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Insecticidal Toxin from *Xenorhabdus nematophilus*, Symbiotic Bacterium Associated with Entomopathogenic Nematode *Steinernema glaseri*

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Abstract Entomopathogenic nematodes are being used for insect control. We purified a toxin secreted by the insect-pathogenic bacterium, *Xenorhabdus nematophilus*, which lives in the gut of entomopathogenic nematodes. Culture broth of *X. nematophilus* was separated by centrifugation and concentrated by ultrafiltration. The concentrated culture broth was applied to a DEAE Sephadex A-50 column, and proteins were eluted stepwise with increasing concentrations of KCl. Fractions with insect toxicity were further concentrated and then applied to a HPLC with a gel filtration column. The molecular weight of purified toxin was 39 kDa on SDS-PAGE, and Fourier transformed infrared (FTIR) spectroscopy indicated that this toxin could be a new protein exhibiting the characteristics of C=O stretching peak near 1650 cm^{-1}.

Keywords. insecticidal toxin, *Xenorhabdus nematophilus*, entomopathogenic nematode, *Steinernema glaseri*, biological pesticide

INTRODUCTION

Insect-pathogenic nematodes of the *Steinernema* and *Heterorhabditis* families, are of interest in developing biological pesticides to replace chemical pesticides, the use of which has raised many environmental and biological concerns [1]. Since they have a wide range of insect hosts which are killed rapidly upon selective infection but are safe for non-targeted organisms such as plants, animals and humans, they are often called entomopathogenic nematodes or beneficial nematodes.

These nematodes are known to be symbiotically associated with insect-pathogenic bacterium of the genus *Xenorhabdus* [2]. It has been observed that an infective, juvenile-stage nematode harbors these bacteria in its gut and, after infecting an insect the nematode burrows through the intestinal wall of the insect and releases the bacteria into the hemolymph. The bacteria avoid the immune response of the insect, multiply rapidly and kill the insect host within 24-48 h, providing suitable conditions for nematode reproduction. Axenic nematodes can still infect an insect but are much less virulent than the *Xenorhabdus* sp.-carrying nematodes [3]. However, completing the life cycle of the nematodes would be hindered without the bacteria. *Xenorhabdus* spp. are Gram negative, facultatively anaerobic, rod shaped bacteria currently assigned to the family Enterobacteriaceae. Only two species had been described (*X. nematophilus* and *X. luminescens*) until Akhurst [4] recognized four subspecies of *X. nematophilus* (*nematophilus, bovienii, poinarii and beddinggi*). The bacteria exist in two forms; primary and secondary forms. The primary form is known to preferably exist in the infective nematodes while the secondary form develops *in vitro* culture medium of the primary form after a long incubation period [5] or when the bacteria exist in soil as free-living organisms [6]. Antibacterial compounds produced by the primary form restrict the growth of other microorganisms, which would reduce the nutritional status of the insect cadaver. The secondary form has a significance for *in vitro* culture of nematodes, because it is detrimental to the final yield as is the case of nematodes grown in infected insects. This aspect is of considerable importance if the nematode-*Xenorhabdus* system is to be employed in the production of a bio-insecticide. The mechanism of conversion from the primary form to the secondary form is unknown but is thought not to involve a plasmid or bacteriophage. In this regard, recent studies of several genera of Gram negative bacteria have found that the expression of numerous virulence associated genes in each species is regulated by a common regulatory locus.

The pathogenicity of *Xenorhabdus* spp. depends on interaction with its nematode associate. The interactions of entomopathogenic nematodes and their bacterial symbionts with the defense systems of insects have been extensively reviewed [7]. The nematode may produce a toxin or a toxic substance that would destroy the inducible enzymatic defense response of the insect. Growth of *Xenorhabdus* is accompanied by the production of exo- and endotoxins. The exotoxin activity is probably linked to the exoenzymatic functions that have been considered as toxins (proteases, phospholipase and lipases) in other bacteria. Like many other Gram negative bacteria, *Xenorhabdus* spp. can also produce endotoxins. The endotoxins are lipopolysaccharide

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components of the bacterial cell wall that are toxic for the haemocytes of insect larvae. It is probable that insects of some species are killed by exotoxin activity whereas others are more susceptible to the endotoxins or nematode toxins.

