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Characterization and Biochemical Analyses of Venom From the Ectoparasitic Wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae)

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During parasitism, the ectoparasitic wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) induces a developmental arrest in host pupae that is sustained until the fly is either consumed by developing larvae or the onset of death. Bioassays using fluids collected from the female reproductive system (calyx, alkaline gland, acid gland, and venom reservoir) indicated that the venom gland and venom reservoir are the sources of the arrestant and inducer(s) of death. Infrared spectroscopic analyses revealed that crude venom is acidic and composed of amines, peptides, and proteins, which apparently are not glycosylated. Reversed phase high performance liquid chromatography (HPLC) and sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the proteinaceous nature of venom and that it is composed mostly of mid to high molecular weight proteins in the range of 13 to 200.5 kilodaltons (kDa). Ammonium sulfate precipitation and centrifugal size exclusion membranes were used to isolate venom proteins. SDS-PAGE protein profiles of the isolated venom fractions displaying biological activity suggest that multiple proteins contribute to arresting host development and eliciting death. Additionally, HPLC fractionation coupled with use of several internal standards implied that two of the low molecular weight proteins were apamin and histamine. However, in vitro assays using BTI-TN-5B1-4 cells contradict the presence of these agents. Arch. Insect Biochem. Physiol. 61:24–41, 2006. © 2005 Wiley-Liss, Inc.

KEYWORDS: wasp venom; infrared spectroscopy; HPLC; SDS-PAGE; size exclusion

INTRODUCTION

Venoms produced by parasitic Hymenoptera possess unique regulatory agents that functionally aid in subduing an insect host. These venom components are known to facilitate host invasion through induction of paralysis, suppression of host immune responses, stimulation or augmentation of the activity of other parasitoid factors (e.g., endosymbiotic viruses), and/or initiation of developmental arrest (Beard, 1963; Coudron, 1991; Piek et al., 1982; Rivers and Denlinger, 1994a; Rivers et al., 2002a; Strand and Pech, 1995; Whitfield and Asgari, 2003). In certain endoparasitic species, other maternally derived secretions (e.g., virus-like particles, polydnaviruses, or ovarian proteins) are also needed for successful parasitism (Beckage, 1998; Webb and Luckhart, 1994; Vinson, 1990). Regardless of the source of regulatory material, only a rudimentary understanding of the mode of ac-

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Abbreviations used: HPLC = high performance liquid chromatography; I.R. = infrared spectroscopy; kDa = kilodalton; LC_{99} = lethal concentration to kill 100% of population; LD = light-dark; MWCO = molecular weight cutoff; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA = trifluoroacetic acid; VRE = venom reservoir equivalent.

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tion or the molecular target sites is available. Consequently, precise pathways triggered by parasitic wasp venoms remain unclear.

The availability of specific venom proteins responsible for disruption of host development and physiology as well as inducers of death would greatly enhance efforts aimed at understanding the mechanisms of action of regulatory proteins found within venoms. Unfortunately, only a limited number of studies have attempted to characterize the composition and biochemical properties of parasitic wasp venoms (Quicke, 1997). Quistad et al (1994) identified several paralytic toxins in the venom from the ectoparasitoid *Habrobracon* (= *Bracon*) *hebetor*, with at least two of the isolated proteins demonstrating high insecticidal activity toward 6 species of lepidopteran larvae. Two mid-range molecular weight proteins (33 and 52 kDa) have been isolated and sequenced from the endoparasitoid *Chelonus* near *circumaculatus* (Jones et al., 1992), and though the 52-kDa protein shares a high degree of homology with an insect chitinase (Krishnan et al., 1994), no functional role in parasitism has been established. By contrast, a 66-kDa protein was isolated from the ectoparasitic wasp *Euplectrus comstockii*. When injected into host larvae, it was capable of inducing developmental arrest that resembled natural parasitism (Coudron and Brandt, 1996). Molecular and biochemical analyses of two wasp species (*Pimpla hypochondriaca* and *P. turionella*) from the genus *Pimpla* suggest that these endoparasitoid venoms possess a large and diverse number of enzymes, including laccase, serine protease, reprolysin-like metalloprotease, phospholipases, and phenoloxidases (Parkinson et al., 2001, 2002a,b, 2003; Uçkan et al., 2004). Uçkan et al. (2004) also observed the presence of noradrenaline, apamin, and melittin in venom from *P. turionella*, consistent with the paralytic action of the venom in multiple life stages of lepidopteran hosts (Kansu and Uğur, 1984). Similarly, an aspartylglucosaminidase-like protein has been identified in the venom of *Asobara tabida* (Moreau et al., 2004), with a speculative role in host paralysis through induction of aspartate-dependent excitatory pathways. Other identified venom proteins are thought to have an immunosuppressive role (Asgari et al., 2003a,b; Parkinson et al., 2001, 2003) or even induce host castration (Digilio et al., 2000).

Nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae), like other species of parasitoids, has been the subject of several studies focused on deciphering how the wasp functions in the parasitic relationship (reviewed by Rivers et al., 1999a). This intensively studied wasp has a well-developed venom system, which produces a proteinaceous venom in the acid gland and stores the venom in active form within a single reservoir (Ratcliffe and King, 1967, 1969). Functionally, venom inhibits host cellular immune responses (Rivers et al, 2002a), depresses respiratory metabolism within 6–8 h (Rivers and Denlinger, 1994b), stimulates increases in lipid levels within hemolymph and fat body (Rivers and Denlinger, 1995; Rivers et al., 1998), and ultimately induces death. In muscoid flies, *N. vitripennis* venom can retard adult fly mobility within 1–2 h when injected artificially, and the flies will succumb to death in less than 24 h (Beard, 1964; Rivers et al., 1993). The venom has been shown to be highly active toward several life stages (wandering larvae, pupae, pharate adults, adults) of flies from at least 4 families (Muscidae, Drosophilidae, Calliphoridae, and Sarcophagidae) (Rivers et al., 1993). Additionally, in vitro assays using cultured insect cells have revealed venom stimulation of G-protein dependent signal transduction pathways that promote mobilization of intracellular calcium from mitochondria and endoplasmic reticulum, elevations in cAMP, and cell death via an oncotic lytic mechanism (Rivers et al., 2002b, 2005).

The diversity of biological functions ascribed to this wasp venom argues that multiple venom proteins or factors are involved in subduing the fly host, a feature that would be consistent with most parasitoid venoms that have been at least partially purified (Digilio et al., 2000; Doury et al., 1997; Jones et al., 1992; Parkinson et al., 2002a). However, despite the accumulation of evidence that has shed light on how venom from *N. vitripennis* operates in vivo and in vitro, scarce information ex-

ists on the chemical composition of the venom, or on how many different proteins may be involved in manipulating the host condition. This lack of knowledge has contributed to the precise target tissues in the host remaining obscure, as have key aspects of the cellular pathways associated with host biochemical changes, developmental disruption, and death. A necessary next step to address these issues is to characterize and identify venom components responsible for the observed host alterations. This study was aimed at biochemical analyses of venom components through high performance liquid chromatography, infrared spectroscopy, and protein gel electrophoresis (SDS-PAGE). We also attempted to isolate active venom proteins by using ammonium sulfate precipitation and size exclusion centrifugal membranes. Isolated venom fractions were then further analyzed electrophoretically and through in vivo and in vitro biological assays.

MATERIALS AND METHODS

Insect Rearing

A laboratory colony of *N. vitripennis* was maintained on pupae and pharate adults of the flesh fly, *Sarcophaga bullata*, as described previously (Rivers and Denlinger, 1994a). Adults and larvae were reared under a 15:9 h light-dark cycle at 25°C. Twenty to thirty females (3–7 days after emergence from host puparia) were placed in a plastic container (15 × 100 mm) with 30–50 nondiapausing pupae (4 days after pupariation at 25°C) of *S. bullata* and a 50% (v/v) honey solution. After 24 h, the adult wasps were removed and parasitized pupae maintained at 25°C, LD 15:9 h. Under these conditions, *N. vitripennis* develops from egg to adult (eclosion) in 12 days.

A colony of *S. bullata* was reared according to Denlinger (1972). Larvae and adults were fed beef liver throughout development at 25°C with a light-dark cycle of LD 15:9 h. To synchronize fly development for assessing host age, third instar larvae that had begun to wander from food (but prior to crop emptying) were collected and placed in a vented glass jar (1 liter) with 1–2 ml tap water. Larvae were held under these conditions for 3 days at 25°C with frequent (3–5 times/day) water changes. This "wet" treatment temporarily inhibits the release of ecdysteroids until the larvae are placed in dry conditions, thereby synchronizing pupariation (Ohtaki, 1966).

