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ORIGINAL PAPER

A neutral protease from *Bacillus nematocida*, another potential virulence factor in the infection against nematodes

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Abstract A neutral protease (npr) (designated Bae16) toxic to nematodes was purified to homogeneity from the strain *Bacillus nematocida*. The purified protease showed a molecular mass of approximately 40 kDa and displayed optimal activity at 55°C, pH 6.5. Bioassay experiments demonstrated that this purified protease could destroy the nematode cuticle and its hydrolytic substrates included gelatin and collagen. The gene encoding Bae16 was cloned, and the deduced amino acid sequence showed 94% sequence identity with npr gene from *B. amyloliquefaciens*, but had low similarity (13–43%) with the previously reported virulence serine proteases from fungi or bacteria, which reflected their differences. Recombinant mature Bae16 (rm-Bae16) was expressed in *Escherichia coli* BL21 using *p*ET30 vector system, and its nematicidal activity confirmed that Bae16 could be involved in the infection process. Our present study revealed that the npr besides the known alkaline serine protease could serve as a potential virulence factor in the infection against nematodes, furthermore, the two proteases with different characteristics produced by the same strain coordinated efforts to kill nematodes. These data helped to understand the interaction between this bacterial pathogen and its host.

Keywords Neutral protease · Nematicidal activity · Virulence factor · *Bacillus nematocida*

Introduction

Biological control of nematodes has been studied as an alternative or complementary approach to physical or chemical nematode control methods (Tunlid et al. 1994). It has been known that diverse bacterial pathogens could infect a variety of evolutionary distinct hosts including plant-parasite nematodes and so nematophagous bacteria have been used extensively as bionematicides due to their rapid culturability and productivity compared with fungi. An obligate parasitic actinomycete, *Pasteuria penetrans*, for example, has been extensively tested for the control of plant-parasite nematodes. Additionally, some other studies have also reported the presence in the rhizosphere of bacteria that exert beneficial effects on plant development by preventing the infection of root-systems by soil pathogens or nematodes (Schroth and Hancock 1981). These bacteria decrease nematode infection either by acting as competitors for space or habitat in or on the root, or by direct killing of nematodes through their ability to produce nematotoxic metabolites. Antagonistic bacteria like *Pseudomonas aeruginosa* (Siddiqui et al. 2000) and *Pseudomonas* spp. (Ali et al. 2002) have shown promising results for the control of *Meloidogyne* spp. *B. firmus* was isolated in Israel from a cultivated soil and formed the basis of the commercial bio-nematicide under investigation (Cox et al. 1981). Therefore, bacteria have shown promising results for the control of plant-parasite nematodes in laboratory and greenhouse experiments.

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Extracellular enzymes, including subtilisin-like serine protease, chitinase and collagenase, corresponding to the main chemical constituents of nematode cuticle and eggshell, have been reported to be involved in the infection as virulence factors (Huang et al. 2004). In the interaction between pathogen and hosts, much experimental evidence supported that serine protease can destroy the integrity of cuticle to help penetration of pathogen (Decraemer et al. 2003; Huang et al.[1] 2005; Niu et al. 2006) and initiate or trap nematophagous fungi (Åhman et al. 2002; Huang et al. 2004). However, serine protease seems not to be the sole virulence factor in the infection because the mortality of nematodes due to the purified serine protease was lower than that due to unpurified virulence mixture though the two mentioned above had similar protease activities (Huang et al.[1] 2005). Furthermore, some proof also illustrated that the nematicidal activity should be a complex process and attribute to other pathogenic factors such as toxic peptides, midterm metabolic products and even some other type of extracellular protease (Åhman et al. 2002; Niu et al. 2006). Thus there is still a need for developing and understanding novel virulence factors.

In our previous work, *B. nematocida* with significant nematotoxic activity was isolated from soil sample in Yunnan Province (Huang et al.[2] 2005) and a virulence serine protease has been purified and cloned (Niu et al. 2006). Now, we obtained a neutral protease (npr) (Bae16) from the same bacterial strain, which hydrolyzed and destroyed the nematode cuticle. We described the purification, biochemical characterization, its nematicidal activity and the cloning of its encoding gene. Moreover, mature Bae16 was expressed in *Escherichia coli* BL21 and the recombinant protease also showed nematicidal activity. Although the nematicidal activity of Bae16 was lower than the activity of the previously reported serine protease, it showed that a synergistic action of several different proteases could be involved in infecting the nematodes. The understanding of the mode of the action is important to propose different alternatives to improve the nematicidal activity for biological control.

Materials and methods

Organisms and culture conditions

Bacillus nematocida with significant nematotoxic activities (Niu et al. 2006) was incubated on yeast extract/peptone/dextrose (YPD) medium. The culture was carried out in 500 ml shaking flask at 37°C and 200 rpm for 3 days on a rotary shaker.

The saprophytic nematode *Panagrellus redivius* was cultured on oatmeal medium (oatmeal: 20 g, water: 80 ml) at 25°C for 7 days, and then refrigerated prior to use.

