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An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes

Xiaowei Huang [1], Baoyu Tian [1], Qiuhong Niu, Jinkui Yang, Lemin Zhang, Keqin Zhang *

Key Laboratory for Conservation and Utilization of Bioresources, Yunnan University, 650091 Kunming, PR China

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Abstract

Brevibacillus laterosporus is an aerobic spore-forming bacterium with the ability to produce canoe-shaped lamellar parasporal inclusions adjacent to spores. An isolate named G4 was identified as a *B. laterosporus* which does not produce parasporal crystals and shows significant toxic activity toward nematodes. Crude extracellular protein extract from culture supernatant of *B. laterosporus* G4 killed the nematodes within 12 h and finally destroyed the targets within 24 h, which suggested possible proteinaceous pathogeny. A homogeneous extracellular protease with nematicidal activities, purified by chromatography, confirmed the hypothesis that it might serve as a pathogenic factor during infection of the G4 strain. Characterization of the purified protease revealed a molecular mass of 30 kDa and optimum activity at pH 10 and 50 °C. The protease hydrolyzed relatively broad substrates including collagen and the cuticle of nematodes, and histopathological observations demonstrated the resulting destroyed nematode cuticle upon treatment by purified protease. Our present study reveals that extracellular protease, but not previously reported parasporal crystals, can be employed in infection against invertebrates by the *B. laterosporus* G4 strain.

Keywords: *Brevibacillus laterosporus*; Nematicide; Extracellular protease; Pathogenic factor

1. Introduction

Brevibacillus laterosporus, previously classified as *Bacillus laterosporus* [13], is an aerobic spore-forming bacterium characterized by its ability to produce canoe-shaped lamellar parasporal inclusion adjacent to spores. As a pathogen against invertebrates, its toxic activities against nematodes have also been described and were found not to be due to its characteristic parasporal inclusion [5,10,14]. At the same time, most of these *B. laterosporus* strains were reported not to produce parasporal crystalline, as *Bacillus thuringiensis* did. Indeed, this was believed to be an important pathogenic factor in *B. thuringiensis* [6,8]. However, Smirnova et al. then obtained two isolates with large parasporal crystals [16,17] and it was proven that these two *B. laterosporus* stains possessed a higher insecticidal activity, associated with parasporal crystals during sporulation rather than with soluble factors present in the culture medium [9]. Interestingly, other evidence from Singer's lab indicated that the nematicidal factor seemed to be a small, proteinaceous heat-stable toxin in some *B. laterosporus* strains [15]. These earlier studies suggested that different virulence factors could be recruited among the different *B. laterosporus* strains [5,10,22].

As a pathogenic factor in the process of infecting nematodes, extracellular proteases have been intensively studied in nematophagous fungi in the last decade. It has been concluded that these hydrolytic enzymes are involved in the stages of penetration of the surface (cuticle) and digestion of the host. Several extracellular proteases in nematophagous fungi have been identified, cloned, and homologously or heterologously expressed, respectively [1,2,12,18,20], and it

* Corresponding author.
E-mail address: kqzhang111@yahoo.com.cn (K. Zhang).
[1] These authors contributed equally to this work.

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has been demonstrated that it is possible to improve pathogenicity by introducing the protease with nematicidal activity into other potential nematicidal organisms [2]. But for nematophagous bacterium, no convincing evidence supports the hypothesis that extracellular proteases serve as pathogenic factors in infection against the host.

Here we report on an isolated *B. laterosporus* strain G4, having strong nematicidal ability but without parasporal crystals. Histological observation showed destruction of the nematode cuticle and final digestion of the entire host in infection of nematodes, thus suggesting the involvement of hydrolytic proteases as potential nematotoxic components. The action mode of this strain differed from that of previously reported parasporal crystal-containing strains, in which the parasporal crystal was demonstrated to be virulent toward invertebrates [9]. After purification, most nematicidal activity was attributed to an extracellular protease. The obtained protease hydrolyzed and destroyed nematode cuticle and even killed nematodes, indicating that it could serve as a potential pathogenic factor in antagonizing nematodes of *B. laterosporus* strain G4.

2. Materials and methods

2.1. Isolation, identification and culture of B. laterosporus *strain G4*

A collection of bacterial strains isolated from soil samples in the Yunnan province were screened for activities against nematodes with the bioassay method described below. The G4 strain with significant nematicidal activity was identified by a combination of morphological, physiological and chemical methods, and collected as No. M203045 by the China Center for Type Culture Collection (CCTCC).

G4 strain was grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose, pH 7.0) at 28 °C on a rotary shaker at 220 rpm.