Cell Culture

BTI-TN-5B1-4 cells (embryos from *T. ni*) (Davies et al., 1993) (also called High Five™) were purchased from Invitrogen (San Diego, CA) and grown in TC-100 (Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma) at 27°C.

Isolation of Crude Wasp Venom and Other Reproductive Tissues

Unless otherwise indicated, crude venom from *N. vitripennis* was isolated from host-fed females in phosphate-isolation buffer [10 mM sodium phosphate (pH 8.0), 0.9% (w/v) NaCl, 15% (w/v) sucrose, 1 mM ethylenediamineteraacetic acid, and 1 mM phenylmethylsulfonyl fluoride] (Rivers et al., 1993) and stored frozen at –70°C. Venom activity was confirmed in vitro and in vivo with BTI-TN-5B1-4 cells and young pharate adults (5 days after pupariation at 25°C) of *S. bullata*, respectively, as described previously (Rivers et al., 1993).

Fluids from calyx tissue, alkaline glands, venom glands (acid gland), and venom reservoirs were collected essentially as described above for crude venom isolation. Fine forceps were used to pull the ovipositor and sheath from host-fed adult females covered in phosphate-isolation buffer. This technique allowed removal of ovaries, oviducts, and accessory glands along with the ovipositor. Specific glands or tissues were dissected free from the remaining tissue using iris scissors and forceps, the identity of each tissue was confirmed using the criteria of Ratcliffe and King (1967), and then the tissue was processed as described above. Extract from each tissue was tested for biological activity

as described below for young pharate adult injections and cell venom assays.

Protein Determination

Total protein in crude venom was determined colorimetrically at 562 nm using a micro-BCA Protein Assay kit (no. 23235, Pierce, Rockford, IL). Bovine serum albumin (Sigma) served as the standard.

Effect of pH on Venom Activity

To determine the optimal pH for maintenance of venom activity (lethality), the pH of crude venom was adjusted using buffers of different buffering capacities. Crude venom was isolated as described above, with the exception that venom reservoirs were homogenized in buffers of different pH [citrate-phosphate (pH 5–6.8), sodium phosphate (pH 7.0–8.0), and Tris-HCl (pH 8.2–9) (Stoll and Blanchard, 1990)], but otherwise the same composition as phosphate isolation buffer. The ability of venom in each buffer to elicit death toward cultured cells and adult flies was assessed as described.

Psoralen Treatment

To determine whether endosymbiotic viruses or other microorganisms may be harbored in wasp venom, and thus responsible for venom biological activity, crude venom was incubated with psoralen, a DNA cross-linking agent. The venom-psoralen mixture was then placed in a UV-cross-link oven for 55 min (Stratelink). Under these conditions, psoralen has been shown to cross-link viral DNA, thereby preventing viral replication and inhibiting all activity (Strand and Noda, 1991).

Infrared Spectroscopy

Infrared spectroscopic analysis was performed essentially as described previously (Uçkan et al., 2004). In brief, a 50-µl sample of lyophoлized crude venom, reconstituted in phosphate isolation buffer (final protein concentration = 1.42 µg/µl), was maintained at 30°C until complete evaporation. The dried material was homogeneously ground with potassium bromide before infrared spectroscopic analyses were performed at room temperature using a Perkin-Elmer Spectrum BX-II infrared spectrometer (Perkin-Elmer, Beaconsfield Buks, England).

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to separate and identify venom components. Reverse-phase liquid chromatography was performed by loading 20 µl of a crude venom solution (150 lyopholized venom reservoirs reconstituted in 150 µl isolation buffer) onto an *Ace*III C_{18} reverse phase column (12.5 cm by 4.0 mm i.d.; MAC-MOD Analytical, Chadds, Ford, PA) as described by Uçkan et al. (2004). The mobile phases were (A) 0.1% trifluoroacetic acid (TFA) in acetonitrile:water (80:20) and (B) 0.1% TFA in water. Crude venom was fractioned by linear gradient (5–80%) using mobile phase A at 40 min. The flowrate was maintained at 1.0 ml/min, and the elution monitored at 280 nm.

