Both the bacterium *Photorhabdus luminescens* alone and its symbiotic *Photorhabdus*-nematode complex are known to be highly pathogenic to insects. The nature of the insecticidal activity of *Photorhabdus* bacteria was investigated for its potential application as an insect control agent. It was found that in the fermentation broth of *P. luminescens* strain W-14, at least two proteins, toxin A and toxin B, independently contributed to the oral insecticidal activity against Southern corn rootworm. Purified toxin A and toxin B exhibited single bands on native polyacrylamide gel electrophoresis and two peptides of 208 and 63 kDa on SDS-polyacrylamide gel electrophoresis. The native molecular weight of both the toxin A and toxin B was determined to be approximately 560 kDa, suggesting that they are tetrameric. NH2-terminal amino acid sequencing and Western analysis using monospecific antibodies to each toxpin demonstrated that the two toxins were distinct but homologous. The oral potency (LD50) of toxin A and toxin B against Southern corn rootworm larvae was determined to be similar to that observed with highly potent Bt toxins against lepidopteran pests. In addition, it was found that the two peptides present in toxin B could be processed *in vitro* from a 281-kDa protoxin by endogenous *P. luminescens* proteases. Polyololytic processing was shown to enhance insecticidal activity.

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have been used for the biological control of soil dwelling pests that include weevils and lepidopteran species (1–3). Strong et al. (4, 5) have provided the best illustration of entomopathogenic nematode predation in the environment by their documentation of the ecological relationship between *Heterorhabditis hepialus* and the ghost moth caterpillar, *Hepialus californicus*. The mechanism by which the entomopathogenic nematodes are able to predate and reproduce in the host involves a mutualistic relationship between the nematode and its symbiotic bacteria, *Photorhabdus* sp. and *Xenorhabdus* sp. (6, 7). Bovien (8) had postulated an association between a steinernematid species and a bacterium during the 1930’s. However, it was not until 1966 that Poinar (9) reported that a single species of bacterium in the family Entorhabididae was present in the anterior region of the infective nematode of this species. Since then investigators have shown that *Steinernema* species carry bacteria of the genus *Xenorhabdus* while *Heterorhabditis* nematodes harbor species of the genus *Photorhabdus* (10, 11). In 1993, *Photorhabdus* bacteria were proposed by Boemare and colleagues (12) for re-classification as a distinct genus from *Xenorhabdus*, based on a variety of phenotypic, ecological, and molecular studies. Recently, increased support for the separation of these two genera was obtained employing 16S ribosomal DNA analysis (13). *Photorhabdus* is represented by a single species, *Photorhabdus luminescens*, so named because of its bioluminescent nature. This phenotype is unique among the Enterobacteriaceae and other bacteria of terrestrial origin.

Once an infective juvenile nematode has penetrated the host hemocoel, the bacterial symbiont is released from the nematode gut, septicemia becomes established, and insect death occurs within 48 h. Although the nematodes may play a role in insect death, in most cases the bacteria alone are sufficient to cause insect mortality following injection into the hemocoel (14, 15). The importance of these bacteria in the life cycle of the nematode has been well documented using axenically reared nematodes (14, 16). Together with the lack of evidence for the free living existence of this bacterium, it has been postulated that the symbiotic association is essential for the survival of both nematode and its symbiotic bacteria. However, one possible exception is the report by Farmer et al. (17) who isolated *P. luminescens* strains from clinical samples with no apparent nematode association (18).

The precise set of mechanisms by which the symbiotic bacterium is able to circumvent the defense host systems in the hemocoel is still under investigation. It has been suggested that the virulence events that lead to bacterial proliferation could involve multiple factors, such as secretion of lipases and proteases, the release of lipopolysaccharide molecules, and the anti-hemocytic properties of the bacterial cell surface (19–28). Recently, Clarke and Dowds (29) speculated that a secreted protease is responsible for the insecticidal activity observed against *Galleria mellonella*. In addition to secreted factors, these bacteria are known to produce intracellular inclusion bodies similar to those produced by *Bacillus thuringiensis* (30, 31). But unlike Bt endotoxin crystals they are not insecticidal and are thought to provide amino acid nutrients for the emerging nematodes (32). In other studies, Bowen (33) reported that a soluble protein fraction derived from *P. luminescens* culture medium possessed sufficient insecticidal activity to kill *Manduca sexta* upon injection. As part of our efforts to find suitable insecticidal proteins that could be employed to produce insect-resistant plants, we initiated a study to further characterize the nature of the oral insecticidal activity. It was found that of *P. luminescens* W-14 fermentation broth showed excellent potency against Southern corn rootworm (SCR)1 neonates,