For the culture of the pine nematode *Bursaphelenchus xylophilus*, the fungus *Botrytis cinerea* was firstly grown on a PDA plate at 25°C. When the fungus colonized the whole plate, *B. xylophilus* was inoculated and then cultured.

The two cultured nematodes were separated by using the Baerman funnel technique (Gray 1984), and an aqueous suspension of the nematode was prepared for use as a working stock.

Protein concentration and protease activity analysis

Protein concentration was determined by the method of Bradford (Bradford 1976) using bovin serum albumin (BSA) as a standard.

According to a modified caseinolytic method (Elmar 1984), 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; pH 7.0) in a 1.5 ml Eppendorf tube and subsequently incubated at 37°C for 10 min, then the reaction was stopped by adding 250 μl 1 M trichloroacetic acid (TCA) and the mixture was kept at 4°C for 10 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was mixed with 2.5 ml 0.55 M sodium carbonate and 0.5 ml Folin-hydroxybenzene agent followed by the incubation at 37°C for another 10 min. The protease activity was measured with a spectrophotometer at 680 nm. A calibration curve using L-tyrosine as a standard was completed to determine emission of L-tyrosine in the reaction. One unit (U) of protease activity was defined as the amount of enzyme that hydrolyzed the substrate and produced 1 μg tyrosine in 1 min under the assay conditions.

Purification of the protease

Five hundred milliliter culture filtrates were collected and centrifuged at 8,000 rpm for 10 min at 4°C. The resultant supernatant was precipitated with ammonium sulfate to 80% saturation with stirrer, the mixture was kept in ice bath for 4 h, and centrifugation was done again at 8,500 rpm for 20 min at 4°C. The precipitate was resuspended in a minimum amount of sodium phosphate buffer (pH 6.0). The solution was dialyzed against 50 mM phosphate buffer at 4°C to conduction bellow 2 ms/cm, designated as crude enzyme and used for further purification.

Filtrated by filtration membrane (0.22 μm), the solution was loaded on a HiTrap™ SP FF column (Amersham) connected to the Amersham FPLC system (ÄKTA™ explorer; pump P-900, detector Variable wavelength Monitor UV-900, fraction collector Frac-900), previously equilibrated with 10 mM phosphate buffer (pH 6.0). The column was eluted with a linear salt gradient (0–0.5 M NaCl) and fractions containing protease activity and nematicidal activity were collected.

The fractions were mixed with 2 M $(NH_4)_2SO_4$ and the pH was adjusted to 7.0. The resultant sample was loaded on a HiPrep™ 16/10 Phenyl FF column (high sub; Amersham Pharmacia Biotech) that had been equilibrated with 10 mM phosphate buffer containing 1 M $(NH_4)_2SO_4$ (pH 7.0). The protease was eluted out from the column by using a linear gradient of $(NH_4)_2SO_4$ (1–0 M, pH 7.0). Fractions of 5 ml were collected at a flow rate of 2 ml min^{-1}. All fractions with absorbance at 280 nm were assayed for protease activity using a caseinolytic method (Elmar 1984) and for nematicidal activity.

Characterization of the purified enzyme

Molecular mass determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) under reducing conditions, by using a Mini-PROTEAN III gel system (Bio-Rad) and 0.5 mm thick slab gels of 12% polyacrylamide, and the proteins were stained with Coomassie blue G-250. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular weight versus the R_f value (relative mobility) using a standard molecular weight marker LMW (Amersham Biosciences, Sweden) (97.4, 66.2, 43.0, 31.0, 20.1 and 14.4 kDa).

Effect of temperature and pH on enzyme activity

Optimal temperature assay was determined by performing a standard activity assay in a temperature ranging from 4 to 80°C. To determine the enzyme stability at different temperatures, the residual activity of the purified enzyme was assayed after incubation at different temperatures ranging from 4 to 90°C for 30 min.

To determine optimal pH, the enzyme activity assay was performed at different pH between 3 and 12 at 55°C. Buffers used were as follows: 0.1 M sodium acetate (pH 3.0–5.5); 0.1 M sodium phosphate (pH 6.0–7.5); 0.1 M Tris–HCl (pH 8.0–9.0); 0.1 M glycine–NaOH (pH 9.5–11.0); and 0.1 M sodium carbonate (pH 11.5–12.0). The pH stability was investigated by adding the purified enzyme to buffers with different pH and incubating at 4°C for 2 h, followed by adjusting pH to 7.0 and then the residual activity was measured.