Venom Injections

Young pharate adults (5 days after pupariation at 25°C) of *S. bullata* were injected with venom lateral to the dorsal midline of the thorax, just behind the neck. To prepare pharate adults for injection, the operculum of each puparium was removed to expose the head-neck region, and then pricked with an insect pin to bleed-off approximately 1–2 µl of hemolymph. The latter was performed to temporarily alleviate the high hemocoelic pressure resulting from longitudinal contraction of the body and shrinkage of the cuticle during puparium formation (Zdarek et al., 1979). Injections were accomplished by means of finely drawn glass capillaries (Rivers et al., 2004). Mortality was assessed at 24-h intervals for 30 days (Rivers et al., 1993). Induction of developmental arrest was determined using the criteria of Rivers and Denlinger (1994a).

In parallel experiments, 48-h-old imagoes (after

eclosion at 25°C) were injected (1 µl/fly) with crude venom lateral to the dorsal midline of the abdomen using finely pulled capillaries. Adults injected with phosphate isolation buffer served as controls.

Venom Assays

BTI-TN-5B1-4 cells were counted with a hemacytometer and seeded (2×10^3 cells/well) into 96-well plates (Falcon) with 100 µl TC-100 containing 10% FBS. Cells were grown at 27°C for 2–3 days. Confluent monolayers were washed with PBS (pH 7.4) by removing spent culture media, adding 100 µl PBS, and then gently rocking the plate for 10–20 sec before discarding the saline. After the wash, 100 µl TC-100 with 10% FBS was added, and wasp venom (0.0003–0.01 VRE/µl) was pipetted into each well. Cell viability was assessed with trypan blue dye exclusion staining (final concentration was 0.04%) as previously described (Rivers et al., 1993).

HPLC analyses suggested that both apamin and histamine were present in crude venom. To confirm the presence of these agents, venom assays were performed with pure apamin (Sigma) and anti-histamine antibodies (Sigma), either alone or in combination with a LC_{99} dose of crude venom from *N. vitripennis* (Rivers et al., 1993). Similarly, phenoloxidases have been identified as major venom components of some wasp species (Dani et al., 2003; Parkinson et al., 2001), as well as components of parasitoid larval secretions, including *N. vitripennis* (Gerling and Legner, 1968; Thompson, 1986; Whiting, 1967). Consequently, parallel experiments were conducted with anti-phenoloxidase antibodies (Research Diagnostics, Flanders, NJ) either alone or simultaneously with crude venom. Cell viability was assessed with trypan blue dye exclusion staining (final concentration was 0.04%) as previously described (Rivers et al., 1993).

Protein Precipitation

The proteinaceous nature of crude venom was examined by mixing a crude venom solution (1 VRE/µl) prepared either from isolated venom glands or venom reservoirs with 3 volumes of ice-cold acetone, a reagent that promotes protein precipitation. The solution was mixed by gentle vortexing for 30 sec, and then placed at –20°C for 24 h to facilitate protein precipitation. The mixture was then centrifuged at 10,000 rpm for 30 min at 4°C. The protein precipitate that formed was washed twice in phosphate isolation buffer (pH 8.0) by centrifugation at 7,000 rpm for 10 min (4°C), followed by re-suspending the pellet in 100 µl (the original volume of crude venom) phosphate isolation buffer. Venom activity was assessed in vivo and in vitro as described above.

The above method coupled with protein electrophoresis confirmed that the active components in crude venom that trigger developmental arrest and death are proteins. Therefore, in an attempt to isolate these active proteins, ammonium sulfate precipitation was performed. Ammonium sulfate was added to crude venom solutions (1 VRE/µl) as described by Englard and Seifter (1990) stepwise until 100% saturation. The solutions were gently stirred for 30 min at 4°C, and then centrifuged at 12,000*g* for 10 min at room temperature. The precipitate was washed twice (centrifugation at 7,000 rpm for 5 min at room temperature) with phosphate isolation buffer, and the final pellet re-suspended in 200 µl phosphate isolation buffer. To facilitate de-salting, these solutions were then loaded onto a 10,000 molecular cut-off filter (Amicon, Bedford, MA) and centrifuged at 3,000 rpm for 30 min at room temperature. The retentate was removed and adjusted to 50 µl with phosphate isolation buffer. The ammonium sulfate supernatants from above were loaded into dialysis membranes (10,000 MWCO, Pierce, Rockford, IL) and dialyzed with several exchanges of 10 mM PBS (pH 8.0) overnight at 4°C. Following dialysis, the samples were concentrated using 10,000 MWCO centrifuge filters (Amicon) as detailed above. Venom fractions were analyzed by biological assays and SDS-PAGE.