2.2. Measurement of protease activity

Measurement of protease activity was performed using 2% casein solution as a substrate. 2% casein in the phosphate solutions (125 µl; pH 7.0) and the tested protease at a given concentration were added to 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 incubation at 37 °C for another 10 min. 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 protease activity unit was defined as emission of L-tyrosine in 1 min at 37 °C.

2.3. Preparation of crude extracellular protein extract

Strain G4 was inoculated into a 500 ml Erlenmeyer flask containing 100 ml YPD medium and grown at 28 °C with rotary shaking at 220 rpm for three days when it was already in stationary phase. Centrifuged at 8500*g* for 15 min at 4 °C, the culture supernatant was collected, then mixed with ammonium sulfate to reach 85% (w/v) saturation and centrifuged again at 8500*g* for another 20 min. Precipitate was dissolved in 20 ml of 50 mM phosphate buffer (pH 7.0) and dialyzed thoroughly against 20-fold volumes (dialysis molecular size: 8000–15 000) at 4 °C. The dialyzed solution designated for crude extracellular protein extract was further filtered with 0.22 µm filter unit (from Millipore Co.) and immediately used in the bioassay.

2.4. Purification of extracellular protease

A 300 ml culture of the G4 stain was collected and bacteria were removed by centrifugation at 8500*g* for 15 min. The resulting supernatant was treated as indicated below for purification:

1. Ammonium sulfate precipitation. After adding ammonium sulfate to 45% saturation (w/v), the mixture was precipitated at 8500 rpm for 20 min at 4 °C and the supernatant was pooled. Then ammonium sulfate was added to reach 70% saturation, centrifugation was again carried out under the same conditions, and precipitate with nematicidal activity was dissolved in 50 mM phosphate buffer for chromatography.
2. Hydrophobic interaction chromatography. Before hydrophobic interaction chromatography, 1 M ammonium sulfate was supplemented in the dissolved precipitate and pH was adjusted to 7. The resulting sample was applied to a HiPrep™ 16/10 phenyl FF column (high sub; Amersham Pharmacia Biotech) that had been equilibrated with 50 mM phosphate buffer containing 1 M ammonium sulfate (pH 7.0). With 50 mM phosphate buffer, the bound proteins were eluted with a liner gradient of 1–0 M ammonium sulfate. The resulting fractions of 2–5 ml were collected and assayed for protease activity and nematicidal activity. Positive fractions were pooled and used for cation-exchange chromatography.
3. Cation-exchange chromatography. Protease and nematicidal activity positive fractions from hydrophobic interaction chromatography were diluted to conduction below 2 ms/cm, adjusted to pH 6.0 and applied to a HiTrap™ SP FF column (1 ml; Amersham Pharmacia Biotech) previously equilibrated with 10 mM phosphate buffer (pH 6.0). Bound proteins were eluted with a linear gradient of increasing NaCl (0–0.5 M, pH 6.0) and fractions containing protease activity and nematicidal activity were pooled and dialyzed thoroughly against 50 mM phosphate buffer. The dialyzed protein sample was used for SDS–PAGE and bioassay.

Concentration of the purified protease solution was determined by the BCA protein assay kit (from Pierce Co.) using bovine serum albumin as a standard.

2.5. SDS–PAGE

The aliquots of dialyzed protein sample were submitted to electrophoresis by sodium dodecyl sulfate (SDS)–12% (w/v) polyacrylamide gel electrophoresis (PAGE). Proteins were visualized after staining with Coomassie brilliant blue R-250. Bovine albumin (66 kDa), egg albumin (45 kDa), rabbit glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine α-lactalbumin (14.2 kDa) were used as molecular mass standards.

2.6. Characterization of the purified protease

The optimum temperature for protease activity of the protein sample was determined by incubating the sample at 4, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80 and 100 °C, respectively, according to the method described for measurement of protease activity. Using the Britton Robinson universal buffer system, optimum pH for protease activity of the protein sample was determined at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 at the known optimum temperature (50 °C), respectively.

The purified protease was incubated for 30 min at the known optimum temperature and pH (50 °C, pH 10.0) with individual inhibitors, and subsequently protease activity was determined. Protease activity was expressed as a percentage of the activity detected without any inhibitor.

Proteolytic activity against protein substrates and purified nematode cuticle, which had been obtained using the method of Cox [4], was performed according to the methods previously described by Anders Tunlid et al. [21].

2.7. Bioassay

The free-living nematode (*Panagrellus redivius*) and plant parasite nematode (*Bursaphelenchus xylophilus*) were selected as the tested nematodes. In bioassay, the nematodes were considered dead when no movement was observed under a light-dissecting microscope, and when gentle tapping of nematodes by a stick did not result in movement. Mortality of nematodes was defined as the ratio of dead nematodes to tested nematodes.

