(2.6)	95(2.6)
Elution(HIC)	2.06	20.00	60(3.7)	90(2.8)	95(2.5)	100(0)
Purified enzyme	1.79	30.80	70(2.2)	90(2.3)	95(3.3)	100(0)
Enzyme(boiled)	1.79	0.72	5(0.2)	5(0.4)	10(1.7)	10(1.9)
Enzyme (PMSF)	1.79	0.00	5(0.2)	5(0.2)	5(0.8)	5(0.4)

Mortality refers to the number of dead nematodes/number of ones tested. For definitions, see Materials and methods. The bioassays were performed with 10 to 15 parallels and repeated at least twice
PU Proteolytic units, *SD* standard deviation

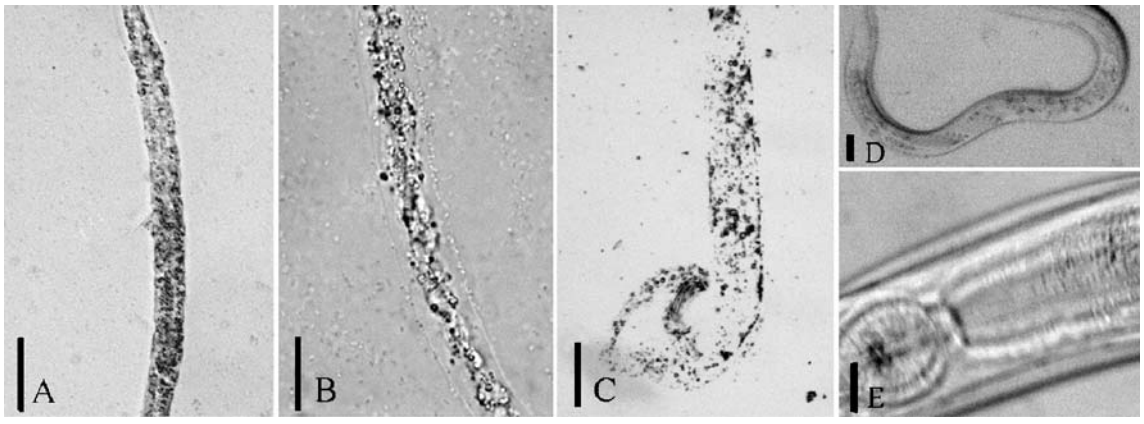

Fig. 3 The action of crude extracellular protease extract against nematode *P. redivivus*. **a** The nematode was dead and the cuticle was not complete when treated for 24 h. **b** The cuticle was degraded when treated for 36 h. **c** Only traces of dead body were visible when treated for 48 h. **d** The nematode was still alive in the control after 48 h. **e** The cuticle was intact and smooth in control after 48 h

Gene clone of the purified protease

The N-terminal amino acid sequence of the purified protease was AQSVPYGVSQ, which had 100% similarity to subtilisin BPN' through BLAST in NCBI. The gene encoding purified enzyme was cloned using PCR with oligonucleotide based on the conserved sequences of subtilisin BPN'. The nucleotide sequence of an amplified fragment was determined and sequenced. The gene encoding the enzyme was submitted in GenBank (AY708655; AAV30845). The deduced protein consisted of a presequence signal peptide of 30 amino acids and a propeptide of 77 amino acids, and the mature protease had 275 residues with a catalytic triad center containing His, Asp, and Ser residues. It was also found that the N-terminal amino acids we sequenced were located at positions 108 to 118 of amino acids of subtilisin BPN', which was coincidentally the beginning of the mature enzyme. The whole amino acid residues showed 62–98% sequence identity between other subtilisins produced by several bacteria, but it showed comparatively low similarity with the cuticle-degrading proteases produced by several fungi such as *Beauveria bassiana*, *Cordyceps brongniartii*, *Metarhizium anisopliae*, etc. The alignment of the available sequences is shown in Fig. 5. It was found that only several residues near the conserved catalytic triad that are probably essential for activity of cuticle degradation are in consensus in all the proteases. The two groups of proteases from bacteria and fungi are only 33% homologous, which reflects their different sources.

Characterization of the purified enzyme

The results of pH studies indicated a broad pH activity range of 6.0–10.0 and optimum pH at 10.0. Less than 20% activity was retained at pH 11.0 after 30 min of incubation. The enzyme was stable at alkaline pH even after incubation for 1 h at pH between 6.0 and 10.0.