Hydrolysis of protein substrates and nematode cuticle assay

The purified protease was incubated with different substrates (casein, skimmed milk, BSA, gelatin, collagen and nematode cuticle) at 55°C and pH 6.5 for 10 min, and protease activity was quantitatively assayed as described above. Nematode cuticle was extracted according to the method of Cox et al. 1981. All extraction steps except those involving SDS were performed on ice. Adult nematodes ($2–3 \times 10^4$/tube) were washed free of bacteria with M9 buffer, suspended in 5 ml of sonication buffer [10 mM Tris–HCI, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF)], and given ten 20-s bursts of a Biosonik III sonifier. Cuticle was collected by centrifugation for 4 min at 300 rpm and washed several times with 10 ml of sonication buffer. Cuticles were then transferred to a 1.5-ml microfuge tube, suspended in 1 ml of ST buffer (1% SDS, 0.125 M Tris–HCI, pH 6.8) and heated for 2 min at 100°C. After several hours of incubation at room temperature, cuticles were spun down in a Brinkmann microcentrifuge (60 s; Brinkmann Instruments, Inc., Westbury, NY, USA) and extracted again with ST buffer, as described above. The disulfide cross-linked proteins of the cuticle were solubilized by heating purified cuticles for 2 min at 100°C in 0.5 ml of ST buffer, 5% ß-mercaptoethanol (BME) and agitating gently for several hours at room temperature. The insoluble cuticle material was extracted again with ST buffer, 5% BME, as described above, washed several times with distilled water, and lyophilized. All protein samples were stored frozen at –20°C. When used, the cuticles were dissolved in axenic water. The concentration used was approximately 2×10^4 cuticles/ml and the cuticles were quantified by using the protease activity assay.

Effect of metal ions on purified enzyme activity

Purified protease was pre-incubated with different metal ions including Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+} for 30 min and then the protease activity was measured.

Effect of protease inhibitors on purified enzyme activity

The effects of various inhibitors on the protease activity were examined by incubating them with the purified enzyme for 10 min at room temperature. Residual proteolytic activity was then measured as a percentage of that in control sample without inhibitors.

N-terminal amino acid sequence analysis

The purified protease was subjected to SDS-PAGE and electroblotted onto polyvinylidene fluoride membrane (Gelman Co.). Cylohexylamino-propane-sulfonic buffer, pH11, containing 20% methanol was used as electrophoretic buffer in a Mini-PROTEAN III gel system (Bio-Rad) (Cheng 1998). Protein bands identified by Coomassie blue were excised and subjected to Edman degradation using a Procise 491 Protein Sequencer (PE/ABI, USA). The N-terminal sequence of the first ten amino acids was blasted in NCBI Genbank.

Cloning of Bae16

Genomic DNA of *B. nematocida* extraction was conducted with the Promega wizard genomic DNA purification kit for Gram-positive bacteria (Sangon Co.) and used as the template for PCR. Oligonucleotide primers were designed based on the npr gene from *B. amyloliquefaciens* (GI: 143248): 5′-GGGGGATTTATGTGGGTTT-3′; and 5′-TAC-AATCCG(A)ACT(A)GCATTCCA-3′. Cycling conditions were: 4 min at 95°C for the first denaturation; 35 cycles of 1 min at 95°C, 1 min at 55°C and 1.5 min at 72°C for amplification; then 10 min at 72°C for extension.

The PCR product of 1,566 bp was inserted into *p*GEM-T vector according to the manual (Promega Co, WI, USA) and sequenced. The deduced amino acid sequence was aligned with other cuticle-degrading proteases that have been reported and other nprs from bacteria using the program of multiple alignments in DNAMAN software.

Heterologous expression of Bae16

According to the modified method of Choi et al. (2004), mature Bae16 was expressed successfully in *E. coli* BL21: The gene encoding the mature Bae16 was amplified by PCR using *Nco*I-linked sense primer (5′-CCATGGCCGCTCAACCGGAACAG-3′) and *Xho*I-linked antisense primer (5′-CTCGAG-CAATCCAACTGCATTCCAGGC-3′). PCR amplification was performed under the following conditions: 30 cycles of 95°C for 40 s, 55°C for 30 s, and 72°C for 1.5 min. The PCR-amplified 900 bp DNA fragment was extracted from agarose gel and then ligated into *p*GEM-T Easy vector (Promega) to generate *p*T-mat-Bae16 plasmid. After digestion with *Nco*I and *Xho*I, the mature Bae16 fragment was inserted into the bacterial expression vector *p*ET 30a. Transformed cells were then grown at 37°C in Luria-Bertani (LB) medium, supplemented with kanamycin (50 μg/ml) to a cell destiny of A_{660}=0.4–0.6. Protein expression was induced by 1.0 mM IPTG (Sigma) and incubation was continued for 3 h at 37°C. The protein expressed in *E. coli* BL21 was obtained from inclusion bodies and dissolved in 20 mM Tris–HCL (pH 7.4) buffer containing 6 M urea, insoluble materials were removed by centrifugation and the proteins in the soluble fraction were resolved by SDS-PAGE. The recombinant proteins were then purified using purification protocol of 6× His-tagged proteins by Ni-NTA affinity chromatography and renatured according to Yokoyama et al. (2002).