2.7.1. Infection of nematodes by B. laterosporus strain G4

This was performed according to the modified dialysis membrane technique [11]. Briefly, pieces of autoclaved cellophane paper were used to cover YPD medium supplemented with 2% agar to avoid nematodes moving into the medium. Bacteria were inoculated onto the cellophane paper and incubated at 28 °C for 7–10 days. Then a block of Oaten medium containing tested nematodes was placed in the middle of the plate. Each plate was plotted into 20 panes and the mortality of nematodes was counted in 5 of 20 panes stochastically every 12 h. Cuticle damage in infected nematodes in each treatment was examined under the light microscope. The experiments were performed with five parallels and repeated at least twice. Negative controls were incubated with non-pathogenic bacteria (*Escherichia coli* strain) and without bacteria under the same conditions.

2.7.2. Toxic effects of crude extracellular protein extract against nematodes

The experiment was performed in a 1.5 ml Eppendorf tube; each tube was added with 150 µl of crude extracellular protein extract. The nematodes were washed thoroughly with 50 mM phosphate buffer (pH 7.0) before being transferred into tubes and about two-hundred nematodes were assayed in each treatment. After incubating the tubes at 25 °C, dead nematodes were determined with a light microscope and mortality was counted every 12 h. Light microscopy or scanning electronic microscopy (SEM) was employed to examine the changes in the nematode cuticle. The experiments were performed with thee parallels and repeated at least five times. Controls were incubated with water, YPD medium, crude protein extract from the YPD medium (prepared as crude extracellular protein extract) and crude extracellular protein extract boiled for 15 min.

2.7.3. Effect of purified protease against nematodes

The effect of purified protease against *P. redivius* nematodes was performed under conditions of pH 7.0, 25 °C with the method described above. Characteristics of body damage to the tested nematodes were examined under SEM after 48 h treatment.

2.8. Preparation of samples for SEM

The filtered nematodes from bioassay specimens were washed in phosphate buffer (pH 7.0) thee times and fixed in 4% glutaraldehyde for 2 h and 2% osmic acid for 40–60 min. Following dehydration in an ethanol series, the material was critical-point-dried overnight, sputter-coated with gold, and observed. Pictures were taken using a scanning electron microscope (SEM) [19].

3. Results

3.1. Nematicidal activity and identification of B. laterosporus strain G4

When screening soil samples from Yunnan Province, strain G4 showed significant nematicidal activity in tests of infection (Fig. 1A–1B): over 20% of the nematodes were killed within 48 h, and about 90% of the nematodes were

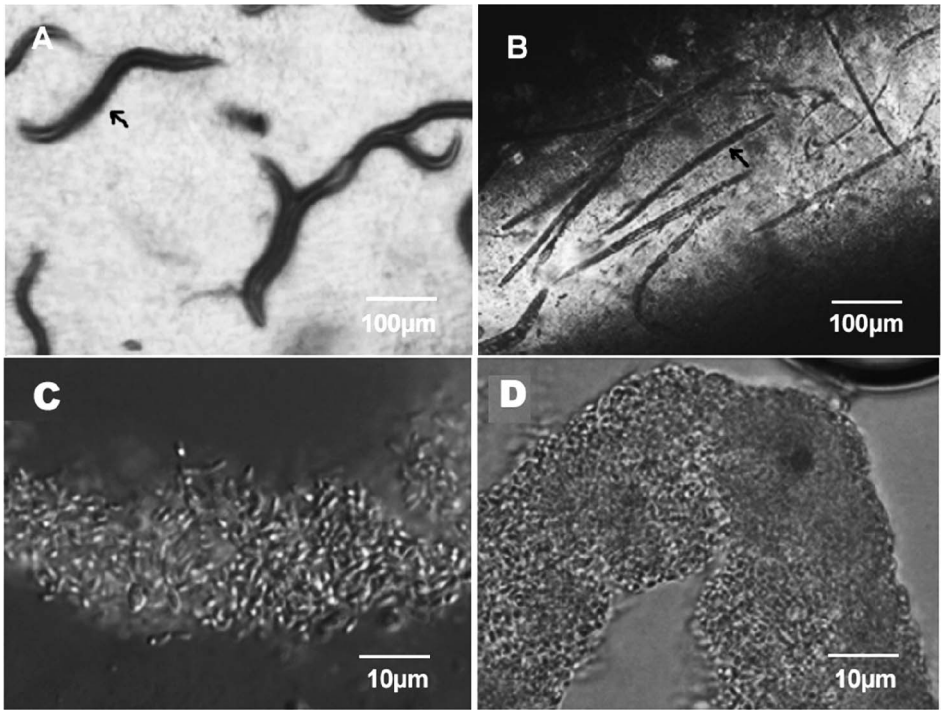

Fig. 1. Infection of nematodes (*P. redivius*) by *B. laterosporus* observed under a light microscope. (A) Within 84 h, most nematodes on the control plate were active. Arrow indicates a living nematode. (B) Within 84 h, over 90% of nematodes were killed on the bacterial plate. Arrow indicates a dead nematode. (C) The nematode was attached by *B. laterosporus* and the bacteria propagated on the body. (D) The outlines of nematodes mainly composed of spores of *B. laterosporus*.

