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Purification and Characterization of a High-Molecular-Weight Insecticidal Protein Complex Produced by the Entomopathogenic Bacterium *Photorhabdus luminescens*

DAVID J. BOWEN† AND JERALD C. ENSIGN*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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***Photorhabdus luminescens* is a gram-negative enteric bacterium that is found in association with entomopathogenic nematodes of the family *Heterorhabditidae*. The nematodes infect a variety of soil-dwelling insects. Upon entering an insect host, the nematode releases *P. luminescens* cells from its intestinal tract, and the bacteria quickly establish a lethal septicemia. When grown in peptone broth, in the absence of the nematodes, the bacteria produce a protein toxin complex that is lethal when fed to, or injected into the hemolymph of, *Manduca sexta* larvae and several other insect species. The toxin purified as a protein complex which has an estimated molecular weight of 1,000,000 and contains no protease, phospholipase, or hemolytic activity and only a trace of lipase activity. The purified toxin possesses insecticidal activity whether injected or given orally. Analyses of the denatured complex by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed it to be composed of several protein subunits ranging in size from 30 to 200 kDa. The complex was further separated by native gel electrophoresis into three components, two of which retained insecticidal activity. The purified native toxin complex was found to be active in nanogram concentrations against insects representing four orders of the class *Insecta*.**

Photorhabdus luminescens is an entomopathogenic, bioluminescent, gram-negative bacterium related, as determined by its biochemical properties (36) and by 16S RNA analyses, to the *Enterobacteriaceae* in the gamma subdivision of the purple bacteria (15, 35). This bacterium was first isolated from a light-emitting insect cadaver infected by entomogenous nematodes (22, 30). Infective juvenile (IJ)-stage nematodes of the family *Heterorhabditae* carry this bacterium in their intestinal tracts (16, 23, 32). The IJ nematodes, enclosed in an outer cuticle layer, do not feed but actively seek out insect prey in the soil. When the IJ nematode encounters a potential victim, it penetrates the outer cuticle of the insect and, upon entering the hemolymph, releases the *P. luminescens* cells from its intestine. The bacteria multiply rapidly, killing the insect within 1 to 2 days. During this time, as the bacterial population reaches a high level, the insect cadaver becomes red in color and visibly bioluminescent. Antibiotics with antifungal and antibacterial activities are produced by *P. luminescens*, and it is thought that these substances prevent invasion of the cadaver by other microorganisms, resulting in ideal conditions for growth and reproduction of the nematode (2, 29). The nematodes feed on the bacteria and dead insect tissue, developing into mature reproductive adults. The nematodes complete several reproductive cycles over a 10- to 20-day period. Then, presumably sensing impending nutrient depletion, the nematodes differentiate into the IJ developmental stage and migrate from the cadaver in search of new insect victims. Detailed descriptions of aspects of *P. luminescens* taxonomy and physiology and the events involved in insect pathogenesis of the bacteria and nematodes are contained in two recent reviews (17, 18).

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin, Madison, WI 53706. Phone: (608) 262-7877. Fax: (608) 262-9865. E-mail: jcensign@facstaff.wisc.edu.

† Present address: Department of Entomology, University of Wisconsin, Madison, WI 53706.

During growth in the insect cadaver or in various complex growth media, the *P. luminescens* cells produce intracellular phase-bright protein inclusion bodies. The inclusions were purified and found to be composed of two distinctive low-molecular-weight proteins (7). These proteins do not possess insecticidal activity, and their functions are unknown. The genes coding for the two proteins were cloned and analyzed (4). The genes do not show a significant degree of homology to any previously described gene.

The 50% lethal dose of *P. luminescens* injected into *Galleria mellonella* larvae was reported as being less than five cells (27). Injection of cells from a 24-h culture of the bacterium used in this investigation, *P. luminescens* W-14, into *Manduca sexta* larvae showed a 50% lethal dose of one cell (unpublished observation). This suggests that *P. luminescens* is an amazingly effective insect pathogen, able to resist completely the antibacterial defense mechanisms of the insect. Several species of the closely related bacterial genus *Xenorhabdus* (6) are also lethal to insect larvae when injected at these low levels (17). *Xenorhabdus* spp. are nematode-borne entomopathogens specifically associated with a different family of nematodes, the *Steinernematidae* (36).

Little is known about the mechanisms by which *Xenorhabdus* and *Photorhabdus* spp. are virulent for insects. Surface components of *P. luminescens* (12) and *Xenorhabdus nematophilus* (13) were shown to be implicated in the evasion of hemocytes in insect hemolymph by these bacteria. Lipid A isolated from *X. nematophilus* cell walls was lethal upon injection into *G. mellonella* larvae (13). Jarosz et al. (21) reported that extracts of homogenates of *G. mellonella* larvae that had been infected with the nematode *Steinernema feltiae* containing *X. nematophilus* cells and also with the nematode *Heterorhabditis bacteriophora* containing *P. luminescens* cells were lethal when injected into larvae of the insect. This publication also reported that injection of samples of cell-free broth cultures of *P. luminescens*, but not *X. nematophilus*, was lethal to the insects. The infected-insect extracts and broth culture samples had no ad-

verse effects when orally supplied to the *G. mellonella* larvae. The toxic activity of the samples was heat labile, and they contained protease activity.