Nematicidal activity assay

The effect of Bae16 and rm-Bae16 on nematodes was investigated according to the method of bioassay of Niu et al. (2006). The free-living nematode (*P. redivius*) and plant parasite nematode (*B. xylophilus*) were selected as the test nematodes. In bioassay, the nematodes were considered dead when no movement was observed under a dissecting microscope and gentle tapping of nematodes by a stitch did not result in movement. Mortality of nematodes was defined as the ratio of dead nematodes over the tested nematodes.

The experiment was performed in 1.5 ml Eppendorf tubes; every tube was added with 150 μl of test protease. The nematodes were washed thoroughly with 50 mM phosphate buffer (pH 7.0) before being transferred into the tubes and about two hundred nematodes were assayed in each treatment. After incubated at 25°C, dead nematodes were affirmed with a light microscope and counted. Nematode mortality was determined 24 h later using eight different concentrations of each protease preparation and the results were analyzed statistically to obtain a half lethal concentration (LC50) value. A light microscope was employed to examine the changes of nematode cuticle. The experiments were performed with three parallels and repeated at least five times. Controls were incubated with water, BSA, YPD medium, phosphate

buffer (pH 7.0) and the corresponding protease boiled for 15 min.

The effect of protease against nematode cuticle was performed under the condition of pH 7.0, 25°C with the method described above. The protease concentration used in this experiment was 2.50 μg/ml. Characters of the tested nematode cuticle were examined under a light microscope.

Results

Protease purification

The protease Bae16 was purified to homogeneity by using $(NH_4)_2SO_4$ precipitation, cation-exchange chromatography (Fig. 1a) and hydrophobic interaction chromatography (Fig. 1b). After cation-exchange chromatography, the fraction of elution peak was assayed for both nematocidal and protease activity. Purification factors and recoveries at each step were summarized in Table 1. About 46.5% protease recovery was achieved with 1.4-fold purification after precipitated by $(NH_4)_2SO_4$, protease activity was observed in the fraction of elution peak in cation-exchange chromatography with 7.3-fold purification. After applied to the HiPrep^{TM} 16/10 Phenyl FF column, the active fraction resulted in 3.8% activity (10.9-fold purification) with a specific activity of 55.6 U mg^{-1} of protein (Table 1).

The purity of the resulting elution was confirmed by single band after Coomassive brilliant blue staining on SDS-PAGE. The molecular weight of the enzyme was estimated to be 40 kDa (Fig. 1c).