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1 The abbreviations used are: SCR, Southern corn rootworm; PAGE, PAGE,

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**Photorhabdus luminescens** W-14 Insecticidal Activity Consists of at Least Two Similar but Distinct Proteins

PURIFICATION AND CHARACTERIZATION OF TOXIN A AND TOXIN B

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a surrogate maize pest. We describe here the isolation and characterization of two distinct but structurally similar protein toxins that are highly potent against SCR larvae. We also show that toxin could be further processed and activated by protease cleavage.

**EXPERIMENTAL PROCEDURES**

**Organism and Growth Conditions**—Stock inoculum of *P. luminescens* strain W-14 (ATCC accession number 55397) was produced by inoculating 175 ml of 2% Proteose Peptone number 3 (PP3) (Difco) liquid media with a primary variant subclone in a 500-ml flask and incubated for 16 h at 28 °C on a rotary shaker at 150 rpm. The production broth was achieved by inoculation of 1.75 ml of the stock inoculum into fresh PP3 medium in 500-ml flasks (175 ml of culture/flask). After inoculation, the culture was incubated at 28 °C for 24 h as above. Following incubation, the broth was centrifuged at 2,600 × *g* for 1 h at 10 °C and vacuum filtered through Whatman GF/D (2.7 μm) and GF/F (1 μm) glass filters to remove debris. The broth was then used for the studies described here.

**Southern Corn Rootworm Bioassay**—Protein fractions were diluted into 10 mM sodium phosphate buffer, pH 7.0, and applied directly in 40-μl aliquots to diet plate wells (surface area 1.5 cm<sup>2</sup>) containing artificial diet (34). The diet plate was then allowed to air dry in a sterile flow hood. The wells were then infested with single, neonate *Diabrotica undecimpunctata howardi* (Southern corn rootworm) hatched from surface sterilized eggs. The plates were sealed, placed in a humidified growth chamber, and maintained at 27 °C for 3–5 days. Mortality was then scored. For quantitation of toxin potency, 16 insects per toxin dose were used, and assays were repeated 2–4 separate times. LC<sub>50</sub> was determined using the toxin concentration needed to cause 50% insect mortality.

**Purification of Insecticidal Activity**—Unless noted, the isolation protocol entailed starting with 5 liters of broth that was concentrated using a vacuum pump (Amicon, Beverly, MA) and then either dialyzed against Buffer A or a Sepharose CL-4B column (1.6 × 50 cm) which was equilibrated with Buffer A. The protein was desorbed with a linear gradient of Buffer C to 10 mM potassium phosphate, pH 7.0, and analyzed by SCR bioassay.

**Protein Determination, Gel Electrophoresis, and Western Analysis**—Protein concentrations were determined according to the method of Bradford (35) with bovine serum albumin as standard. Native and SDS-PAGE analyses were performed on either 10% or 4–20% gradient gels (36). Western blotting was performed using ECL Western blotting detection reagent according to the manufacturer’s instructions (Amersham).

**NH<sub>2</sub>-Terminal Amino Acid Sequencing of Purified Toxins and Related Peptides**—The purified toxin A and toxin B, as well as partially purified active gel filtration fractions, were separated on a 10% SDS-PAGE gel and blotted to a Bio-Rad polyvinylidene difluoride membrane according to the manufacturer’s procedure. The protein bands were localized by staining with Amido Black 1 for 1 min (0.1% Amido Black 10 B [Sigma] in 10% acetic acid) followed by destaining for 1 min with 5% acetic acid. Blots were sent for NH<sub>2</sub>-terminal sequencing at Cambridge ProChem (Lexington, MA). NH<sub>2</sub>-terminal sequences are described under “Results.”