Schmidt et al. (33) purified an alkaline metalloprotease from culture broths of *P. luminescens* and suggested that the protease might be involved in the virulence of the organism. No evidence supporting this idea was presented. Yamanaka et al. (40) reported injectable insecticidal activity for *Spodoptera litura* larvae in culture broths of two of four species of the genus *Xenorhabdus* and a low level of toxicity in one of three *Photorhabdus* strains. All of the extracts of both genera contained high levels of protease activity, leading the authors to conclude that proteases are not involved in virulence.

A lipase activity purified from broth cultures of *Photorhabdus* sp. strain K122 showed toxicity for larvae of *G. mellonella* (8). Culture broths of *Escherichia coli* that had been transformed with the plasmid-borne lipase gene acquired injectable toxicity for the insect larvae. The lipase was not reported to be toxic when administered orally. Involvement of lipase activity in the virulence of *X. nematophilus* is also suggested by a report that the autoinducer molecule of bioluminescence in *Vibrio harveyi*, *n*-β-hydroxybutanoyl homoserine lactone, increased lipase activity in avirulent mutants of *X. nematophilus* coincident with restoration of virulence for *G. mellonella* larvae (11).

In avirulent mutants of *X. nematophilus* obtained by Tn*5* mutagenesis, no relationship between virulence and lecithinase, lipase, or hemolysin activity was evident (39). A later study of Tn*5*-derived avirulent mutants of the same bacterium revealed no relationship between extracellular enzyme activities and insect virulence (14). There was also no difference in removal of the virulent and avirulent mutants by *G. mellonella* hemocytes.

This report describes the purification and characterization of a high-molecular-weight protein complex produced by *P. luminescens* during growth in peptone broth. The purified protein complex, which has no protease activity and negligible lipase activity, is lethal when injected into or fed to a variety of insect larvae.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The *P. luminescens* strains used in this investigation were W-14 (ATCC 55397; isolated from South Florida garden soil during this study), Hm (obtained from G. Poinar, University of California, Berkeley), NC-19 (obtained from W. Brooks, University of North Carolina), and Hb (isolated from Hb nematodes purchased from Gardens Alive, Lawrenceburg, Ind.). Stocks were maintained on petri plates containing 2% Proteose Peptone no. 3 (PP3) and 1.5% agar (Difco Laboratories, Detroit, Mich.). The cultures were incubated at 28°C for 4 days and then stored at room temperature for up to 1 month. Primary-form colonies, selected on the basis of colony morphology, bioluminescence, pigmentation, and inclusion protein production, were inoculated into 1-liter flasks containing 200 ml of 2% PP3 broth supplemented with 0.5% polyoxyethylenesorbitan monostearate (Tween 60; Sigma Chemical Co., St. Louis, Mo.). The cultures were incubated for 48 h at 30°C on a rotary shaker at 250 rpm.

Insect rearing. *M. sexta* larvae were reared from eggs (Carolina Biological Supply Co., Burlington, N.C.) or supplied by N. Beckage or W. Goodman, Department of Entomology, University of Wisconsin, Madison. Larvae were reared using a 16-h light–8-h darkness photoperiod at 25°C and fed a gypsy moth wheat germ diet (ICN Pharmaceuticals Inc., Costa Mesa, Calif.). *G. mellonella* larvae (obtained from H. Coppel, Department of Entomology, University of Wisconsin, Madison) were reared by the procedure of Mohamed and Coppel (28). German and American cockroaches, obtained from S. C. Johnson Wax Co., Racine, Wis., were reared on dry rat chow (Ralston Purina, St. Louis, Mo.) and tap water. Pharaoh ants, also obtained from S. C. Johnson Wax, were reared on 10% sucrose, tap water, and dead frozen houseflies. A colony of meal worms (*Tenebrio* sp.) was maintained in our laboratory on a diet of dry oatmeal, apples, carrots, potato slices, granola, and tap water.