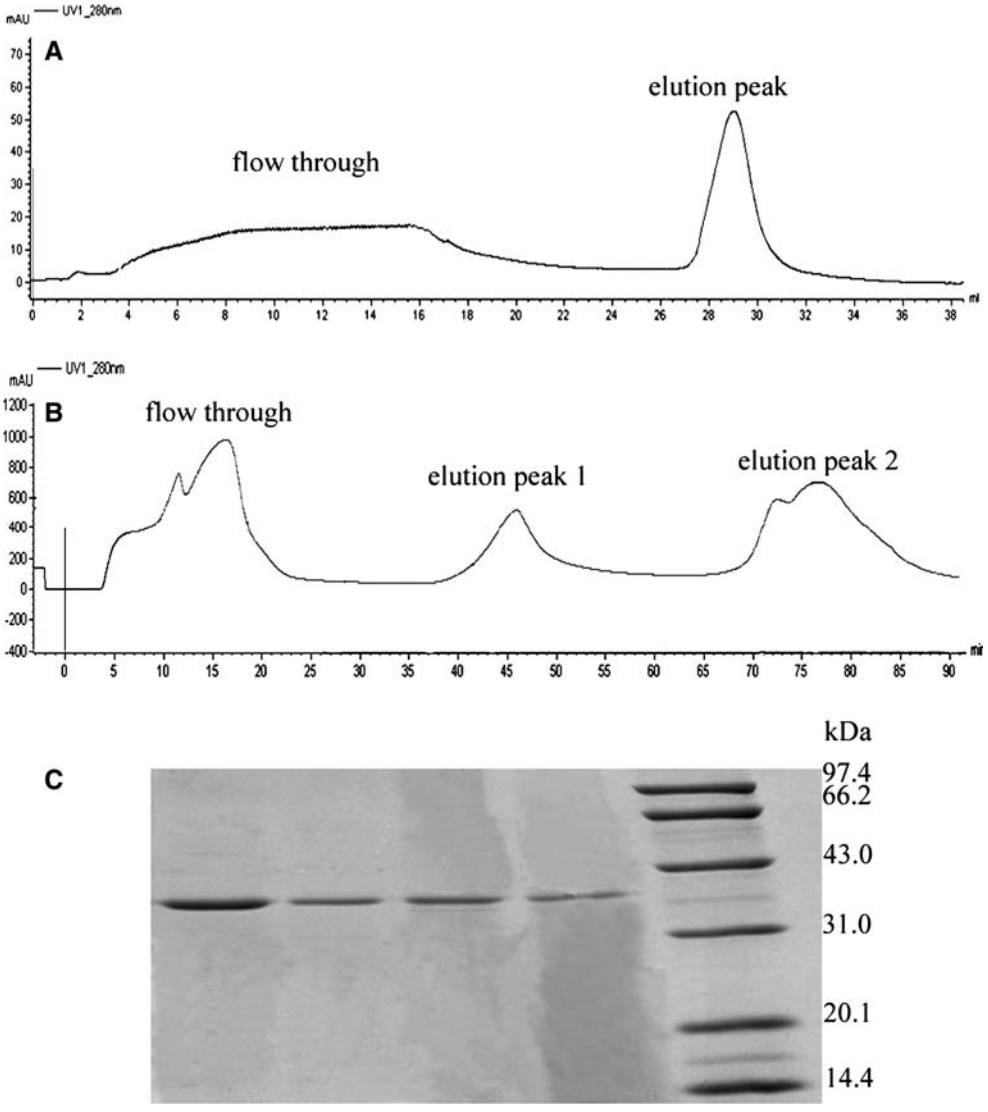

Fig. 1 **a** Result of cation-exchange chromatography. Only fraction of elution peak showed protease and bioassay activity; **b** result of hydrophobic interaction chromatography. Fractions of elution peak 1 and elution peak 2 were both detected protease activity and nematicidal activity; **c** SDS-PAGE (12%) of the fraction of elution peak 1 from hydrophobic interaction chromatography yielded a single protein band with a molecular mass about 40 kDa

Table 1 Purification of the neutral protease (npr) (Bae16) from *Bacillus neatocida* sp. nov

Purification procedure	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification factor	Recovery (%)
Culture filtrate	60.3	300.1	5.1	1.0	100
Crude enzyme	19.2	137.1	7.2	1.4	46.5
HiTrap SP FF	6.7	249.2	37.2	7.3	12
HiPrep phenyl FF (Bae16)	5.0	278	55.6	10.9	3.8

Characterization of purified Bae16

Effect of temperature and pH on enzyme activity

The optimum temperature of the enzyme was determined to be 55°C. The enzyme activity was stable at 30–60°C for 30 min incubation, but completely inactivated at temperature over 70°C after 1 h incubation.

The purified protease had the maximum hydrolytic activity at pH 6.5. Protease activity decreased from pH 6.5 to pH 10, and no activity was detected at pH 11 and pH 12. The enzyme was stable at pH between 5 and 8, while less than 40% activity was retained after incubation for 1 h at pH 4.

Hydrolysis of protein substrates and nematode cuticle assay

The protease showed high hydrolytic activity against denatured casein and skimmed milk, moderate hydrolysis of gelatin, BSA and nematode cuticle. The activity against native collagen was considerately low (Table 2).

Effect of metal ions on purified enzyme activity

Among the tested metal ions, 0.1 M Ca^{2+} enhanced enzyme by 60%, Cu^{2+} had almost no effect on protease activity. Hg^{2+} significantly inhibited the enzyme activity (>60%), while Mg^{2+}, Zn^{2+} and Al^{3+} moderately reduced it, respectively (Table 3).

Table 2 Hydrolysis of various protein substrates by the purified enzyme

Substrate	Relative activity (%) (OD_{680})
Denatured casein	100.0 (0.6)
Skimmed milk	59.8 (0.4)
Gelatin	8.9 (0.05)
Denatured collagen	11.5 (0.07)
Collagen	1.3 (0.01)
Bovine serum albumin (BSA)	8.7 (0.05)
Nematode cuticle	2.8 (0.02)

Effect of protease inhibitors on purified enzyme activity

In order to determine the nature of the purified enzyme, activity was measured in the presence of different protease inhibitors (Table 4). The protease activity was simulated up to 29% in the presence of EDTA. PepstainA increased protease activity up to 14%, but 1.0 M PMSF and 5.0 mM DTT inhibited protease activity up to 28 and 17%, respectively. In contrast, almost no inhibition was observed with 0.1 mM Leupeptin and 0.02 μg Aprotinins.

Cloning of the gene bae16 and its sequence analysis

The N-terminal amino acid sequence of the purified protease was AAATGTGTTL, which had 90% similarity to the npr from *B. amyloliquefaciens* (AAT TGTGTTL) through BLAST in NCBI. The gene encoding Bae16 was cloned using PCR with oligonucleotide based on the conserved sequences of the npr from *B. amyloliquefaciens*. The nucleotide sequence of an amplified fragment was determined and sequenced. The gene for the enzyme was submitted in GenBank (AY: 708654; AAV: 30844). The deduced protein was

Table 3 Effect of metal ions on the protease enzymatic activity

Metal ions	Concentration (M)	Enzyme activity as percentage of control (SD)
Control		100.0 (0.3)
Ca^{2+}	0.1	159.9 (1.7)
Mg^{2+}	0.1	74.5 (7.4)
Cu^{2+}	1.0	97.3 (19.6)
Zn^{2+}	0.1	65.3 (9.2)
Al^{3+}	0.1	60.0 (5.1)
Hg^{2+}	1.0	38.7 (8.0)