Protease is considered as one of the major toxic factors contributing to the pathogenicity of the nematode/bacteria complex [8]. It might play a role both in inactivation of the insects's defense systems and in the digestion of the insect. Recently, Ryu *et al.* [9] purified and characterized an extracellular protease produced by the primary form of *Xenorhabdus nematophilus* cultured *in vitro* medium The protease activity increased in parallel with cell growth then decreased significantly for the extended culture period of the bacteria. However, it was observed that the culture supernatant of the bacteria was still highly toxic and killed the host insect larva within 30 h when injected into the third instar larva under the conditions of low protease activity. Bowen *et al* [10] also reported several toxins lacking protease activity purified from the culture supernatant of *Photorhabdus luminescens* W14 They purified a few toxin complexes with high molecular weights, which consisted of a series of four native complexes encoded with four toxin complex loci. Smigielski and Akhurst [11] identified a new class of toxins from the *Xenorhabdus nematophilus* A24, and a DNA fragment encoding one of these toxins was isolated and characterized by sequencing The toxin gene consisted of an 834 base pair open reading frame, which translates into a 278 amino acid protein. In this work, we report a new extracelluar toxin produced by *Xenorhabdus nematophilus,* which was isolated from *Steinernema glaseri.*

MATERIALS AND METHODS

Organisms and Culture Conditions

An insect-pathogenic bacterium, *Xenorhabdus nematophilus,* isolated from *Steinernema glaseri* was used for this study [12]. The culture was maintained at 25°C on NBTA agar medium in which the primary and secondary forms of the symbiotic bacterium can be distinguished by green and dark red color, respectively, depending on the adsorption of bromothymol blue. The NBTA medium contained 20 g nutrient agar (Difco), 25 mg bromothymol blue (Sigma), and 40 mg triphenyltetrazolium (Sigma) in 1 L distilled water The primary form of the bacterium in green color on an agar plate was selectively transferred to a 250-mL flask containing 100 mL liquid medium. The flask was incubated at 200 rpm in a rotary shaker at 28 °C for 24 h. Subsequently, the cultured medium was inoculated into 4 L medium in a 7-L jar fermentor containing 200 g yeast extract (Merck), 20 g NaCl, 2 g K_2HPO_4, 2 g $NH_4H_2PO_4$, and 0.8 g $MgSO_4 \cdot 7H_2O$. The main culture was kept at 28 °C for 24 h in an agitation of 500 rpm with an air flow rate of 0.8 L/min.

Purification of Toxin

The culture broth was centrifuged at 6,000 rpm for 30 min at 4 °C to remove the cells. The supernatant was concentrated by ultrafiltration with a PM-10 membrane filter of molecular-weight-cutoff of 10,000 (Amicon), then applied to an anion exchange column which was packed with DEAE Sephadex A-50 (Pharmacia). The column was eluted stepwisely with buffers (pH 8.1) containing 10 mM Tris-HCl and various concentractions of KCl The samples resulting from each step were collected and frozen at –20 °C for toxicity assay. Fractions containing toxins were pooled and concentrated by ultrafiltration with an Amicon 10,000-molecular-weight-cutoff filter, then the concentrated sample was applied to HPLC with a gel filtration column (Progel-TSK G3000SWXL). The HPLC column was eluted with a buffer (0.3 M KCl, 0.05 M KH_2PO_4, pH 6 9) at the flow rate of 0.2 mL/min. A peak containing the toxin was collected and desalted by a Sephadex G-25 column (Sigma) The desalted sample was freeze-dried for further analysis All purification steps were performed at 4°C.