Temperature optimization studies at pH 10.0 showed that at 50°C, the purified protease had the maximum hydrolytic activity. As for the stability of temperature, the purified protease still possessed about 50% activity at 50°C after 20 min of incubation, instead of half times of 3–4 min at 50°C for subtilisin BPN' previously reported (Jacobs et al. 1985).

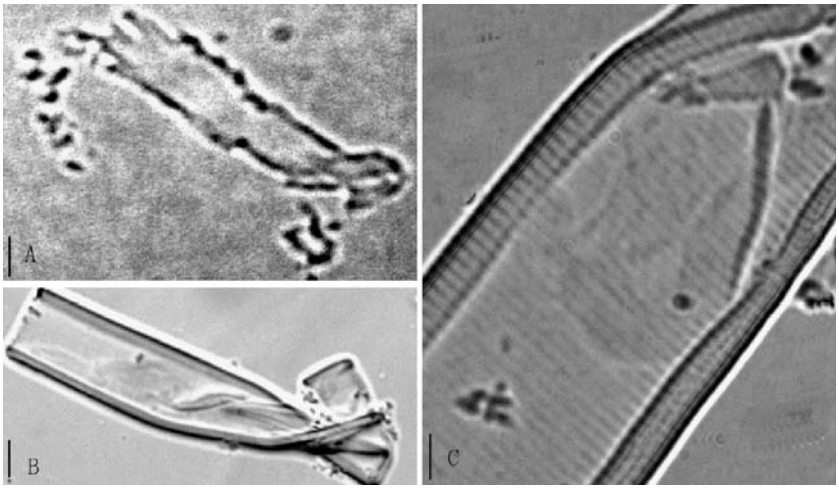

Fig. 4 The action of purified protease against cuticle observed under a dissecting microscope. **a** The cuticle was degraded when treated with purified enzyme for 5 days. **b** The cuticle was intact when treated with water for the same time. **c** The cuticle was complete when treated with BSA within the same time. *Scale bar*=20 μm (in Fig. 1, Fig. 4, Fig. 5)