Size Exclusion Separation of Crude Venom

Size exclusion membranes (centrifuge filters) were used to estimate the molecular size of venom

components responsible for host developmental arrest and death. Crude venom samples (30–50 ml, 1.51 μg protein/μl) in phosphate isolation buffer were loaded onto low-protein binding (cellulose acetate) centrifuge membrane filters of different molecular weight cut-off exclusion limits [10,000 to 100,000 MWCO (Spectrum, Gardena, CA or Viviscience, Hannover, Germany)]. Filters were centrifuged at 12,000*g* (4°C) for 30–45 min, or until the volume had been reduced to approximately 10 μl. Retentates were transferred to a microcentrifuge tube (0.5 ml) and the volume adjusted to 25 μl with phosphate isolation buffer. Retentates and filtrates were then tested for biological activity in vivo and in vitro as described above, and also subjected to SDS-PAGE (below).

Protein Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the modified Laemmli method described by Garfin (1990) using Tris-HCl pre-cast gels [10% and linear (4–15%)] (BioRad, Hercules, CA) in a Mini Protean III apparatus (BioRad). After electrophoresis, gels were placed in fixative [40% methanol (w/v), 10% trichloroacetic acid (w/v)] for 1 h at room temperature on a Labnet agitator (Orbit P4), followed by Coomassie blue staining (Garfin, 1990). Gel images were captured using a Versadoc image analysis system (BioRad) connected to a Macintosh G-4 computer (Apple) equipped with Quantity One 1-D analysis software (V. 4.4, BioRad). Molecular weights of protein bands were estimated with reference to molecular weight markers (Kaleidoscope standards, BioRad).

Statistical Analyses

Means were compared using one- and two-way analysis of variance (ANOVA) and Student Newman-Keul's multiple comparisons tests using StatView statistical software (v. 5.01, $\alpha = 0.05$). Percentage data was arcsine transformed prior to analysis.

RESULTS

Source of Biological Activity

Upon encountering a fly host in nature, females of *N. vitripennis* inject secretions into pupae and pharate adults via the ovipositor that evoke a developmental arrest and eventual host death (Rivers and Denlinger, 1994a). The source of the arrestant and inducer of death appears to be the venom gland and venom reservoir as evidenced by injection of material from either tissue into young pharate adults of *S. bullata* inducing a developmental arrest of the fly reminiscent of natural parasitism that eventually culminated in death (Table 1). Similarly, incubation of BTI-TN-5B1-4 cells with fluids from venom glands and reservoirs resulted in cell death (Table 1). Prior to death, the plasma and nuclear membranes swelled, which was followed by cell lysis, a series of events previously shown for crude wasp venom (Rivers et al., 1999b). By contrast, saline, calyx fluid, and fluids from alkaline glands displayed no biological activity in vivo or in vitro (Table 1).

Psoralen and Acetone Treatments

Incubation of psoralen with crude venom, followed by UV cross-linking did not alter wasp venom's ability to trigger developmental arrest or its toxicity toward young pharate adults of *S. bullata* (Table 1).

When venom (collected from either venom glands or venom reservoirs) was incubated with ice-cold acetone, a precipitate formed. Following centrifugation and pellet washes with PBS, the resuspended protein pellet was injected into fly hosts. This fly treatment induced host arrestment and death consistent with natural parasitism (Table 1). In contrast, the supernatant resulting from acetone precipitation displayed no biological activity toward pharate adults of *S. bullata*.

In parallel experiments, the supernatant and resuspended pellets were incubated in vitro with confluent monolayers of *T. ni* cells and yielded similar results to those observed in vivo: the

TABLE 1. Sources of Insecticidal Activity From Tissues and Fluids of *Nasonia vitripennis**