Toxin assays. Test fractions were filter sterilized with a 25-mm-diameter, 0.2-μm-pore-size cellulose acetate syringe filter (Nalgene-Nalge Co., Rochester, N.Y.). For determination of the activity of toxins administered orally to *M. sexta* larvae, various samples, consisting of 20 to 100 μl of solution, were applied to the surfaces of 1-cm^3 blocks of gypsy moth wheat germ diet. Bioassays for other insects that were administered toxins orally were performed with a high-performance liquid chromatography (HPLC)-purified toxin fraction in 100 mM KPO_4 buffer, pH 6.9, at a concentration of 2 mg of total protein/ml. Cockroaches were each fed a 1-cm^3 block of gypsy moth wheat germ diet to which 100 μl of toxin solution had been applied. The wax moth larvae were fed a diet of dry food to which 100 μl of toxin solution had been added/g. Mealworms were fed several rolled oats which had been moistened with the toxin solution (approximately 10 μl/oat). Pharaoh ants were fed the toxin solution in a 10% sucrose solution. The intrahemocoelic injections of 5-μl toxin samples were performed through the first proleg of fourth- or fifth-instar *M. sexta*, wax moth, or mealworm larvae. The intrahemocoelic injection of 5-μl toxin samples into the cockroaches was done through the abdomen. A 25-μl gas-tight syringe (Hamilton Co., Reno, Nev.) equipped with a 30-gauge, no. 4-point needle was used for all injections. Different syringes were used for injections of live bacteria and filtered samples. Between injections, each syringe was rinsed three times with sterile deionized-distilled H_2O (ddH_2O), three times with 70% ethanol, and finally three times again with sterile ddH_2O. Each larva was examined for weight, cessation of growth, and death at 24-h intervals for up to 7 days.

Preparation of concentrated culture supernatants. Cultures of the *P. luminescens* strains grown for 48 h in 2% PP3 plus 0.5% Tween 60 at 30°C were centrifuged at 10,000 × *g* for 20 min. A sample of the supernatant fluid of each culture was filter sterilized and centrifuged for 4 h at 500 × *g* and 4°C in a Centriprep 100 ultrafiltration device with a molecular mass cutoff of 100 kDa or a Centricon 30 apparatus with a molecular mass cutoff of 30 kDa (Amicon Inc., Beverly, Mass.). An equal volume of sterile 100 mM Tris-HCl, pH 7.0, was added to the retentate chamber of the Centriprep tube; this was followed by a 1-h centrifugation as described above. The high-molecular-weight material retained by the filters was removed with a pipette and tested for toxicity to *M. sexta* larvae by oral administration or injection of samples.

Column chromatography. All buffers and stock solutions for column chromatography were filtered through 0.2-μm-pore-size filters and autoclaved before use. Two liters of a 48-h culture grown in 2% PP3 plus 0.5% Tween 60 was centrifuged at 10,000 × *g* for 20 min. The supernatant fraction was adjusted to 50 mM K_2HPO_4 with 1.0 M K_2HPO_4, and the pH was adjusted to 8.6 by the addition of a small amount of 5.0 M KOH. The supernatant was then mixed with a 250-ml slurry of DEAE-Sephacel (Pharmacia Biotech, Uppsala, Sweden) which had been autoclaved as suggested by the manufacturer and equilibrated with 50 mM K_2HPO_4, with no pH adjustment. The toxin activity was adsorbed to the DEAE resin under these conditions. This mixture was poured into a 2.6- by 40-cm column and then washed with 500 ml of 50 mM K_2HPO_4. The column was next washed with 150 mM KCl prepared in ddH_2O, pH 6.3, and applied at a flow rate of 30 ml/h at room temperature until the effluent had a steady baseline absorbance at 280 nm. The column was then eluted with 300 mM KCl prepared in ddH_2O, pH 6.3. Fractions were collected and tested for insecticidal activity and for the presence of a high-molecular-weight protein complex by HPLC molecular sieving (see below). Fractions containing toxic activity were pooled, filtered through a 0.2-μm-pore-size filter, and concentrated in a Centriprep 100 device.

The retentate was washed by several centrifugations with 100 mM KPO_4 buffer, pH 6.9. The protein content of the retentate fraction was finally adjusted to a concentration of 1 to 2 mg/ml. A 3-ml sample of the concentrated material was applied to a Sephacryl S-400 HR (Pharmacia Biotech) gel filtration column (2.6- by 95-cm bed). The column was washed with eluent buffer (100 mM KPO_4, pH 6.9) at a flow rate of 17 ml/h at 4°C. Fractions containing toxic activity were pooled and concentrated in a Centriprep 100 device to a final protein concentration of 2 mg/ml.

HPLC. HPLC was performed with a 0.75- by 60-cm TSK-GEL G-4000 SW molecular sieve column (Toso Hass, Montgomeryville, Pa.). The column was eluted with 100 mM KPO_4 (pH 6.9) at a flow rate of 0.4 ml/min at room temperature. The sample loading volume was 250 μl. The eluent absorbance at 218 and 280 nm was routinely monitored. The column was calibrated with proteins of known molecular mass which were dissolved at 2 mg/ml in 100 mM KPO_4, pH 6.9. The protein standards (Pharmacia Biotech) were thyroglobin (669 kDa), aldolase (158 kDa), and ovalbumin (42.7 kDa).