Table 4 Effect of various protease inhibitors on the enzyme activity

Inhibitors	Concentration	Enzyme activity as % of control (SD)
PMSF	1.0 mM/0.1 mM	71.8/77.0 (7.2/6.6)
EDTA	1.0 mM	128.7 (12.3)
Aprotinins	0.02 ml (1 μg/ml)	94.3 (8.6)
Leupeptin	0.1 mM	96.6 (9.5)
DTT	5.0 mM	82.9 (11.7)
PepstainA	0.01 mM	113.5 (9.1)

composed of a presequence signal peptide of 27 amino acids, a propeptide of 194 amino acids and the mature protease had 300 residues without the catalytic triad center of serine proteases, which ensured that Bae16 should not be identified as a serine protease. Coincidentally the first 10 N-terminal amino acids we sequenced were located at 222–231 of amino acids, which is also the beginning of the mature npr of Bae16. The whole amino acids residues showed 82–98% sequence identity between other nprs produced by several bacteria, but different with the other reported cuticle-degrading serine proteases.

Heterologous expression of Bae16

Culture of transformed *E. coli* BL21 cells was induced with 1.0 mM IPTG at 37°C, which led to the production of major protein of 42 kDa in inclusion body of rm-Bae16, as shown in Fig. 2. As the recombinant protein was produced mainly as insoluble inclusion body, active processing steps, i.e., isolation, solubilization and refolding were carried out and inclusion body turned to active protein in vitro. In Fig. 2, A 42 kDa band corresponding to mature Bae16 plus expressional vector *p*ET 30a was found in rm-Bae16, meaning that pre- and pro-sequences were deleted to produce mature Bae16. Proteolytic enzyme activity of rm-Bae16 was assayed, but a lower activity than that of Bae16 was observed, probably due to halfway renaturation, which is a complex process.

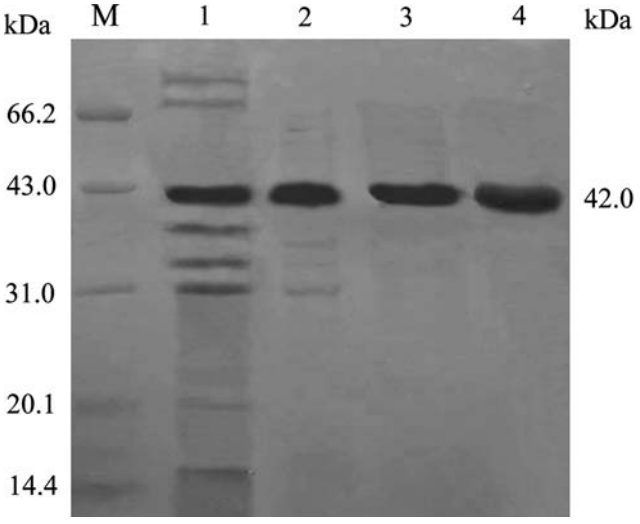

Fig. 2 SDS-PAGE (12%) of rm-Bae16. The expressed protein as insoluble inclusion body was dissolved in denatured buffer, 20 mM Tris–HCl (pH 7.4) containing 6 M urea (*line 1*), and dialyzed against the same buffer at 4°C for overnight and insoluble materials were removed by centrifugation (*line 2*). After purifying and renaturing, the active recombinant protein rm-Bae16 was shown respectively in *lines 3* and *4*

Nematicidal analysis

The result of Table 5 showed LC50 of crude enzyme, purified protease and expressed mature protease against the nematodes *P. redivivus* and *B. xylophilus*. LC50 of crude protease extracts in Eppendorf tubes against *P. redivivus* and *B. xylophilus* were 2.13 and 2.56 μg/ml within 24 h, respectively. Whereas in the negative controls with sterilized water and PBS (pH 7.0), it was shown that mortalities were below 20% using the maximum concentration. After chromatography of cation exchange, only the fraction of elution peak had nematicidal activity, and LC50 against *P. redivivus* was 1.72 μg/ml/24h, and *B. xylophilus* 2.35 μg/ml/24h. However, it lost the ability to kill nematodes after being boiled. Following HIC chromatography, the resultant purified protease was obtained by collecting fraction of Elution Peak 1, and then the bioassay showed 50% nematode could be killed within 24 h at the concentration of 1.69 μg/ml and 2.26 μg/ml for *P. redivivus* and *B. xylophilus*, respectively. While less than 10% of dead nematodes in the control of boiled Bae16. Regarding to the rm-Bae16, 50% *P. redivivus* and *B. xylophilus* were dead at the concentration of 1.70 and 2.28 μg/ml (Table 5), which indicated that expressed protease rm-Bae16 had similar weaker nematicidal activity than purified Bae16. But it was demonstrated that treatment of Bae16, simultaneously with the previously reported pathogenic serine protease, provided a possible more effective means to accelerate the death of nematodes. The same thing happened to rm-Bae16.