**Determination of Molecular Weight by Matrix-assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy**—Protein molecular mass using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy was determined on a Voyager Biospectrometry workstaton with delayed extraction technology (PerSeptive Biosystems, Framingham, MA). Typically, the protein of interest (100–500 pmol in 5 μl) was mixed with 1 μl of acetonitrile and diazylized for 0.5 to 1 h on a Millipore VS filter having a pore size of 0.025 μm (Millipore Corp., Bedford, MA). Dialysis was performed by floating the filter on water followed by adding the protein/acetonitrile mixture as a droplet to the filter surface. After dialysis, the purified protein was removed using a pipette and then mixed with a matrix consisting of sinapinic acid and trifluoroacetic acid according to the manufacturers’ instructions. The protein and matrix (4 μl total volume) were allowed to co-crystallize on a ~3 cm (2) gold-plated sample plate (PerSeptive Biosystems). Excitation of the crystals and subsequent mass analysis was performed using the following conditions: laser setting of 3050, pressure of 4.55e-07, low mass gate of 1500.0, negative ions off; accelerating voltage of 25,000, grid voltage of 90.0%, guide wire voltage of 0.010%, linear mode, and a pulse delay time of 350 ns.

**Production of Peptide-specific Antibodies**—The genes for toxin A and toxin B described in this paper were subsequently cloned (gene cloning to be presented elsewhere) into a new expression system for peptides A1, A2, and B2 (peptides to be described under “Results”). The following peptides were synthesized according to the deduced amino acids sequences: NPNNSSNKLMLFPYVQVQSINT (for peptide A1), VSQGSQGASGQNLLAFG (for peptide A2), and FDSSQSLYEE-NINAGEQR (peptide B2). The corresponding antibodies to the above three peptides were generated in Genemed Biotechnology Inc. (San Francisco, CA). The crude sera were purified using a SufoLink<sup>TM</sup> matrix (Pierce) column. The captured column (Pierce) was eluted with a matrix that specifically binds to the gel following the protocol described by the manufacturer. The respective antisera was applied to the column at the rate of 0.5 ml/min. The column was subsequently washed with phosphate-buffered saline, pH 7.6. The purified antibodies were eluted from the column using 0.05 mM sodium acetate, pH 3.0, and they were immediately neutralized to pH 7.0 with 1 M Tris, pH 8.0. The three purified peptide-specific antibodies were named synA1Ab, synA2Ab, and synB2Ab, and specifically recognized peptide A1, A2, and B2, respectively.

**Protein Treatment of Proteins**—The standard reaction consisted of 40 μg of purified *P. luminescens* W-14 toxin B protease as described under “Results,” and 0.1 M Tris buffer, pH 8.0, in a total volume of 100 μl. For control reactions, protease was omitted. The reaction mixtures were incubated at 37 °C overnight. At the end of the reaction, 10 μl was removed and then boiled with an equal volume of 2 × SDS-PAGE sample buffer for SDS-PAGE analysis. The remaining 90 μl of reaction mixture was serially diluted with 10 mM sodium phosphate buffer, pH 7.0, and analyzed by SCX bioassay.
**RESULTS**

*P. luminescens W-14 Insecticidal Activity Is Proteinaceous—*

The fermentation broth of *P. luminescens* W-14 strain presented a broad spectrum of oral activity against a variety of insects (data not shown), including SCR. Because of our interest in controlling corn rootworm in maize, SCR bioassays were used to follow the *Photorhabdus* insecticidal activity in this study.

Consistent with prior studies by Ensign (33), it was found that the majority of the activity was retained upon extensive dialysis or upon concentration with devices containing 100-kDa molecular mass filters, indicating that the majority of activity was associated with large molecular mass material. Because of the large size, an Amicon M-12 filtration unit equipped with a 100-kDa membrane was used to enrich activity from 5 to 10 liters of broth. Following a single step elution from an ion-exchange column, the bulk of the activity was further resolved by gel filtration using a preparative Sepharose CL-4B column (Fig. 1A). The native molecular mass of the activity appeared to be in the range of 700 to 900 kDa, as judged by comparison to molecular weight standards. SDS-PAGE analysis of this active protein fraction indicated the presence of more than 10 major peptides (Fig. 1B).