Determination of protein concentrations. Protein concentrations were determined with bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.), using bovine serum albumin as a concentration standard. The protein concentration of HPLC-purified toxin was estimated both by the bicinchoninic acid method and by comparison of the integrated peak areas (absorbance at 280 nm) of the toxin samples with integrated peak areas of standard proteins at a known concentration (thyroglobin, aldolase, ovalbumin, and bovine serum albumin, each at 2 mg/ml).

Electrophoresis. Nondenaturing native agarose gel electrophoresis with high-resolution Metaphor agarose (FMC BioProducts, Rockland, Maine) was performed by the method of Dean et al. (9). The 15-cm by 12-cm by 4-mm-thick 1.9% agarose resolving gel, buffered with 200 mM Tris-borate (pH 8.3), was allowed to solidify at 4°C. A 5-cm section was cut from one end of the resolving gel and replaced with the 1.5% agarose stacking gel, buffered with 100 mM Tris-HCl (pH 7.0) and containing 0.05% phenol red as a tracking dye. The stacking gel was allowed to solidify at 4°C for 30 min. Protein samples, buffered in 50 mM Tris-HCl (pH 7.0), were then loaded into wells at the cathode end of the gel. The cathode buffer was 0.025 M Tris–0.192 M glycine, and the anode

buffer was 1.0 M Tris-HCl, pH 8.3. The gel was electrophoresed horizontally at 6.5 mA with a constant current on a Peltier-cooled platform at 15°C until the tracking dye reached the anode end of the gel.

To recover the native proteins, 5-mm-wide strips were cut from the edges of the gels and protein bands in the strips were visualized by staining for 5 to 10 min with 0.1% Coomassie brilliant blue in a solution consisting of 60% ddH_2O, 30% methanol, and 10% acetic acid followed by washing with a solution containing 60% ddH_2O, 30% methanol, and 10% acetic acid until the protein bands became visible (5 to 15 min). The strips were then placed alongside the gel, and areas corresponding to the protein bands were precisely excised.

Agarose gel electrophoresis was also performed with agarose gels in the presence of Triton X-100 (Sigma Chemical Co.). This procedure was the same as described above except that the gel buffers and electrode buffers contained 0.1% Triton X-100 and the sample buffer contained 1% Triton X-100 (10). The protein samples were incubated in the sample buffer at room temperature for 4 h before being applied to the gel.

Samples separated by column chromatography, native agarose gel electrophoresis, or Triton-agarose gel electrophoresis were analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (19) (see Fig. 2A and E) or by a protocol (37) designed for high resolution of proteins in the 5- to 30-kDa range, involving the use of an 18% polyacrylamide gel (see Fig. 2C). Proteins were stained with Coomassie brilliant blue R-250 or silver stained (silver stain kit; Stratagene, La Jolla, Calif.) in accordance with the manufacturer's instructions.

Analysis of native-gel fractions for insecticidal activity. Proteins separated in native gels were either used directly or eluted into buffer by incubation of macerated sections for 16 h at 30°C in an equal volume of sterile 50 mM Tris-HCl, pH 7.0, in a microcentrifuge tube. Following removal of gel pieces by centrifugation, the buffer (containing the eluted proteins) was removed with a pipette.

For SDS-PAGE analysis, the eluted protein samples or macerated gel pieces were mixed with SDS sample loading buffer, boiled for 5 min, and loaded directly into wells while the agarose was still molten. For oral toxicity analyses, the eluted protein samples or macerated gel pieces were applied to the surface of the insect diet which was fed to *M. sexta* larvae.

Enzymatic activity assays. HPLC-purified toxin was assayed for the presence of proteolytic activity (38), type C phospholipase activity (25), and nuclease activity (24) in accordance with published procedures. Lipase was assayed by cutting wells in Tween agar (34) and filling the wells with toxin samples. A positive result was a zone of precipitation in the agar around the well. Lipase activity was also determined by a colorimetric assay (8). Hemolysin was assayed by cutting wells in sheep erythrocyte blood agar and filling the wells with toxins samples. A positive reaction was a zone of hemolysis (clearing of the erythrocytes) in the agar around the well.

RESULTS

Discovery of insecticidal activity. Approximately 100 cells of *P. luminescens* NC-19, Hm, or W-14 were injected into each of 10 fourth-instar *M. sexta* larvae. This number of bacteria was lethal to the larvae within 24 h. At 48 h, the larvae were brick red in color and bioluminescent, and microscopic examination of a sample of hemolymph from each insect revealed large numbers of *P. luminescens* cells. These cells were easily recognized by the presence of phase-bright intracellular protein inclusion proteins. The moribund larvae were then ground in a tissue grinder in 2.5 ml of sterile saline. The homogenates were then centrifuged, the supernatant fractions were passed through 0.2-μm-pore-size filters, and a 5-μl sample of filtrate was injected into each of 10 insect larvae. All of the insect larvae were dead 24 h later. A similarly prepared homogenate of an insect larva that had not been injected with the bacteria did not kill or inhibit growth following injection into control larvae. The three strains of *P. luminescens* had produced a toxic material during growth in the larvae.