In the meantime, under the light microscope, we noticed that the cuticle of nematode was degraded gradually, and the cuticle of nematode was almost digested completely after treatment with purified protease Bae 16 for 2 days (Fig. 3a), expressed protease rm-Bae16 for 3 days (Fig. 3c). When the time of treatment with Bae16 was extended to 2.5 days or with rm-Bae16 to 3.5 days, only interior contents and traces of nematode corpse were visible (Fig. 3b, d). However, in the negative controls, most of the nematodes were living when treated for the same time (Fig. 3e). Even though dead nematodes were observed in the negative controls, the cuticles were almost complete (Fig. 3f).

Furthermore, we purified the cuticle from nematode *P. redivivus* and treated again with the purified protease Bae16 and expressed protease rm-Bae16 to verify the hydrolysis of nematode cuticle in vitro. When

Table 5 Half lethal concentrations (LC50) of protease extracts and rm-Bae16 against nematodes

Samples	Protease activity (U ml^{-1})	LC50 against *Panagrellus redivius* (μg ml^{-1} 24 h^{-1}) (SD)	LC50 against *Bursaphelenchus xylophilus* (μg ml^{-1} 24 h^{-1}) (SD)
Culture filtrate	88.5	3.09 (1.6)	7.87 (1.9)
Crude enzyme	106.9	2.13 (1.8)	2.56 (2.1)
HiTrap SP FF	137.0	1.72 (2.7)	2.35 (1.3)
Bae16	250.5	1.69 (2.1)	2.26 (2.3)
Serine protease	308.0	1.28 (2.0)	1.94 (1.7)
Boiled Bae16	0	–	–
Rm-Bae16	190.5	1.70 (1.4)	2.28 (1.2)
Boiled rm-Bae16	0	–	–
Bae16 + serine protease	820.0	0.99 (1.8)	1.40 (1.5)
Rm-Bae16 + serine protease	673. 6	1.17 (2.3)	1.88 (2.6)
BSA	250.0	–	–

treated with either of the proteases, several incomplete and degraded fragments were observed, and large flaws and scars appeared in the cuticle. While in the negative controls, the cuticles were intact and the striae were clear (data not shown).

Discussion

Cuticle of nematodes, which completely surrounds the animal except for small openings into the pharynx, is a very rigid but flexible multilayered extracellular structure exoskeleton and an effective barrier preventing nematodes from being environmentally damaged (Cox et al. 1981; Maizels et al. 1993). It may have extremely variable structures depending on different taxa, sexes and developmental stages. The most common structure features a triple-layered epicuticle on the external surface: a cortical zone, a median zone and a basal zone (Åhman et al. 2002). Structure components (collagen), soluble protein (glycoproteins) and lipids are made up of the cuticle. Additionally, the cuticle surface may be covered with a proteinaceous membrane (Gray 1984; Maizels et al. 1993). Therefore, protease, collagenase and chitinase are being emphasized in biological control of nematodes.

At present, fungal pathogens are thought as promising biological means of controlling plant-parasite