Table 1
Mortality of nematodes *P. redivius* and *B. xylophilus* killed by crude extracellular protein extract of *B. laterosporus*

Samples	Mortality of *P. redivius* % (SD)			Mortality of *B. xylophilus* % (SD)		
	12 h	24 h	48 h	12 h	24 h	48 h
Crude extracellular protein extract	84(2.3)	95(0.6)	100(0)	33(3.5)	58(5.9)	92(4.1)
Crude protein extract from YPD medium	12(1.6)	20(1.3)	25(2.5)	10(3.7)	16(4.2)	25(10.3)
YPD medium	6(0.6)	12(1.1)	13(0.9)	5(0.8)	8(2.1)	17(1.9)
Crude extracellular protein extract, boiled	10(2.2)	12(3.1)	30(2.7)	12(3.0)	24(2.5)	30(4.6)
Water	5(0.3)	13(0.7)	15(0.7)	6(0.3)	8(1.5)	10(0.6)

Death was determined by nudging nematodes with a stick under a light microscope; the immobilized nematodes were treated as dead nematodes. Mortality of nematodes = number of dead nematodes/all tested nematodes $\times 100\%$.

killed within 84 h. Five days later, most of the dead nematodes were degraded and only white traces were left. But in two negative controls, one including non-pathogenic bacterium (*E. coli*) and one without any bacterium, most of the tested nematodes were mobile, grew and propagated normally; furthermore, the death rate remained below 10%. Light microscopy recorded the infectious process of the nematodes by *B. laterosporus* strain G4 (Fig. 1C–1D). After attaching to the epidermis of the host body, it was easily observed that the bacterium G4 destroyed the nematode cuticle. In the early stage, the typical shape of the degraded tissue was a circular hole full of bacteria until the whole host was digested and the bacteria propagated and formed spores on the trail of the nematode.

Strain G4 was sent to the China Center for Type Culture Collection for identification and deposition (CCTCC No. M203045). According to the morphological, physiological and biochemical characteristics, this stain was identified as *Brevibacillus laterosporus* (previously classified as *Bacillus laterosporus*). The bacterium was characterized by its ability to produce oval, lateral canoe-shaped lamellar parasporal inclusions adjacent to spores, and two strains were reported to produce parasporal crystals with obvious toxic effects toward invertebrates. However, our strain failed to form such parasporal crystals.

3.2. *Effects of crude extracellular protein extract of* B. laterosporus *strain G4*

As shown in Table 1, the crude extracellular protein extract from culture supernatant of strain G4 showed high toxinogenic activity toward the free-living nematode (*P. re-*