An experiment was designed to determine if the bacteria produced insecticidal activity when grown in a culture medium. The three *P. luminescens* strains were grown for 48 h in PP3 broth. The cultures were then filter sterilized, and 5-μl samples of each were injected into 10 fourth-instar *M. sexta* larvae. All of the insects ceased feeding 24 h later, and more than one-third of the larvae injected with each sample were dead at 72 h. The results of testing the toxicity of PP3 culture broths by injection into *M. sexta* larvae are shown in Table 1. Detectable toxin appeared in the three cultures at 24 h, which coincides with the late exponential-early stationary growth phase. The maximum level of toxicity was reached at 48 to 72 h. It appeared that strains Hm and W-14 were equally potent and more potent than NC-19. The insecticidal activities of NC-19 and W-14 were heat labile. The activities of NC-19 and Hm were retained by a 30-kDa molecular size separation filter (Centricon 30). In later experiments, the activities of W-14, Hm, and NC-19, administered orally or by injection, were found to be retained by the 100-kDa filters (data not shown). These data suggest that the insecticidal activities of the three organisms are attributable to a large protein or proteins.

TABLE 1. Summary of effects of *P. luminescens* cell-free[a] culture broth injected into newly molted fourth-instar *M. sexta* larvae

Strain	Sample[b]	% Mortality
NC-19	19 h	0
	24 h	50
	48 h	33
	72 h	67
	72 h, heated at 60°C for 15 min	0
Hm	19 h	0
	24 h	83
	48 h	100
	72 h	92
	72 h, heated at 60°C for 15 min	50
	>30-kDa fraction	100
	<30-kDa fraction	16
W-14	48 h	100
Control	2% PP3 broth	0

[a] A 2-μm-pore-size filter was employed.
[b] Each larva was injected with 5 μl of sample broth; $n = 12$ larvae for each sample group.

The toxicity of each of the *P. luminescens* strains upon oral administration was tested by feeding fourth-instar *M. sexta* larvae an insect diet to which concentrated culture broth had been added. A 50-μl sample of each of the filter-sterilized PP3-Tween culture broths, concentrated with Centriprep 100 ultrafiltration devices with a 100-kDa-cutoff separation membrane, was applied to the surface of the insect's diet. Larval growth was noticeably inhibited by some culture broths, but no death was observed other than in W-14 samples. The results of oral toxicity testing of retentate fractions of Centriprep 100-concentrated growth broths of four *P. luminescens* strains are shown in Table 2. All of the concentrated samples showed growth-inhibitory activity, but none (except strain W-14) were lethal doses. The insect larvae were significantly more susceptible to the toxic activity of strain W-14 than to the other test strains, since the 15-fold concentrate retained by the Centriprep 100 device killed 70% of the larvae. Samples of the 15-fold-concentrated broth that had passed through the 100-kDa-cutoff membrane filter inhibited growth, but no lethal activity was observed. Insect larvae receiving growth-inhibitory but sublethal doses of toxin tended to cease feeding, and their reduced frass output was darker than that of the control larvae. The decision to direct continuing efforts to the most potent of the toxins produced by strain W-14 was made.

Purification of insecticidal toxin. Advantage was taken of the apparent large size of the insect-killing activity in broth cultures. Growth liquor from a 48-h PP3-Tween 60 broth culture of strain W-14 was concentrated 15-fold by using a Centriprep 100 device. The concentrate was then applied to a large-pore molecular sieving HPLC column. The column re-

TABLE 2. Toxicity of orally administered concentrated culture broths of *P. luminescens* for newly molted fourth-instar *M. sexta* larvae

Sample[a]	Fold concentrated	% Mortality[b]	Initial wt (g) ± SE	Final wt (g) ± SE	Fold weight gain[b]
Control (PP3-Tween)		0	0.20 ± 0.01	2.00 ± 0.18	10
Strain Hm	15	0	0.22 ± 0.02	0.63 ± 0.04	2.8
Strain Hb	25	0	0.23 ± 0.01	0.57 ± 0.13	2.4
Strain NC-19	15	0	0.19 ± 0.01	1.36 ± 0.06	7.2
Strain W-14	10	0	0.14 ± 0.01	0.22 ± 0.04	1.5
W-14 HPLC toxin (5 μg)		30	0.17 ± 0.01	0.16 ± 0.01	0.9

[a] A 50-μl sample was applied to 1 cm^2 of insect diet; $n = 10$ larvae for each sample group.
[b] At 4 days posttreatment.