Electrophoresis

Reducing SDS-PAGE was performed according to the Laemmli's method [12]. A slab gel consisting of a stacking gel (3.8% acrylamide) and a resolving gel (12.5% acrylamide) were used. Electrophoresis was performed at a constant current of 20 mA for about 1 h. After electrophoresis was completed, the gel was removed and washed with gentle agitation for 2 h in 2.5% Triton X-100 to remove SDS. The gel was stained overnight in methanol-acetic acid-water (40 7 53 by volume) containing 0 025% Coomassie briliant blue R-250, then destained in methanol-acetic acid-water (7 5.5.87.5 by volume).

FTIR-spectroscopy

The freeze dried purified toxin was mixed evenly with KBr. The mixture was palletized then applied to FTIR-spectroscopy (model:ATI Mattson Genesis Series FTIR™). Data were collected in the wavenumbers from 400 to 4,000 cm^{-1}.

Insect Toxicity Test

Insect toxicity of the sample solutions during the purification procedure was examined using 3rd instar *Galleria mellonella* larvae as described by Park *et al.* [14]. 3 μL of the sample solution was injected into the hemolymph of the larva using a microsyringe. For each sample, four or five *Galleria mellonella* larvae were used. After injection, each insect was placed on a 9 cm filter paper in an individual petri dish (100 by 15 mm), incubated at 25°C and observed for mortality for 3 days Controls consisted of 5 larvae injected with 3 μL of sterile buffer (pH 8.1) containing 10 mM Tris-HCl and 1 M KCl, 5 larvae pierced with a needle alone, and 5 uninjected. For oral toxicity test, the cells were separated from culture broth by centrifugation at 6,000 rpm for 30 min at 4°C. The toxicity of *X nematophilus* to *G. mellonella* was assessed by applying different doses of cell pellets to 20 g of artificial diet. The weight and life cycle of surviving *G. mellonella* larvae exposed to diet

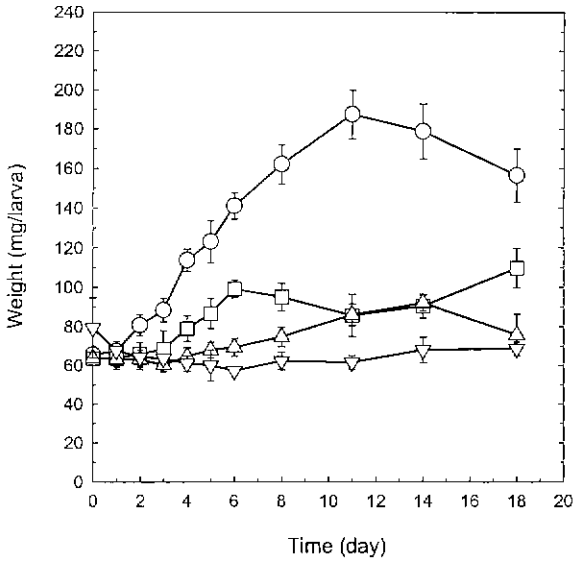

Fig. 1 Oral toxicity of *Xenorhabdus nematophilus* isolated from *Steinernema glaseri* to *Galleria mellonella* Dose (mg/ 20 g feed), ○, control; □, 72; △, 144; ▽,720.

treated with the cell pellets were measured at various incubation times at 25°C

RESULTS AND DISCUSSION

To investigate the cause of toxicity of entomopathogenic nematode to insect pests, we isolated an insect-pathogenic bacterium, *Xenorhabdus nematophilus*, from *Steinernema glaseri*. As shown in Fig. 1, *Xenorhabdus nematophilus* isolated was orally toxic to *G. mellonella*. At doses as low as 72 mg pellets/20 g feed after 6 days, larvae gained only 50% of the growth weight of the untreated controls The artificial diet containing the higher doses of *X. nematophilus* caused severe growth retardation in the surviving larvae. Fig. 2 shows changes in the mortality data and life cycle of *G. mellonella* in a diet treated with *X. nematophilus*. Clearly, *X. nematophilus* was responsible for causing abnormal life cycle of *G. mellonella*, at the dose of 144 mg pellets/20 g feed, 50% of the larvae was found to be dead after 30 days.