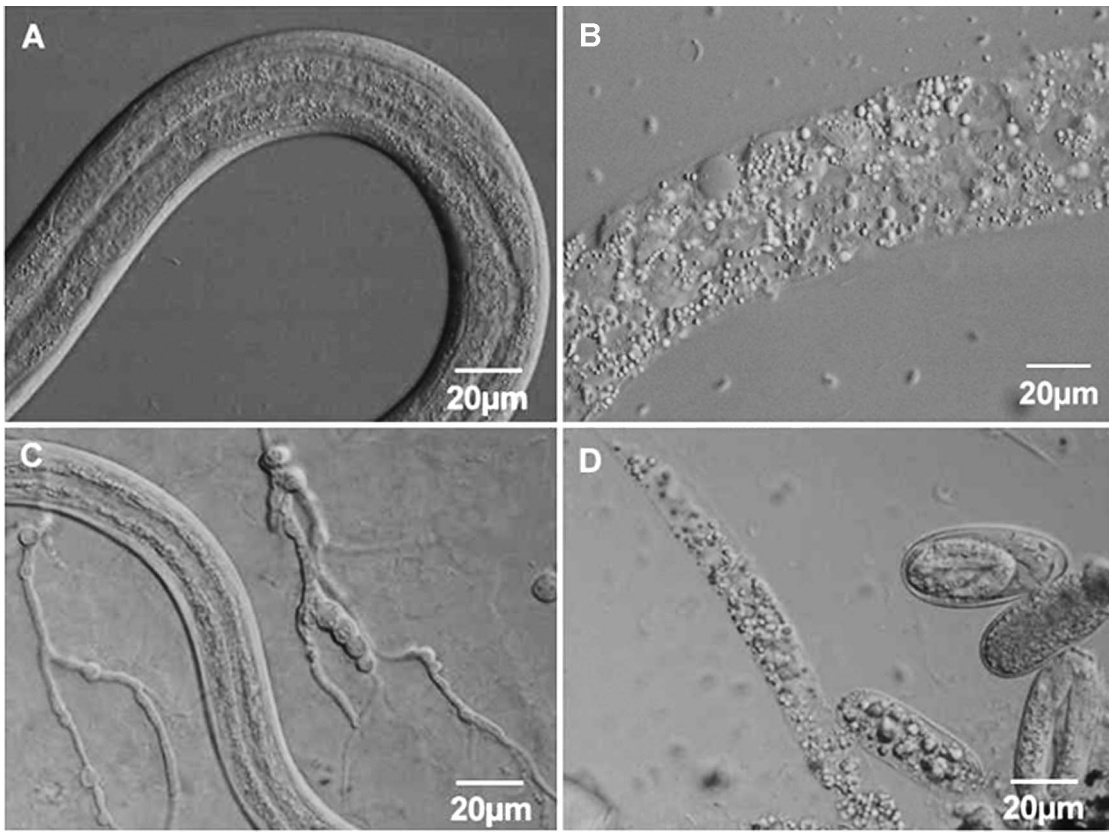

Fig. 2. The action of crude extracellular protein extract against the nematodes *P. redivius* and *B. xylophilus* observed under a light microscope. (A) The cuticle of the nematode (*P. redivius*) in the control was intact and smooth within 12 h. (B) The cuticle of the nematode (*P. redivius*) treated with crude extracellular protein extract was destroyed within 12 h. (C) The cuticle of the nematode (*B. xylophilus*) in the control was intact and smooth within 48 h. (D) The cuticle of the nematode (*P. redivius*) treated with crude extracellular protein extract was degraded within 48 h.

divius) and the plant parasite nematode (*B. xylophilus*). In the test against *P. redivius*, the mortality of the nematode was 85, 95 and 100% within 12, 24 and 48 h, respectively, whereas in the controls with YPD medium, crude protein extract from YPD medium, boiled crude extracellular protein extract and water, mortality of nematodes was below 30% up until 48 h. In the test against *B. xylophilus*, the mortality of nematodes was 33, 58 and 92% within 12, 24 and 48 h, respectively, while the mortality of nematodes remained below 30% in all controls.

Histopathological changes in nematodes were observed both with the light microscope (Fig. 2) and SEM (Fig. 3). The breakdown in the nematode cuticle and the absence of nematode inclusion were visible within 12–24 h and about 95% of dead nematodes had been completely destroyed within 24 h in the test of crude extracellular protein extract against *P. redivius* (Fig. 2B). In the test of crude extracellular protein extract against *B. xylophilus*, no apparent changes in the nematode cuticle were observed under the light microscope within the initial 24 h. However, the image after 48 h indicated a similar devastating phenomenon compared with that of *P. redivius* (Fig. 2D). While, nematode bodies remained well in all controls (Figs. 2A, 2C). More detailed changes in the nematode cuticle could be observed with SEM. Nematodes in controls had a smooth surface with distinct striaes and lateral lines (Figs. 3A, 3B); however, with treatment by crude extracellular protein extract, the cuticles of nematodes were almost totally destroyed (Figs. 3C–3F). Initially, the outer layers of the nematode cuticle were exfoliated (Fig. 3C). As treatment continued, damage became more severe, the outer membrane covering the nematode was continuously exfoliated and the fibrillar layer appeared (Fig. 3D). Finally, a large flaw appeared (Fig. 3F), inclusions of nematodes exuded and only the broken cuticle remained (Fig. 3E).

Concerning *B. laterosporus* strain G4 without parasporal crystals, its nematicidal activity from crude extracellular protein extract demonstrated that most pathogenic factors were present in secreted proteinaceous materials to a greater extent than for previously reported parasporal crystalline. The destroyed nematode cuticle and digestion of the whole nematode were visible in bioassay of extracellular protein extract, which has been speculated to be associated with protease in nematophageous fungi [12,20]. However, results indicating that the similar efficacy of *B. xylophilus* takes longer than that of *P. redivius* suggested that nematicidal activity of this extracellular protease was different among disparate hosts.