solved the concentrated liquor into numerous-size fractions (Fig. 1A). Samples of each fraction were tested for insecticidal activity, and activity was found only in the small peak eluted from the column at approximately 31 to 34 min. This result provided two pieces of information: the insecticidal activity is large, since it elutes early from the column, and the low UV absorbance of these fractions suggests that the toxin is present in very small amounts. To obtain larger quantities of toxin, a DEAE-Sephacel chromatography purification step was developed. Culture broth was mixed batchwise with the DEAE-Sephacel (so that the toxin bound to the resin); the mixture was poured into a column, and the toxic activity was eluted with KCl. This step greatly increased the insecticidal activity present in the sample while reducing other proteins (Fig. 1B). Insect toxicity was found exclusively in the peak eluting at 33.8 min, which had increased in size (absorbance) relative to the other peaks present. The material in this KCl fraction from the DEAE column was then applied to a Sephacryl S-400 column (Fig. 1C). Just one major peak, which contained the insecticidal activity, was eluted. The material in this fraction was next passed through a 4000 SW size exclusion column (Fig. 1D). One sharp peak of protein, which eluted from the column at 33.5 min, contained the toxic activity. Injection or feeding of 5-μl samples of this fraction killed all of 10 *M. sexta* larvae within 48 h (data not shown). Based on the elution times of the standard proteins (Fig. 1E), we estimated the size of the toxic protein molecule to be approximately 1,000 kDa. Calculations based on the amount of protein in the 33.5-min peak fraction (Fig. 1D) indicate that the organisms produce and secrete approximately 2.5 mg of toxin per liter of 72-h culture broth. The toxin activity, purified by the procedure described above, was considered sufficiently pure for further studies of the activity.

Analysis of the purified toxin by SDS-PAGE (Fig. 2A) revealed that the high-molecular-mass complex was composed of three major proteins 55 to 65 kDa in size, several proteins significantly larger than 100 kDa, and one major protein of approximately 31 kDa. The purified toxin was separated by native agarose gel electrophoresis into three fractions (Fig. 2B). The protein in each of these gel bands was eluted from the gels and tested for toxicity. A 5-μl sample of the band 1 fraction killed 60% of the insects when injected into *M. sexta* larvae. Injection of the same quantity of the band 2 fraction was less potent, killing 20% of the larvae, but the survivors were markedly inhibited in growth. The band 3 sample showed no toxicity. The three fractions were tested for toxic activity on the larvae by oral administration. The band 1 fraction did not kill the larvae but significantly inhibited their growth. No killing or growth-inhibitory activity was found for the orally introduced band 2 and 3 samples.

The protein contents of the native-gel-separated fractions were analyzed by SDS-PAGE. Band 1 contained several proteins greater than 212 kDa in size and three distinct proteins in the 60- to 70-kDa size range (Fig. 2C). The diffuse protein bands of <30 kDa may have resulted from protein degradation during the native-gel manipulation. Band 2 contained several

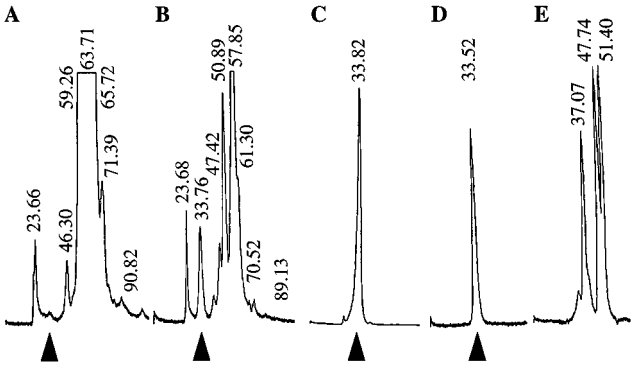

FIG. 1. HPLC chromatograms, from the HPLC 4000 SW molecular sieve column, of the toxic fraction following each step of the purification. (A) Crude culture broth; (B) DEAE-Sephacel; (C) Sephacryl S-400HR; (D) HPLC 4000 SW molecular sieve column; (E) standard proteins (37.07 min, thyroglobin, 669 kDa; 47.74 min, aldolase, 158 kDa; 51.40 min, ovalbumin, 42.7 kDa). The arrows below the chromatograms indicate fractions containing the insecticidal activity. The numbers on the chromatograms indicate the elution times in minutes. Absorbance was monitored at 280 nm.