To examine toxic substances produced by *X. nematophilus*, the cells were cultivated in a 7-L jar fermentor. The production of insecticidal toxins by *X. nematohpilus* was confirmed by measuring the toxicity of the supernatant of culture broth of the bacteria after centrifugation 3 μL of culture supernatant was sufficient to kill an insect larva within 30 h, suggesting that major toxic substances come from the extracellular products secreted by *X. nematophilus*. Thus, the culture supernatant was further concentrated by ultrafiltration by a factor of 10. Twenty mL of the concentrated supernatant was diluted to 100 mL with a buffer solution (10 mM Tris-HCl, pH 8.1) then applied to an anion-exchange chromatography column (2.6 × 30 cm) packed with DEAE Sephadex A-50 The column was eluted

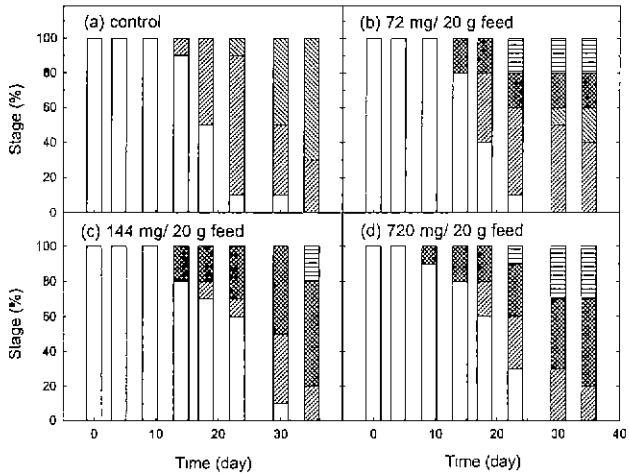

Fig. 2. Life cycle of *Galleria mellonella* larva in a diet treated with *Xenorhabdus nematophilus* isolated from *Steinernema glaseri*, □, larva; ▨, pupa; ▧, moth, ▩, dead larva; ▤, dead pupa.

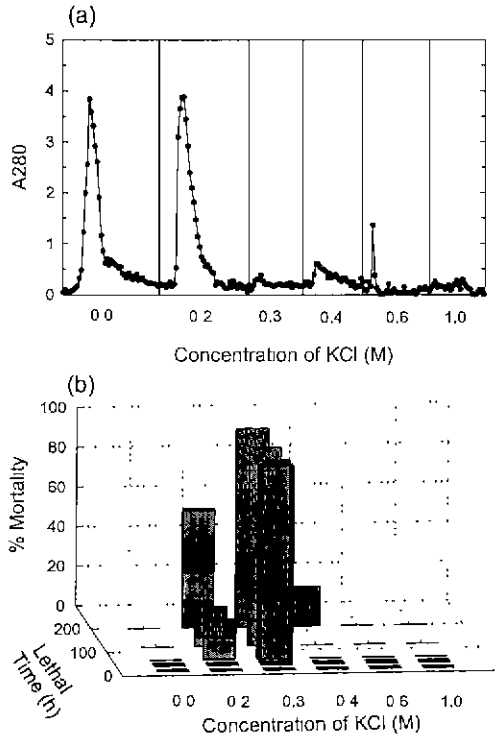

Fig. 3. First anion-exchange chromatography of the culture supernatant of *X. nematophilus* on a DEAE Sephadex A-50 column (a) Stepwise elution pattern by 0 0, 0.2, 0 3, 0 4, 0.6, and 1 0 M KCl buffers, (b) Insecticidal toxicity of the combined fractions corresponding to each KCl concentration.

eluted step-wisely using buffers of increasing concentrations of 0, 0.2, 0.3, 0.4, 0 6, and 1.0 M KCl (first anion-exchange chromatography). Fig. 3(a) shows the elution profile from the column as measured by the absorbance of each fraction at 280 nm Fractions corresponding to each KCl concentration were collected, concentrated by ultrafiltration to 20 mL, then tested for