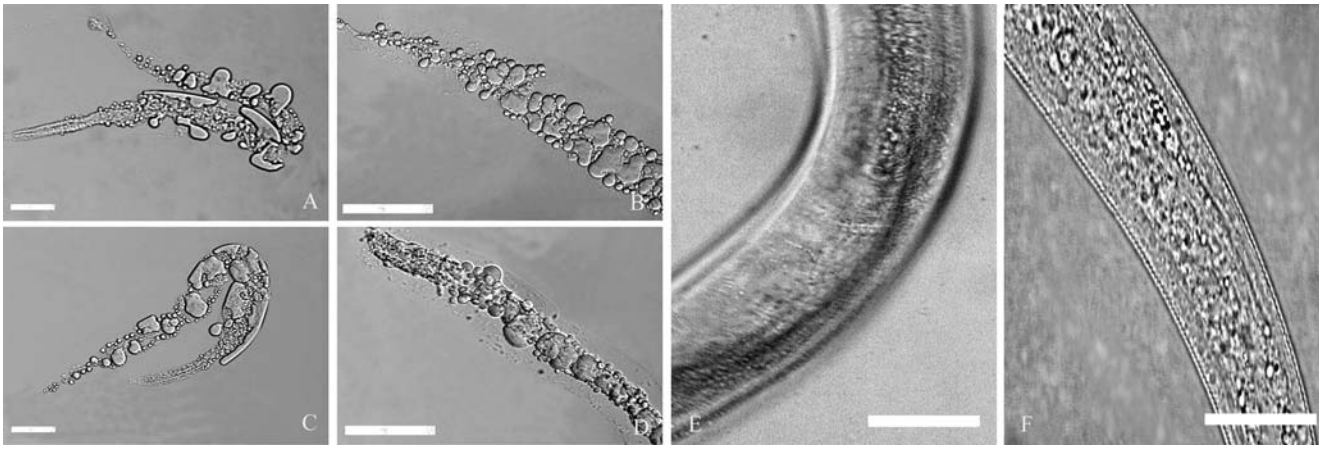

Fig. 3 The action of purified enzyme Bae16 and expressed enzyme rm-Bae16 against nematode *Panagrellus redivius* under a light microscope, among which **a** and **b** showed the action of purified enzyme Bae16; **c** and **d** showed the action of the recombinant Bae16. **a** The cuticle of nematodes was degraded and the outer layers of nematode cuticle were exfoliated when treated with 2.50 μg/ml purified enzyme Bae16 for 2 days. **b** The cuticle of nematodes was degraded completely and only inclusions remained when treated with 2.50 μg/ml purified enzyme Bae16 for 2.5 days. **c** The outer membrane covered around the nematode was continuously exfoliated and inclusions were exposed when treated with 2.50 μg/ml expressed enzyme rm-Bae16 for 3 days. **d** Only traces of dead body were visible when treated with 2.50 μg/ml expressed enzyme rm-Bae16 for 3.5 days. **e** The nematode was still live and cuticle was complete in the controls after 3.5 days. **f** Although the nematode was dead in the controls after 3.5 days, the cuticle was intact. *Scale bar* 20 μm

nematodes and other agricultural pests for their ability to directly penetrate the cuticle to infect hosts. On the basis of studies of entomopathogens, penetration of cuticle has been assumed to be due to the combination of mechanical force and hydrolytic enzymes (Leger 1995). Then the extracellular proteases probably contribute to the following processes of infection against nematodes: facilitating penetration by solubilizing the cuticle, digesting the host tissue and releasing nutrients for pathogen growth (Niu et al. 2006; Schroth and Hancock 1981; Tunlid 1994). Up to now, a variety of serine proteases have been purified and cloned from pathogenic fungi, such as PrI, PП, pSP-3, VCP-I and DEP-I etc. However, the exploration of virulence factors against nematodes from bacteria may have important and unique advantages. Huang et al. (2005a) reported on a purified extracellular serine protease (BLG4) with the capacity to hydrolyze and destroy nematode cuticle from *Brevibacillus laterosporus* strain G4; Niu et al. (2006) also reported another serine protease identified as a pathogenic factor in infection against nematodes from *B. nematocida* (Niu et al. 2006), which suggested that the extracellular serine proteases from bacteria, as from nematophagous fungi, could play an important role in infection against nematodes. However, there is a paucity of knowledge about whether other proteases are also associated with the infection process.

As indicated previously (Niu et al. 2006), *B. nematocida* could function as a potential pathogenic strain against nematodes because it can first attach to the epidermis of its host, then penetrate and degrade the cuticle, propagate and form spores on the nematodes, and eventually break them down. In this work, we purified another extracelluar protease Bae16 from the same bacterial strain and confirmed it contributed to the pathogenesis by the fact that the purified protease and heterologously expressed mature protease could immobilize nematodes.

When this new virulence protease was characterized, the insensitivity to the inhibitor PMSF indicated that it was not a serine protease. The gene encoding this protease was cloned and the deduced amino acids had 98% identity with npr gene from *B. amyloliquefaciens*. Based on the alignment of the amino acid sequence in NCBI, the enzyme should belong to npr. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Due to their intermediate rate of reaction, bacterial nprs generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry (Rao et al. 1998). While we have found the new function of bacterial nprs: Bioassay of the purified enzyme showed its capability to degrade the nematode cuticle.

Compared with the serine protease, the purified npr had weaker nematicidal activity. Surprisingly, the addition of the npr Bae16 to bioassay experiments exposed to serine protease increased significantly the nematicidal efficacy, as shown in Table 5. Also, the npr Bae16 showed minor proteolytic activity (250.5 U ml^{-1}), but when added to serine protease, they immediately presented considerably protease activity (820.0 U ml^{-1}). These phenomena might illustrate that serine protease and npr synergize to kill nematodes. We found that proteolytic activity was in proportion to nematicidal capability, i.e., too little proteolytic activity would result in a reduction in nematicidal activity. and increased levels of proteolytic activity corresponded with increased toxicity of *B. nematocida* against nematodes. Although Bae16 showed comparatively lower nematicidal activity than the serine protease, it indicated that it is possible to develop *B. nematocida* as a model to study the interaction between host and parasite for increasing the knowledge of the infection process, by identifying virulence factors and characterizing the factors that control their activities. Further studies, such as localization of pathogenic factors, relationship between structure and function, key domain of amino acids to degrade cuticle of nematode, are currently done in our laboratory.

The objectives of this study were (1) to compare the nematicidal efficacy of npr with serine protease. (2) To enrich the knowledge about the virulent factor of *B. nematocida*. (3) To extend the understanding of the mechanism of nematode infection by *B. nematocida*. (4) To assess the influence of *B. nematocida* on the biological control of nematodes.

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