It was found that essentially all of the insecticidal activity could be eliminated at temperatures above 60 °C or upon treatment with protease K. Similar results were obtained with broth samples (data not shown). These results are consistent with the toxic activity being proteinaceous. In order to further determine the biochemical nature of the protein toxin, samples were treated with a variety of reversible and irreversible protease inhibitors which included E-64 (cysteine inhibitor), 3,4-dichloroisocoumarin (serine inhibitor), leupeptin (serine inhibitor), and pepstatin (aspartic inhibitor). In all cases, no effects were observed on the biological activity of the toxin. In addition, aliquots of toxin complex treated with inhibitors of metalloenzymes, including 1,10-phenanthroline and EDTA, did not affect the insecticidal potency.

As shown in Table I, NH2-terminal amino acid analysis of selected peptides from the SDS-PAGE (Fig. 1B) demonstrated that each was distinct, but several peptides appeared to have substantial sequence similarity (50–67%). The amino acid sequence for a 64-kDa peptide corresponded to *groEL*, an *Escherichia coli* chaperonin (37), whose concentration varied for each preparation. The occurrence of related peptides suggested that several toxins may be present in the 860-kDa fraction.

**Purification and Characterization of Two Individual Toxins from the 860-kDa Photorhabdus Fraction—**The 860-kDa fraction was applied to a Mono Q column and resolved by a linear salt gradient. It was found that the insect activity was broadly eluted throughout the gradient. Each fraction was bioassayed by serial dilution in order to identify those with the highest SCR activity. Two peaks were identified with high SCR toxicity: one that eluted at approximately 0.2 M NaCl (peak A) and the other at approximately 0.3 M NaCl (peak B) (Fig. 2). Each activity peak was pooled separately and further purified by a series of hydrophobic and ion-exchange chromatography columns. The toxin purified from peak A was denoted toxin A while the toxin from peak B was named toxin B. Both purified toxin A and toxin B contained two predominant bands on a 4–20% SDS-PAGE gel (Fig. 3A). Each activity peak was pooled separately and further purified by a series of hydrophobic and ion-exchange chromatography columns. The two toxins were purified to homogeneity and the native molecular mass of the activity appeared to be in the range of 700 to 900 kDa, as judged by comparison to molecular weight standards. SDS-PAGE analysis of this active protein fraction indicated the presence of more than 10 major peptides (Fig. 1B).

The subunit molecular weights of both toxins were assessed by MALDI-TOF mass spectrometry and SDS-PAGE as presented in Table II. When the SDS-PAGE gel was quantified by densitometry, the relative band intensity of the large and small peptides in both toxin preparations was found to be approximately 3 to 1 ratio, respectively. Given their molecular masses, these results suggested that these subunits were represented in the purified toxins in equal molar amounts. Toxin A and toxin B showed single stained bands in native PAGE, with the smaller peptides of the two toxins being proteinaceous. In order to further determine the biochemical nature of the protein toxin, samples were treated with a variety of reversible and irreversible protease inhibitors which included E-64 (cysteine inhibitor), 3,4-dichloroisocoumarin (serine inhibitor), leupeptin (serine inhibitor), and pepstatin (aspartic inhibitor). In all cases, no effects were observed on the biological activity of the toxin. In addition, aliquots of toxin complex treated with inhibitors of metalloenzymes, including 1,10-phenanthroline and EDTA, did not affect the insecticidal potency.