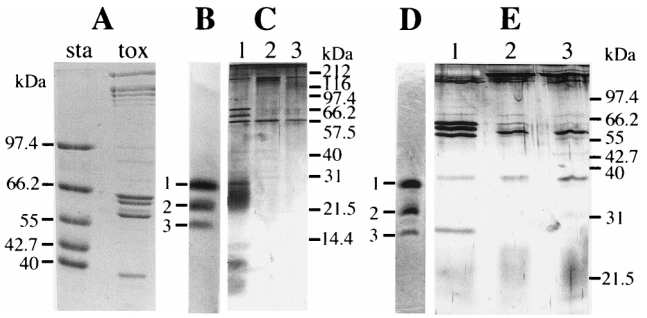

FIG. 2. Analysis of protein components of the insecticidal toxin complex of *P. luminescens*. (A) SDS–10% PAGE analysis of HPLC-purified *P. luminescens* insecticidal toxin preparations. The gel was stained with Coomassie brilliant blue. Sta, size standard; tox, purified toxin sample. (B) HPLC-purified toxin separated by native agarose gel electrophoresis; 5 μg of total protein was loaded. The gel was stained with Coomassie brilliant blue. (C) SDS–18% PAGE analysis of protein bands separated by native agarose gel electrophoresis. Lanes: 1, band 1; 2, band 2; 3, band 3. The gel was silver stained. (D) HPLC-purified toxin separated by agarose gel electrophoresis in the presence of 0.1% Triton X-100; 4 μg of total protein was loaded. The gel was stained with Coomassie brilliant blue. (E) SDS–10% PAGE analysis of protein bands separated in native agarose gels in the presence of 0.1% Triton X-100. Lanes: 1, band 1; 2, band 2; 3, band 3. The positions of molecular mass markers are indicated.

TABLE 3. Toxicities of injected or orally administered HPLC-purified toxin from *P. luminescens* W-14 for fourth-instar *M. sexta* larvae

Sample[a]	% Mortality[b]	Initial wt (g) ± SE	Final wt (g) ± SE	Fold weight gain[b]
Fed[c]				
Control buffer	0	0.23 ± 0.02	6.15 ± 0.48	26.7
0.2 μg	0	0.26 ± 0.02	2.74 ± 0.50	11.0
2.0 μg	0	0.25 ± 0.03	0.68 ± 0.14	2.7
20 μg	75	0.24 ± 0.03	0.20 ± 0.01	0.8
Injected[d]				
Control buffer	0	0.56 ± 0.02	8.02 ± 0.30	14.4
0.05 μg	58	0.52 ± 0.03	1.02 ± 0.07	1.9
0.5 μg	100	NA	NA	NA[e]
5 μg	100[f]	NA	NA	NA

[a] $n = 12$ larvae for each sample group.
[b] At 6 days posttreatment.
[c] Toxin was applied to 1 cm^2 of diet in 50 μl of KPO_4 buffer, pH 6.9.
[d] Toxin was injected in 5 μl of KPO_4 buffer, pH 6.9.
[e] NA, not applicable (all larvae died).
[f] All larvae were dead by 24 h postinjection.

TABLE 4. Enzymatic activities of culture broth versus HPLC-purified *P. luminescens* insecticidal toxin

Activity	Activity of[a]:	
	Culture broth	HPLC-purified toxin
Protease	0.68	0
Lipase		
Ca precipitate	+	−
Colorimetric	5.5	0.2
Hemolysin	+	−
Phospholipase C	+	−
Nuclease	+	−

[a] +, activity present; −, no activity present. Units of activity for protease: change in optical density of an azocasein solution, 2 h; units of activity for lipase (colorimetric): micromoles of *p*-nitrophenol liberated from *p*-nitrophenyl palmitate in 30 min.

proteins of sizes greater than 200 kDa and one major protein of 60 kDa. Band 3 appeared to contain less of the 200-kDa-range proteins but was very similar to band 2. Two proteins, with approximate sizes of 60 and 200 kDa, were present in all three fractions.

We attempted to separate the purified toxin complex by agarose gel electrophoresis in the presence of Triton X-100, a technique used to dissociate membrane-associated proteins (10). The toxin was separated into a three-protein band pattern that was similar to that observed in the native agarose gels, suggesting that the complexes are not associated with membrane fragments (Fig. 2D). SDS-PAGE analysis of each of these bands (Fig. 2E) revealed that each band fraction had a protein profile similar to that resulting from separation on native agarose. These fractions were not tested for insect toxicity.

Properties of the insecticidal toxin. The insecticidal activity appears to be proteinaceous since it is heat labile (Table 1) and large in size. This supposition was confirmed by exposing the purified toxin to proteolytic digestion. Both the injectable and oral toxicities to larvae were completely eliminated by protease K digestion (data not shown).

The potency of the purified toxin was estimated by testing dilutions for activity through both injection and oral administration. As shown in Table 3, injection of 5.0- or 0.5-μg samples into *M. sexta* larvae (12 larvae each) resulted in 100% mortality. Seven of the 12 larvae injected with 0.05 μg of toxin were dead at 144 h, and the average weight of the survivors increased only 12% over that of the controls. Growth of larvae fed 2.0 or 0.20 μg of toxin was reduced; a weight gain of 3.0- or 11.1-fold was determined, compared to the 26.7-fold weight gain of the controls. Oral administration of 20 μg of toxin killed 75% of the larvae, and the survivors lost weight (0.8 times the initial weight).