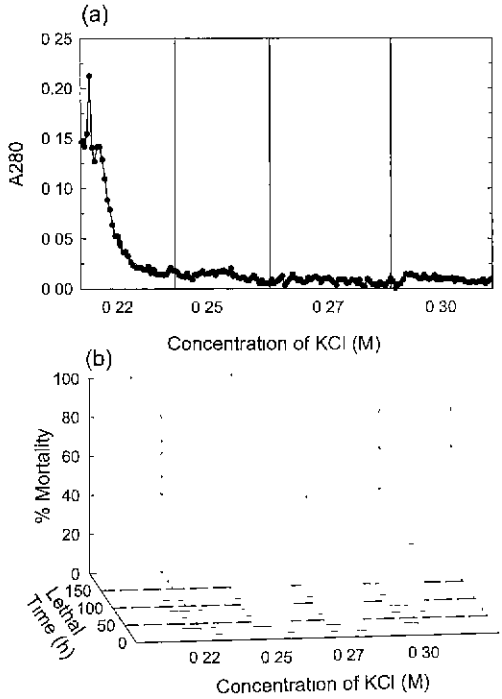

Fig 4. Second anion-exchange chromatography of the culture supernatant of *X nematophilus* on a DEAE Sephadex A-50 column (a) Stepwise elution pattern by 0 22, 0 25, 0 27, and 0 30 M KCl buffers The column was initially washed with 0.2 M KCl buffer, (b) Insecticidal toxicity of combined fractions corresponding to each KCl concentration

insect toxicity. As shown in Fig. 3(b), the concentrated sample solution eluted with 0.2-0.3 M KCl buffer revealed that the highest toxicity against *G. mellonella* larva while others including the controls exhibited negligible toxicity In particular, the major peaks shown in Fig 3(a) did not show any toxicity. In order to find the sharp KCl concentration range from the anion-exchange column, the DEAE A-50 column loaded with the concentrated culture supernatant as described above was washed with 0.2 M KCl buffer. Then, the column was eluted stepwise with buffers containing 0.22, 0.25, 0.27, and 0 3 M KCl (second anion-exchange chromatography). Fig. 4(a) shows the elution profile of the second anion-exchange chromatography Fractions corresponding to each KCl concentration were again pooled, concentrated by ultrafiltration to 20 mL, then tested for toxicity. As shown in Fig. 4(b), the concentrated sample solution eluted with 0 25 M KCl buffer was the most pathogenic to the *G. mellonella* larvae, although the sample solutions eluted with 0.27 and 0.30 M KCl buffer showed about 50% and 25% mortality, respectively. For further purification of the toxin, the concentrated fractions obtained by the second anion-exchange chromatography (fractions eluted with 0 25 M KCl buffer) were applied to HPLC with a gel filtration column. Figs 5(a) and (b) show the HPLC chromatogram and insect toxicity of each fraction The peak at an elution time of 47 min on Fig. 5(a) shows the highest toxicity Finally, the purified toxin with an elution time of 47 min was investigated by FTIR-spectroscopy and SDS-PAGE. Fig. 6 shows the FTIR-spectroscopy of the