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**TABLE I**

| Peptide molecular mass | Amino acid sequence
|------------------------|----------------------|
| 201 kDa                | FIOQGSDLEGN-A        |
| 190 kDa                | LSIGNQFPG*SA         |
| 175 kDa                | MQGQTFPGVEG         |
| 165 kDa                | MQDSFESVITTTL        |
| 175 kDa                | MSLTMEQKLNESQRDA     |
| 108 kDa                | MNLASPL5RTE          |
| 80 kDa                 | MNLDINEQNKIVMV       |
| 68 kDa                 | SESLFTQTLK EA-ROALVA |
| 64 kDa                 | AAKDVFQSGDARVKMLGVRN|
| 51 kDa                 | AAKDVFQSDARVKMLGVRN |

*The symbols used are: *, N/D/P/R/E amino acid; †, no determination; |, identical amino acids; :, similar amino acids.*
Since toxin B was an order of magnitude less active than toxin A against SCR neonates, the possibility that the activity observed in toxin B was due to a small amount of contamination of toxin A in the final purified toxin B material was examined by Western analysis using antibodies specific to synthetic peptides corresponding to either toxin A or toxin B. Three peptide-specific antibodies used for analysis were synA1Ab and synA2Ab, which specifically recognized peptides A1 and A2 in toxin A, respectively, and synB2Ab, which specifically recognized peptide B2 in toxin B. Western analysis of purified toxin A and toxin B (5 μg of protein/lane) was performed using the above three antibodies. As shown in Fig. 5, immunological analysis showed that only toxin A but not toxin B reacted when either synA1Ab or synA2Ab was used (Fig. 5, A and B). Conversely, only toxin B but not toxin A reacted when synB2Ab was used (Fig. 5C). This indicated that no detectable cross-contamination existed in the purified toxin A and toxin B fractions. Therefore, the insecticidal activities observed for each toxin were independent of each other.

To eliminate the possibility that protease activity was responsible for the insecticidal activity, samples of isolated toxins were examined for enzymatic activity using fluorescein isothiocyanate-dervatized substrates. Compared with controls and endogenous proteases, no significant protease activity was found for either toxin fraction (data not shown). A second biological activity that has been associated with certain classes of bacterial toxins is the ability to lyse different cell types. In order to examine the general lytic properties of these toxins, in vitro assays were performed using rabbit erythrocytes incubated with up to 0.7 and 2 μg of purified toxin A or toxin B, respectively. Using protein sample buffer as controls, no cell lysis was found with either toxin, while erythrocytes treated with the appropriate units of α-hemolysin from Staphylococcus aureus, a positive control, were completely lysed.

Processing and Activation of Toxin B—In some cases, it was noted that when Photorhabdus W-14 broth was rapidly purified as described previously, a 281,040 kDa peptide (determined by MALDI-TOF MS) was present in the final purified toxin B fraction as the major component instead of the two peptides typically observed (Fig. 6A). It was also observed that in some broth productions that toxin A was similarly present as a large molecular weight species. The same purification protocols were used to isolate the toxin B 281-kDa peptide from Photorhabdus W-14 broth as described earlier. Western analysis of the 281-kDa peptide fraction using the peptide-specific antibody synB2Ab indicated that the 281-kDa peptide was related to the smaller toxin B peptide (Fig. 6B). The 58-kDa signal detected by the antibody indicated the presence of small toxin B subunit that was not readily visible by Coomassie Blue staining. These data strongly suggested that the small and large peptides were derived from a single 281-kDa peptide, a protoxin peptide, possibly by protease(s) present in Photorhabdus W-14 broth. In diet bioassays, the 281-kDa peptide was found to be approximately 10-fold less active than the cleaved species.

Two distinct metalloproteases, 38 and 58 kDa, have been purified from W-14 fermentation broth (to be reported elsewhere). The effect of the 38-kDa metalloprotease on the 281-kDa peptides was evaluated in vitro by incubation with the protoxin peptide (see “Experimental Procedures”). In Fig. 7A, the result of SDS-PAGE analyses of protease treatment of toxins are shown. After overnight incubation, the 281-kDa material was converted into 201- and 58-kDa peptides as judged by SDS-PAGE analysis. Western analysis using synB2Ab verified that the 58-kDa peptide was generated from the 281-kDa peptide (Fig. 7B). Bioassays of protease-treated and untreated (control) toxin fractions showed that the biological activity (LD_{50}) against SCR was improved to ~150 ng/cm² diet upon cleavage (Fig. 7C). The 58-kDa protease also had a similar effect on the cleavage and activation of the 281-kDa
peptide as the 38-kDa protease (data not shown). These data demonstrated that the two peptides in toxin B originated from a single protoxin of 281 kDa by protease cleavage.