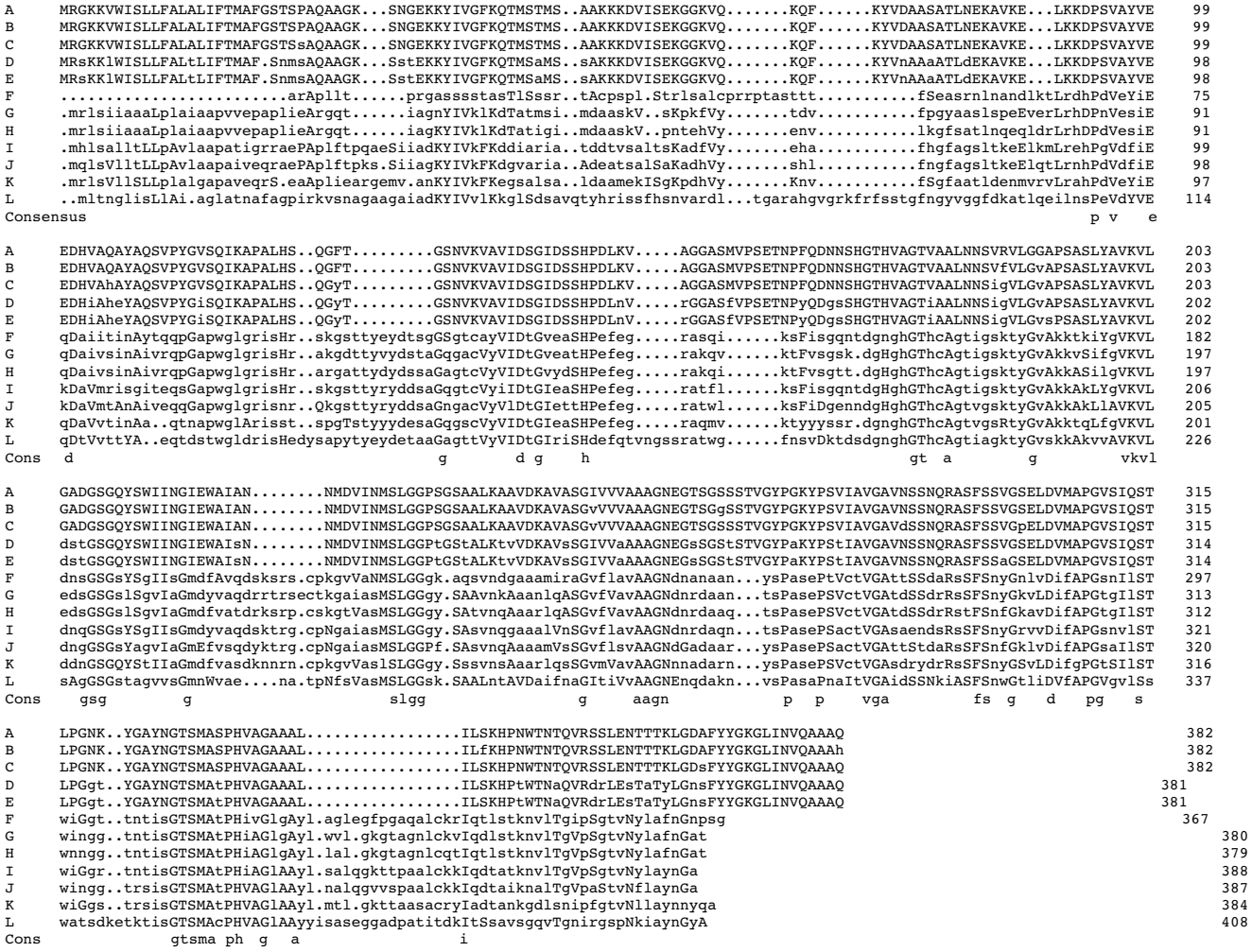

Fig. 5 Multiple sequence alignment of amino acid sequences of 12 proteases. *A*, amino acid sequence of protease by B16; *B*, subtilisin from *B. subtilis*; *C*, subtilisin BPN'; *D*, subtilisin NAT precursor; *E*, subtilisin E precursor; *F*, pSP-3 from *Paecilomyces lilacinus*; *G*, cuticle-degrading protease from *C. brongniartii*; *H*, cuticle-degrading protease from *B. bassiana*; *I*, PR1I from *M. anisopliae*; *J*, VCP-I; *K*, alkaline serine protease from *Tritirachium album*; *L*, amino acid sequence of PII. The GenBank accession numbers of proteases A-L are AAV30845, AAC63365, SUBSN, P35835, SUBSI, AAA91584, AAR97273, AAL55578, CAC95049, CAD20583, CAA32820 and CAA63841, respectively

Among the tested metal ions, 0.1 M Ca^{2+} showed strong enhancement of the enzyme activity; however, Zn^{2+}, Al^{3+}, Hg^{2+}, Cu^{2+}, and Mg^{2+} inhibited the protease activity moderately.

To determine the nature of the purified enzyme, activity was measured in the presence of different protease inhibitors (Table 3). No activity was detected when serine protease inhibitor PMSF (1.0 mM) was added, which

Table 3 Effect of various protease inhibitors on the purified enzyme

Inhibitors	Concentration	Enzyme activity as % of control (SD)
PMSF	1.0 mM	0.00 (0.00)
EDTA	1.0 mM	88.72 (13.08)
Aprotinins	0.02 ml(1 μg/ml)	84.07 (8.55)
Leupeptin	0.1 mM	109.74 (9.00)
DTT	5.0 mM	98.24 (10.86)
Pepstatin A	0.01 mM	90.52 (8.63)

EDTA Ethylenediaminetetraacetic acid, *DTT* dithiothreitol, *SD* standard deviation

Table 4 Hydrolysis of various protein substrates by the purified protease

Substrate	Relative activity (%)
Casein (denatured)[a]	100
Skimmed milk	94.99
Gelatin	69.32
Denatured collagen[a]	50.24
Bovine serum albumin	23.6
Collagen	3.57

[a]Denatured by heating at 100°C for 20 min

suggested the purified enzyme has a serine type protease activity.

In addition to the utilization of substrates, the purified protease showed relatively high hydrolytic activity against casein, skimmed milk, gelatin, denatured collagen, and BSA. However, native collagen was hydrolyzed by the protease at considerably slower rate (Table 4).

Discussion

At present, although some successful natural enemies have been used in the biocontrol of nematodes, disadvantages such as cultural technologies and limited hosts still existed. For example, fungi are not produced easily and are inhibited by soil; most bacteria are only effective to larvae and eggs of plant-parasitic nematodes; and *Pasteuria nishizwae* cannot be successfully cultured axenically. All of these encouraged the researchers to explore new or alternative strains for biological control. Here we reported the isolation of a new strain of *Bacillus* sp. representing remarkable nematocidal activity, with a serine protease serving as the important pathogenic factor, which opens the new possibilities for studies on bacterial biocontrol agents to control plant-parasitic nematodes.