The results of testing the purified toxin for enzymatic activities are shown in Table 4. The 72-h culture broth contained protease, lipase, phospholipase C, hemolysin, and nuclease activities. The purified toxin contained none of these activities except for a low level of lipase activity, detected by the sensitive colorimetric assay but not by the calcium precipitate assay. A 1:100 dilution of the purified fraction used in these analyses was lethal when injected into *M. sexta* larvae, and this diluted sample showed no lipase activity in the colorimetric assay.

The purified toxin did not lose any activity upon subjection to several freeze-thaw cycles or storage for 3 months at 4°C (data not shown).

The HPLC-purified toxin was tested for activity against six insect species by oral administration and injection, and the results are summarized in Table 5. When administered orally, the toxin was lethal to all of the insects except the American cockroach; when injected, it was lethal to all of them, including the cockroach. We found that injection of toxin into ants was too technically demanding, so this application was not tested.

DISCUSSION

Two families of nematodes, each carrying a specific genus of bacteria, are pathogenic for a wide range of agriculturally important insect pests (for a review, see reference 31). We have discovered that one of these bacteria, *P. luminescens*, secretes into the growth medium a high-molecular-weight protein complex that is lethal when injected into or fed to members of at least five insect genera representing four orders of the class *Insecta*. The significance of our discovery is that it makes feasible the protection of plants by direct application of the toxin or by transformation of the toxin gene or genes into plants as a strategy for the control of insect pests. Attempts to exploit the *Heterorhabditis* and *Steinernema* nematodes in pest control have been made (for reviews, see references 3 and 31). Problems inherent in mass cultivation of nematodes, their stability during transport and storage, and application of the live nematodes make the use of entomopathogenic nematodes for pest control, especially in large-scale agriculture applications, impractical. Research directed to cloning of the *P. luminescens* genes and their transformation into plants, with the goal of obtaining transgenic insect-resistant plants, is now in progress.

It is likely that the virulence of *P. luminescens* for insects is a complex and multifaceted process. It is important to point out that the protein toxin that we purified and characterized shows none of the enzymatic activities, such as lipase, protease, and phospholipase, that others have suggested to be associated with virulence. These enzymes may be virulence factors also, but only the protein complex that we have purified has been shown to be toxic to insects upon oral administration. Work

TABLE 5. Insects killed by *P. luminescens* HPLC-purified toxin

Common name	Order	Genus and species	Method of toxin introduction	
			Oral[a]	Injection[b]
Tobacco hornworm	Lepidoptera	*Manduca sexta*	+	+
Wax moth larva	Lepidoptera	*Galleria mellonella*	+	+
Mealworm	Coleoptera	*Tenebrio molitor*	+	+
Pharaoh ant	Hymenoptera	*Monomorium pharaonis*	+	ND[c]
German cockroach	Dictyoptera	*Blattella germanica*	+	+
American cockroach	Dictyoptera	*Periplaneta americana*	−	+

[a] $n = 10$ insects/group. +, >30% mortality; −, no insect mortality.
[b] $n = 10$ insects/group. +, 100% mortality.
[c] ND, not determined.

now in progress indicates that the *P. luminescens* toxin causes disruption of the midgut epithelium of *M. sexta* larvae (5).

The heat lability and protease susceptibility of the purified toxin complex indicate that its lethality to insects is associated with a protein(s) in the complex. This activity is clearly different from the lipopolysaccharides and lipid A, which were shown to be associated with virulence of *Xenorhabdus* and *Photorhabdus* spp. (12, 13).

Many bacterial toxins, including the insecticidal toxin of *Bacillus thuringiensis*, are known to be composed of multiple protein subunits (20, 26), and many of these toxins require enzymatic processing or activation by the producing cells or cells of the target organism to be fully active. The *Photorhabdus* insecticidal toxin is composed of a complex of proteins of various sizes that separate on native agarose gel electrophoresis into three subcomplexes. The proteins in these complexes may represent various stages of processing of a single large protein, or they may be derived from three or more individual gene products. Our data indicate clearly that the toxic activity is due to one protein or a combination of proteins that were purified as a multimeric complex from the culture supernatant.

Cells of *P. luminescens* are often observed to go through a phase variation process in which variants, termed secondary forms, appear at a high frequency (1, 17). These secondary variants have lost many characteristics of the original primary culture, including bioluminescence, pigmentation, dye reduction activity, protease activity, antibiotic production, and intracellular crystalline proteins. We found the secondary variants to be as virulent as primary cells when injected into *M. sexta* larvae, and the insect toxin was detected in the growth medium (unpublished observations).

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