**DISCUSSION**

*B. thuringiensis* (Bt) endotoxins have been used as sprayable microbial insecticides for nearly 3 decades with limited success in the commercial marketplace (38, 39). The effective, reliable use of these proteins to control insect pests in the field remained elusive until advances in plant transformation technology allowed the stable introduction of these genes in a variety of crop species. Today, concerns about the development of insect resistance to Bt transgenic plants and the narrow biological activity of Bt proteins have spurred a renewed effort to discover orally active insecticidal proteins. Early discovery programs focused on growth inhibitory proteins, such as lectins and protease inhibitors (40–43). Their low oral activity and lack of insect mortality proved to be a severe impediment to their use in transgenic products, since very high levels of expression are required for control. In general, the loss of insect control in transgenic plants can be attributed to the premise that it is necessary to maintain a pest control threshold which is sufficient to control at least 90% of the insect pests during an active infestation. We have arbitrarily defined the pest control threshold as the value of expressed insect control protein (E), in units of micrograms per milligram of soluble protein, divided by toxin potency (P), whose units are micrograms required for LD90 in bioassays; pest control threshold = E/P. Therefore, it is advantageous to discover oral protein toxins that are highly efficacious, resulting in high pest control threshold values. Such toxins may prevent failures due to reduced expression as a result of environmental factors such as stress, senescence, or other physiological events. However, the idealized pest control threshold value required for plant resistance is currently being defined as scientists evaluate efficacy of Bt and other pest control transgenic plants in the field.

Although the availability of orally active insecticidal proteins outside the Bt endotoxin family that meet the efficacy hurdles required for pest control has been limited to date, several academic and industrial laboratories have reported interesting new leads. For example, agricultural companies such as Novartis have discovered several new bacterial toxins referred to as VIPs, or vegetative insecticidal proteins, from a variety of *Bacillus* species. One, VIP3A, an 88-kDa protein, has excellent activity against black cutworms and armyworms, both lepidopteran pests (44, 45). Another bacterial toxin includes cholesterol oxidase derived from *Streptomyces* broth, that has shown to have selective, high potency against cotton boll weevil.
(46). The engineered cholesterol oxidase gene has been transformed into cotton plants and is currently under evaluation for insect resistance. Bowen (33) first reported the presence of a novel, very large protein derived from the bacterial genus, *Photorhabdus*, that was orally toxic to *M. sexta*. It is too early to know whether the second wave of insecticidal proteins will be able to withstand insect pressure in the field and provide the desired insect control to be commercially competitive with chemical sprayable products.

We have demonstrated here that a high molecular weight protein fraction can be routinely isolated from the fermentation broth of *P. luminescens* strain W-14. This protein fraction represents the majority of insecticidal activity in the fermentation broth and contains at least 10 distinct peptides that can vary with each fermentation. The 860-kDa fraction was shown to be sensitive to heat and protease K treatment, suggesting that it is proteinaceous in nature and distinct from other reported insecticidal agents such as lipopolysaccharides, which are stable at higher temperatures (47). Amino acid sequencing of the NH₂ terminus of partially purified toxin peptide fractions indicated that each peptide is distinct; however, several peptides have significant homologies. These results suggest that there may be several genetically related toxins produced by the *P. luminescens* strain W-14, and therefore, the partially purified fraction (Fig. 1) represents a complex of several toxins. Evidence for this hypothesis was supported by the discovery of two remarkably similar but distinct SCR protein toxins that are composed of equal molar amounts of a small and large peptides. Amino-terminal analyses of the small subunits suggested that the toxins are highly related in their amino acid identity (82%) and similarity (91%). In Table I, a third peptide, 61 kDa had 50% homology over a 10 amino acid sequence with both small peptides of toxin A and toxin B. This suggests the possibility that other toxin(s) are present in the toxin complex. This finding together with the broad elution profile of insect activity from ion exchange resin has led to continued investigation of other related protein toxins. The presence of multiple toxins is not surprising, based on observations that *B. thuringiensis* organisms typically contain more than one endotoxin (48, 49).

The isolation of multiple toxins by *Photorhabdus* organisms may be a necessary attribute to assure survival of the juvenile nematodes whose host is vulnerable to predation by other insects and nematodes.