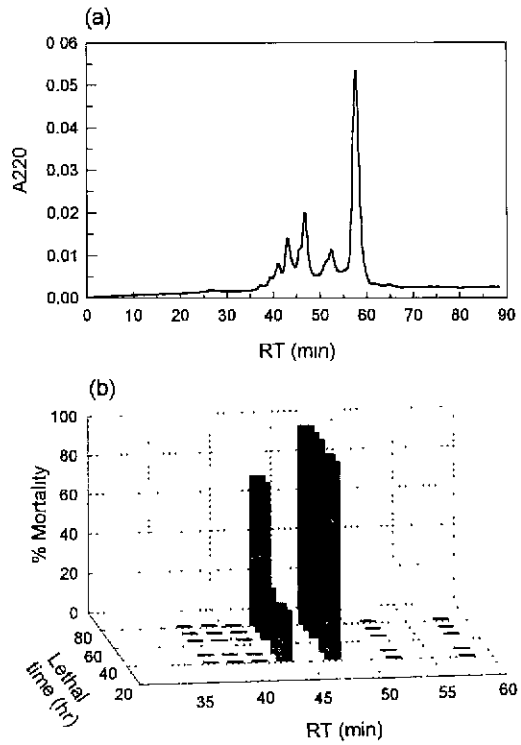

Fig. 5. HPLC of the insecticidal toxin produced by *X. nematophilus* using a gel filtration column. (a) Elution profile at 220 nm, (b) Insecticidal toxicity of each fraction

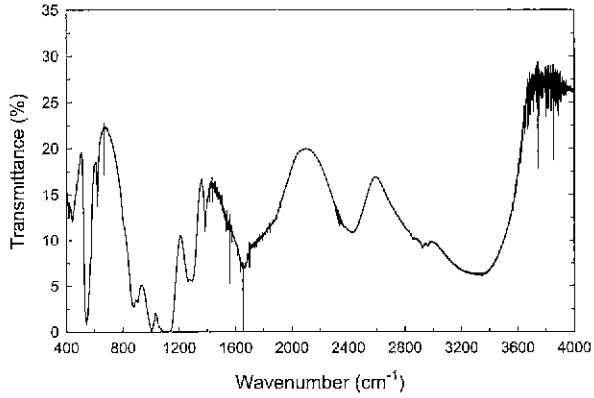

Fig. 6. Fourier-transformed infrared spectroscopy of the purified toxin showing the characteristics of C=O stretching peak near 1650 cm^{-1}

purified toxin, rerevealing that the toxin material could be a new protein exhibiting the characteristics of C=O stretching peak near 1650 cm^{-1}. The amino acid sequence of its N-terminal region was also analyzed by the Edman degra-dation procedure with a pulsed liquid phase sequencer (data not shown). However, it was unsuccessful, suggesting that the N-terminal of this toxin may be blocked. The partial digestion of the toxin and their sequence alignments are under investigation. Fig. 7 shows the reducing SDS-PAGE of the purified toxin, revealing that the purified toxin is a molecule with an approximate molecular weight of 39 kDa The

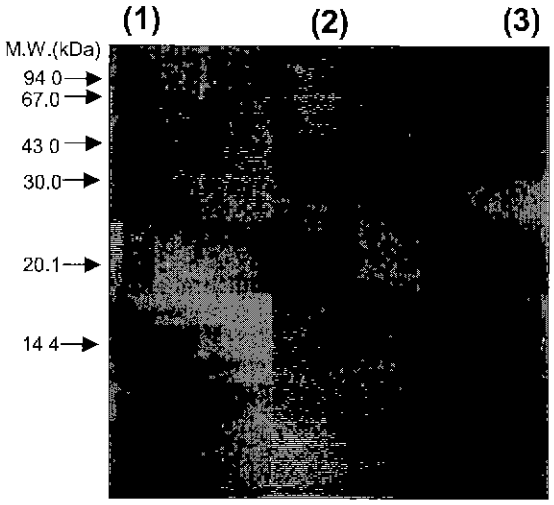

Fig. 7. SDS-polyacrylamide gel electrophoresis of the purified toxin. Lane 1: molecular weight standards (phosphorylase b, 94,000, albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400). Lane 2: purified toxin Lane 3 horseradish peroxidase (Type II from Sigma) of a molecular weight of 44,000 as a marker

molecular mass is different from those of the toxins purified by Bowen *et al.* [10]. They purified toxins from *Photorhabdus luminescens*, another symbiotic bacterium of entomopathogenic nematodes; these toxins were at least four protein complexes with high molecular weights between 30,000 to 200,000 Therefore, the toxin we purified from the culture broth of *X. nematophilus* could be a much simple toxin which can be a potential alternative to the development of biological pesticides or transgenic plants expressing insecticidal toxins. Further studies are needed on characterization of the peptide segment and to clarify cloning the gene encoding this toxin.

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