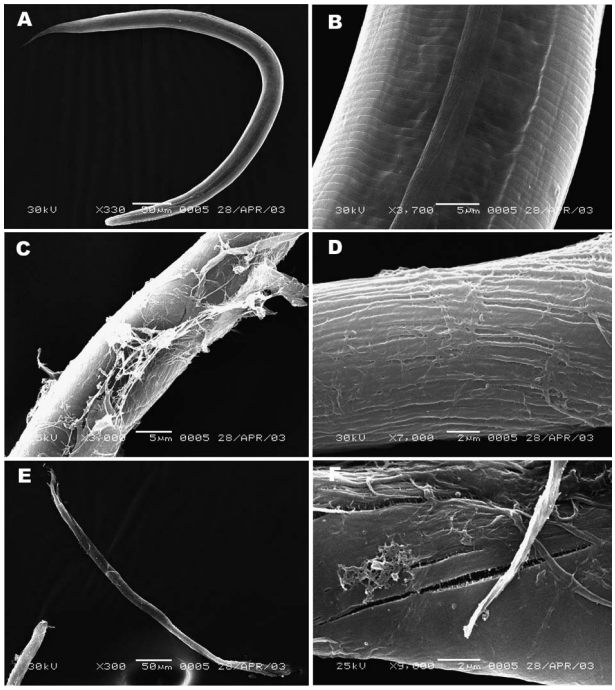

Fig. 3. The action of crude extracellular protein extract against the body of nematodes (*P. redivius*) observed under SEM. (A–B) Nematodes in all controls had smooth undisturbed surface, striae and lateral lines of the cuticle were clear. (C) After treatment with crude extracellular protein extract for 12 h, the cuticles of nematodes were degraded and the outer layers of nematode cuticle were exfoliated. (D) After treatment with crude extracellular protein extract for 18 h, the outer membrane covering the nematode was continuously exfoliated and the fibrillar layer appeared. (E) After treatment with crude extracellular protein extract for 24 h, the cuticle of nematode was broken, inclusions of nematodes exuded and only the broken cuticle of the nematode was left. (F) With the severe degradation of crude extracellular protein extract against nematode cuticles, large flaws and scars appeared in the cuticle.

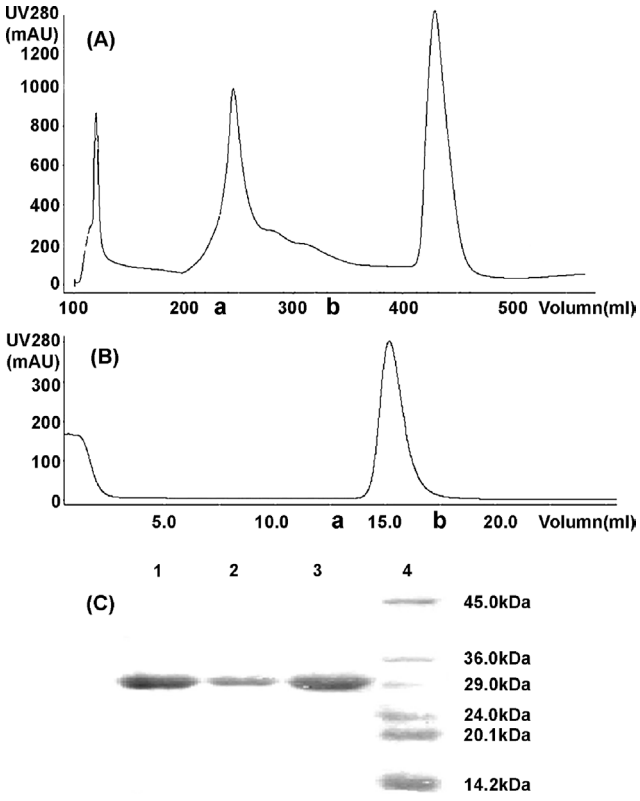

Fig. 4. Purification of extracellular protease from culture supernatant of *B. laterosporus* strain G4. (A) Hydrophobic interaction chromatography on a HiPrep 16/10 phenyl FF column of the dissolved precipitate from the culture supernatant of *B. laterosporus* strain G4. Protease activity and nematicidal activity were detected from the peak between (a) and (b). (B) Cation-exchange chromatography on a HiTrap SP FF column of fractions containing protease activity and nematicidal activity from hydrophobic interaction chromatography. Protease activity and nematicidal activity were also detected from the peak between (a) and (b). (C) SDS–PAGE (12%) of fractions containing protease activity and nematicidal activity from cation-exchange chromatography yielded a single protein band with a molecular mass about 30 kDa. Lines 1–3 represent 3 aliquots of purified protease in parallel; line 4 represents the protein marker.