The isolated protein toxins were unusual in their gross protein biochemical properties with respect to other insecticidal proteins described previously. Most notably, unlike *Bt* endotoxins which are monomeric with molecular sizes ranging from 25 to 130 kDa, both *Photorhabdus* toxins described appear to be very large oligomers, perhaps tetramers, of approximately 860 kDa. Furthermore, the protein toxin is highly soluble at neutral pH environments, in contrast to *Bt*, where the toxin molecules natively exist in crystalline inclusion bodies (48). Other biochemical aspects, such as the large sizes of toxin subunits, ~280 kDa for the protoxin, are not unusual. For example, protein subunits for *Clostridium difficile* toxins A and B have been calculated to have molecular masses of 308,057 and 269,709 Da, respectively (50). Furthermore, *Photorhabdus* toxins appear to have similarities to other generic A-B type bacterial toxins that are proteolytically processed to the mature form (51). Interestingly, the *Photorhabdus* broth contains two distinct metalloproteases that are capable of specifically cleaving the native protoxin into large (208 kDa) and small (63 kDa) molecular mass subunits that are not held together via a disulfide bridge (data not shown). The two subunits added together, ~271 kDa, did not account for the total molecular size of the protoxin, 281 kDa. Also, a 216-kDa species was observed in protease-treated samples, suggesting that additional trimming of the larger subunit occurs (data not shown). Both the unprocessed and processed species had a similar native molecular size, but the potency of the oligomeric protoxin B was 5–10-fold less active than fermented or *in vitro* processed species. These results suggest that the toxin molecule is first assembled as an oligomer in the bacterial cell or during secretion, and subsequently processed to the mature form in the media. It is of interest to note that one peptide that has been observed in the 860-kDa toxin fraction is a homolog of GroEL, a chaperonin (37). However, this chaperonin homolog was not present in the purified toxins, and therefore, not necessary for toxicity.

The potential similarity to other bacterial toxins that are known to contain enzymatic subunits provided the impetus to perform a set of experiments to examine the toxins for proteolytic activity. For example, the hemolysin toxin from *Erwinia chrysanthemi* is known to contain a metalloprotease moiety (52). However, no proteolytic activity of the *Photorhabdus* toxins, either the partially purified fraction or purified toxins, was observed using a variety of protease substrates. Furthermore, the use of irreversible and reversible inhibitors to metal ions and serine and cysteine residues failed to inactivate to the insect activity. These results indicate that the toxin does not appear to be a protease or an enzyme with a serine or cysteine at the active site. Furthermore, we examined the toxin for hemolytic activity which is known for certain types of bacterial enzymes.
toxins and found that neither toxin A or B adversely affected rabbit erythrocytes in vitro (data not shown). In toto, these studies indicate that some general biochemical features appear to be related to bacterial toxins, but at this time no definitive correlation was observed. The genetic relationship of these toxins to other bacterial toxin genes will be addressed in a separate article describing the isolation of their genes.

We further examined the effects of other protease preparations, such as trypsin and a variety of crude protein extracts from guts of several species of insects, on the 281-kDa peptide. Under appropriate conditions, the 281-kDa peptide can be processed into two peptides in the similar fashion as observed with the purified metalloproteases as judged by SDS-PAGE. However, it was also observed that treatment with different insect gut protease preparations had differential effects on the insecticidal activity against SCR, ranging from loss of activity to more than 10-fold activation. This suggests that the precise cleavage of the protoxin by proteases impacts the degree of insecticidal activity. Furthermore, we have observed similar effects on toxin activation upon treatment with plant-derived protease preparations. Currently, we are using mass spectrometry to dissect the molecular differences among cleavages by different proteases to elucidate the precise mechanism of activation. In conclusion, to date these data suggest that there is a general proteolytically sensitive region in the protoxin that is accessible to a variety of proteases, however, it is unclear whether different proteases affect the degree of toxin activation upon cleavage.