It has been reported that the bacterial-feeder *P. redivivus* was attracted to four bacterial species tested (Zuckerman 1984). In our study, we found that the *Bacillus* strain B16 has attraction to nematode *P. redivivus* and that it killed the most nematodes within 84 h and digested them at last. The fact that the crude extracellular protein extract from the bacteria exhibited strong nematocide suggests that the extracellular proteases, as a potential pathogenic factor, may be involved in the infectious process. Therefore, we purified the protease that is toxic to nematodes. The purified protease extract killed about 90% of the tested nematodes within 24 h and finally destroyed the targets within 48 h. In vitro, the purified cuticle of nematodes could be effectively hydrolyzed by that homogeneous protease. Consequently, it was manifested that the obtained extracellular protease should be an important cause for the death of the nematodes and serve as the pathogenic factor in the infection. This study confirmed the potential use of *Bacillus* sp. and its virulence protease in the biological control of plant-parasitic nematodes.

It was well known that the cuticle of nematodes is rigid and composed of proteins and chitin, especially the outer part that is covered by a layer of proteinaceous membrane, which is an effective barrier protecting nematodes from damage from the environment (Tunlid et al. 1994). Therefore, protease, collagenase, and chitinase have been emphasized in biocontrol of nematodes. Hydrolytic enzymes probably play a role in the process of infection against nematodes by their natural enemies: releasing nutrients, mainly amino acids or small peptides, for pathogen growth and propagation; facilitating penetration by solubilizing the cuticle; and inducing cytotoxic effects on nematodes (Leger et al. 1987). The roles of extracellular protease, especially serine protease including P32, PII, pSP-3, and VCPI, have been studied extensively in nematophagous fungi in the infection of nematodes. Extracellular protease has also been described to play important roles in nematocidal action in previous reports (Singh et al. 2001; Balaji et al. 2002; Beg and Gupta 2003).

When the virulence protease was characterized in our study, the sensitivity of the purified enzyme to the inhibitor PMSF indicated that it was a serine protease. The gene encoding this protease was then cloned, and the deduced amino acids had 98% identity with subtilisin BPN' from *B. amyloliquefaciens*. Based on the alignment of the amino acid sequence in NCBI, the enzyme probably belongs to the subtilisin family of enzymes, subtilisin BPN' (EC 3.4.21.14, also known as Novo, or Nagarse). The presequence signal peptide directs the secretion of subtilisin from the interior of cells. The propeptide acts as a chaperon to facilitate the folding process of the active protease; the folding process is intramolecularly and intermolecularly accessible, and the mature subtilisin, after autoprocessing of the precursor, has 275 residues, with a catalytic triad center and two calcium binding sites for stabilizing the three-dimensional structure (Day et al. 2003). Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protein Nagarse, have been identified. The enzymatic reaction mechanism of subtilisin BPN' has been studied well (Huang et al. 1997). The structural and functional roles of its unique propeptide in relation to the enzyme activity during the maturation process have also been understood in detail. Subtilisin is used worldwide in commercial detergents by several tons annually (Day et al. 2003). In this study, we have found the new function of bacterial serine protease subtilisin BPN': Bioassay of the purified enzyme showed its capability to degrade the nematode cuticle. The significance of our discovery is that it makes feasible the protection of plants by direct application of the protease or by transformation of the toxin gene or genes into plants as a strategy for the control of nematodes.

It is necessary to note that the molecular mechanism of action against nematodes is a complex process that is not clearly known. Nematocidal activity probably attributes to many factors, including extracellular proteases, toxic peptide, or midterm metabolic products. It is necessary to further detect these virulence factors to better understand the mechanism.

Acknowledgements We express our gratitude to Dilantha Fernando and Zhang L.M. for their invaluable discussions and assistance in preparing the manuscript. We are also grateful to Zhou W. for her invaluable help in facilitating the work and to Wang M., Sun H., Xu J., Wang R.B., Lin C., and Li J. for their helpful advices in the study. We thank Li W.J. and Zhang Y.Q. for helping identify the bacterium strain B16.

This work was supported by the projects from National Natural Science Foundation Program of P. R. China (30470067), Ministry of Science and Technology of P. R. China (2002BA901A21), and Department of Science and Technology of Yunnan Province, P. R. China (No. 2004C0004Q and 2004C0001Z).

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