3.3. Purification and properties of extracellular protease with nematicidal activity

Purification of protein with nematicide activity from culture supernatant of *B. laterosporus* strain G4 was performed by ammonium sulfate precipitation, hydrophobic interaction chromatography and cation-exchange chromatography. Following hydrophobic interaction chromatography, protease activity and nematicidal activity were assayed in each peak. One peak shown in Fig. 4A was detected as containing protease activity as well as nematicidal activity, but for the others, no obvious nematicidal activity was visible. The peak with the two activities was further purified by cation-exchange chromatography. Both protease activity and nematicidal activity were detected in only one peak (Fig. 4B). The fraction was pooled and SDS–PAGE of the aliquots yielded a single protein band with a molecular mass about 30 kDa (Fig. 4C).

Biochemical activity assays of the protease showed stable activity at a wide range of temperatures from 25 to 55 °C, and more than 70% of activity remained after incubation at 60 °C for 30 min at pH 7.0. Protease activity continuously increased from 25 to 50 °C and subsequently declined until no activity was detected at 100 °C. Thus, at the optimum temperature of 50 °C, proteolytic activity was assayed within a range of pH from 3.0 to 12.0. Protease activity increased from pH 4.0 to 10.0 and immediately declined, and no activity was detected at pH 3.0 and 12.0. Thus, the maximum proteolytic activity was obtained at 50 °C and pH 10.0. At a protease concentration of 71.6 μg/ml, the highest protease activity of 1360 U/ml was obtained using conditions of pH 10.0, 50 °C.

The effect of different protease inhibitors chosen on the basis of their selective inhibition is summarized in Table 2. Most inhibitors tested did not significantly influence proteolytic activity; phenylmethylsufonylfluoride (PMSF), however, was able to completely inhibit activity at 1 mM, which suggested that protease belongs to the serine protease class.

Table 2
Effect of protease inhibitors on purified protease from *B. laterosporus* strain G4

Inhibitor	Concentration	Proteolytic activity as % of control
PMSF	1.0 mM	0.0
EDTA	1.0 mM	97.9
Leupeptin	10.0 μg/ml	97.1
Aprotinin	1.0 μg/ml	98.7
Pepstatin A	10.0 μg/ml	103.5
DTT	1.0 mM	108.7

PMSF, phenylmethylsufonylfluoride; EDTA, ethylene diaminotetraacetic acid; DTT, dithiotheitol.

Table 3
Hydrolysis of protein substrates by the purified protease from *B. laterosporus* strain G4

Substrate	Relative activity (%)
Denatured casein	100.0
Skimmed milk	87.0
Gelatin	24.3
Bovine serum albumin	45.3
Denatured collagen	35.6
Collagen	31.6
Nematode cuticle	14.6

In addition, the purified protease had a relatively broad substrate specificity. It showed strong hydrolytic activity against denatured casein and skimmed milk. It could also moderately hydrolyze gelatin, BSA, denatured collagen, and even native collagen. Furthermore, the protease we obtained also possessed relatively strong activity toward the purified nematode cuticle (data was shown in Table 3).

3.4. Bioassay of the purified protease

To confirm that protease can serve as the pathogenic factor in infection against nematodes, *P. redivius* were treated with the purified protease described above at pH 7.0, 25 °C. The concentration and activity of protease used for bioassay were 1302 μg/ml and 2800 U/ml, which was close to the activity of the crude extracellular protein extract. The results showed that with treatment of the purified protease, about 71% of nematodes were killed within 24 h, but there were less than 15% of dead nematodes in controls using boiled protease and water. After 48 h, all tested nematodes were nearly dead and degraded. Concomitantly, SEM was performed and revealed similar damage to nematodes with the crude extracellular protein extract. After treatment with purified protease, exfoliation of outer layers could be easily observed and many severe flaws and scars appeared on the cuticles of nematodes. In contrast, no visible damage was observed in the controls. The cuticles of nematodes remained intact and the striae of cuticles were clear.

4. Discussion

B. laterosporus has been showed to be toxic toward certain invertebrate organisms and may even have more than one target, including parasitic nematodes [15]. Some reports also reported that the insecticidal activity of *B. laterosporus* strains was 1000 times lower than that of *B. thuringiensis* [5,10]. For example, Favret and Yousten found that almost half of the 29 strains of *B. laterosporus* that they tested were not toxic toward mosquito larvae [5]. Low efficacy in insecticidal activities impeded their application in biocontrols. However, genotypic diversity among *B. laterosporus* and isolation of strains with parasporal crystals having significant insecticidal activity encouraged endeavors to screen more new strains having toxic activities [9,22].