One aspect of these protein toxins that is unique is their high potency against corn rootworm species, including Western corn rootworm (to be reported elsewhere). Both toxin A and toxin B had high potency against SCR, 5 and 87 ng/cm² diet, respectively, which is in the range of the most potent Bt toxins against lepidopteran species. In contrast, Bt endotoxins have been reported to have relatively low activity against corn rootworm species, LC₅₀ of 10 to 1000 µg/cm² diet (53). In addition, toxin A was also found to have good efficacy against tobacco hornworm, a lepidopteran insect. This activity is unusual, given the general inability of Bt toxicity to cross insect orders (48). We have also determined that insecticidal activity of W-14 Photorhabdus toxins extends to several other insect orders beside coleopteran and lepidopteran (data not shown).

The high potency of both toxins makes them attractive candidates as potential insect control genes for transgenic plants. Another advantage for transgenic applications is the finding that a single protoxin was responsible for the insect toxicity, suggesting that only a single gene may be required for biological activity. Our gene cloning results and heterologous expression studies (to be reported elsewhere) corroborated the biochemical studies described herein. Recently, Bowen et al. (54) have described the isolation and cloning of similar toxin proteins and genes by alternate methods. Their results are also consistent with the observations reported in this article. Furthermore, gene disruptions of proteins identical to toxin A and toxin B directly reflected the biological potencies of the two toxins against tobacco hornworm (54). In the process of our collaborations, we have agreed to use a standardized nomenclature for the toxins discovered. Toxin A and toxin B described here are identical, based on our cloned genes, to peptides denoted TdaA and TcbA (54), respectively. Cumulatively, these research data verify that the purified toxins reported here are critical components of insecticidal toxic activity observed in Photorhabdus broth.

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21. Under appropriate conditions, the 281-kDa peptide can be processed into two peptides in the similar fashion as observed with the purified metalloproteases as judged by SDS-PAGE. However, it was also observed that treatment with different insect gut protease preparations had differential effects on the insecticidal activity against SCR, ranging from loss of activity to more than 10-fold activation. This suggests that the precise cleavage of the protoxin by proteases impacts the degree of insecticidal activity. Furthermore, we have observed similar effects on toxin activation upon treatment with plant-derived protease preparations. Currently, we are using mass spectrometry to dissect the molecular differences among cleavages by different proteases to elucidate the precise mechanism of activation. In conclusion, to date these data suggest that there is a general proteolytically sensitive region in the protoxin that is accessible to a variety of proteases, however, it is unclear whether different proteases affect the degree of toxin activation upon cleavage.

One aspect of these protein toxins that is unique is their high potency against corn rootworm species, including Western corn rootworm (to be reported elsewhere). Both toxin A and toxin B had high potency against SCR, 5 and 87 ng/cm² diet, respectively, which is in the range of the most potent Bt toxins against lepidopteran species. In contrast, Bt endotoxins have been reported to have relatively low activity against corn rootworm species, LC₅₀ of 10 to 1000 µg/cm² diet (53). In addition, toxin A was also found to have good efficacy against tobacco hornworm, a lepidopteran insect. This activity is unusual, given the general inability of Bt toxicity to cross insect orders (48). We have also determined that insecticidal activity of W-14 Photorhabdus toxins extends to several other insect orders beside coleopteran and lepidopteran (data not shown).

The high potency of both toxins makes them attractive candidates as potential insect control genes for transgenic plants. Another advantage for transgenic applications is the finding that a single protoxin was responsible for the insect toxicity, suggesting that only a single gene may be required for biological activity. Our gene cloning results and heterologous expression studies (to be reported elsewhere) corroborated the biochemical studies described herein. Recently, Bowen et al. (54) have described the isolation and cloning of similar toxin proteins and genes by alternate methods. Their results are also consistent with the observations reported in this article. Furthermore, gene disruptions of proteins identical to toxin A and toxin B directly reflected the biological potencies of the two toxins against tobacco hornworm (54). In the process of our collaborations, we have agreed to use a standardized nomenclature for the toxins discovered. Toxin A and toxin B described here are identical, based on our cloned genes, to peptides denoted TdaA and TcbA (54), respectively. Cumulatively, these research data verify that the purified toxins reported here are critical components of insecticidal toxic activity observed in Photorhabdus broth.

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