In this report we isolated *B. laterosporus* strain G4 showing high nematicidal activity. In the infectious experiments, *B. laterosporus* strain G4 killed about 90% of the tested nematodes within 48 h, which confirmed its potential application in biological control of nematodes. But contrary to two previously reported strains with large parasporal crystals, the G4 strain, without any virulent parasporal crystal, appeared to depend upon some other pathogenic factor to infect hosts.

Experiments employing infection of nematodes by *B. laterosporus* strain G4, profiled with severe damage to cuticles, and the crude extracellular protein extract exhibiting strong nematicidal activity, seemed to suggest that extracellular proteases, as potential pathogenic factors, may be involved in the infectious process. This result is coherent with evidence from Singer [15], in which it was revealed that the nematicidal activity of their isolated *B. laterosporus* strains appeared to be due to a small proteinaceous toxin. Thus we sought to purify the protease with toxin to nematodes and such a protease was obtained. Evidence that the purified protease killed the tested nematodes, hydrolyzed the substrates including collagen and nematode cuticle, and severely damaged the cuticle of the nematode confirmed that the obtained virulent extracellular protease is an important cause of nematode death.

Thus, our current studies extend the understanding of the mechanisms of infection of nematodes by *B. laterosporus*. Smirnova [16,17] and Orlova [9] purified parasporal crystals from two mosquitocidal *B. laterosporus* strains and demonstrated that the larvicidal activity of these *B. laterosporus* strains was associated with parasporal crystals. The action mode of this crystal was similar to that of the Cry protein of *B. thuringiensis.* It is known that the Cry protein of *B. thuringiensis* needs to be ingested by targets; this results in pore formation in midgut cell membranes, ionic imbalance, and consequent septicemia [6]. However, the action mode of strain G4 is different from that of the above crystal-forming strains. In our study, it has been demonstrated that extracellular proteases, in addition to the previously reported parasporal crystals, could also serve as an important pathogenetic factor in *B. lat-*

erosporus. These results were consistent with previous research on the action mode of *Arthobotrys oligospora* in which the death of nematodes occurred during penetration of the cuticle [21].

The role of extracellular proteases, and especially serine protease, in infection of nematodes, has been studied extensively in nematophagous fungi. It is well known that the cuticle of the nematode is a very rigid but flexible exoskeleton composed mainly of proteins and chitin; in particular, the outer part is covered by a layer of proteinaceous membrane which is an effective barrier preventing nematodes from being infected [3,4,7]. The extracellular protease not only degraded the outer proteinaceous membrane of the cuticle, but also destroyed the protein matrix, which connects different chitin microfibrils to the cuticle of the nematode and leads to effusion of inclusions [21]. Thus, the proteases may also contribute to pathogenesis, along with being involved in hydrolysis of protein into amino acids or smaller peptides as nutrients to support bacterial propagation. It was demonstrated that hydrolytic protease participated in infection by releasing nutrients for pathogen growth, and facilitating penetration of microorganisms or toxins by solubilizing the cuticle [18]. Another possibility is that the detrimental effect of protease arises from damage to the nematode gut by concurrent uptake of protease. In the infectious test against *P. redivius*, in addition to the destroyed cuticle, histological observation recorded other significant damage to the heads of nematodes, especially the stoma where only a few thin layers with pores remained (data not shown). In addition, we noted that a similar percentage of killed nematodes had spent less time in the infectious test of crude extracellular protein extract than that of bacterium. The reason for faster efficacy is probably that the large quantity of virulent extracellular protease could easily be swallowed by living nematodes, in addition to the fact that the whole nematode immediately was in contact with virulence factors present in crude extracellular protein extract. Further studies on the action mode of virulent proteases are underway.

It has also been noted that, although the protease activities of the two are similar, the mortality of nematodes (*P. redivius*) due to purified protease (71%, 24 h) is lower than that due to crude extracellular protein extract (95%, 24 h), indicating that most (but not all) nematicidal activity was due to extracellular protease. Thus, it cannot be excluded that other pathogenic factors such as toxic peptide or midterm metabolic products also contribute to infection against nematodes in *B. laterosporus* strain G4. The identification of other virulent factors is needed to better understand the mechanism of infection against nematodes by *B. laterosporus* strain G4.

This is the first report of a purified protease with the capacity to kill nematodes from nematophagous bacteria, and which suggests that the extracellular protease, as in nematophagous fungi, could play an important role in infection of hosts. The differing infectious mechanism employed by the nematophagous bacterium *B. laterosporus* G4 provides a solid foundation for further understanding the relationship between pathogeny and host, and is a possible tool for improving nematicidal activity in biological control.

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