	% Response				
	In vivo			In vitro	
Source	N	Developmental arrest	Death	N	Death
Saline (isolation buffer)	45	0[a]	0[a]	9,781	0[a]
Alkaline gland	45	0[a]	0[a]	8,643	0[a]
Venom gland	45	95.9 ± 2.7[b]	100[b]	10,175	100[b]
Venom reservoir	45	98.7 ± 3.0[b]	100[b]	7,421	100[b]
Calyx fluid	45	0[a]	0[a]	7,704	0[a]
Acetone precipitate					
Venom gland	45	78.1 ± 4.6[c]	87.4 ± 2.2[c]	10,305	86.3 ± 1.8[c]
Venom reservoir	45	75.4 ± 6.8[c]	83.6 ± 1.8[c]	8,895	88.6 ± 3.2[c]
Psoralen treatment					
Venom gland	45	97.8 ± 4.2[b]	100[b]	9,547	100[b]
Venom reservoir	45	96.0 ± 2.9[b]	100[b]	9,036	100[b]

*Fluids were collected by dissecting tissues from host-fed adult females in isolation buffer [10 mM sodium phosphate (pH 8.0) containing 15% (w/v) sucrose, 0.9% (w/v) NaCl, and 1 mM EDTA]. In vivo assays were performed by injecting samples with finely drawn glass capillaries into the dorsal surface of adults of the flesh fly, *S. bullata*. In vitro assays used confluent monolayers of BTI-TN-5B1-4 cells grown in 96-well plates at 27°C.

acetone supernatant was not lethal toward BTI-TN-5B1-4 cells but the re-suspended precipitate displayed high cytotoxicity (Table 1).

Effect of pH on Venom Activity

The stability of the arrestment and lethal activity of crude wasp venom at different pH levels was examined using solutions with differing buffer capacities. Toxicity of crude venom toward adult flies and cultured insect cells was highest at alkaline pH values at or near pH 8.0 (Fig. 1). As the pH became more alkaline than 8.2 or more acidic than 7.4, the toxicity of venom in vivo declined steadily. In contrast, in vitro lethality decreased much more slowly with increasing or decreasing buffer pH (Fig. 1).

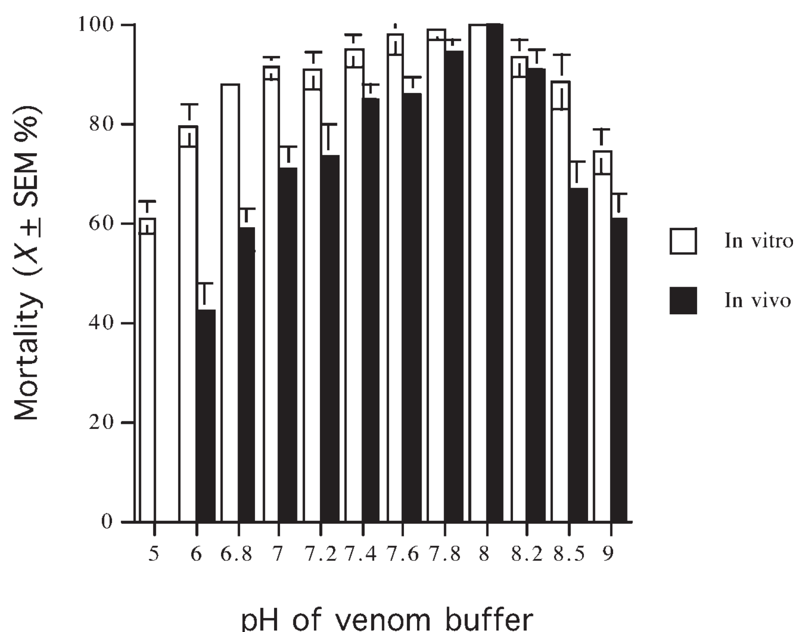

Fig. 1. Effect of pH on insecticidal activity of crude venom from *N. vitripennis*. A LC_{99} or LD_{99}, respectively, dose of crude venom was either incubated with confluent monolayers of BTI-TN-5B1-4 cells at 27°C, or artificially injected into adults of the flesh fly, *S. bullata*. Mortality was assessed 24 h later.

Total Protein Determination

Total protein in crude venom extracted from venom glands and venom reservoirs was determined colorimetrically using bovine serum albumin as standards. The mean protein content of venom reservoirs ($X \pm$ SEM = 1.48 ± 0.3 µg, n = 8 samples containing 450–500 venom reservoirs in phosphate isolation buffer) was significantly higher (t = 25.7, df = 7, $P < 0.001$) than that of venom glands (0.78 ± 0.2 µg, n = 8). However, this may simply reflect the difference in tissue size, and hence fluid volume, between glands and reservoirs.

TABLE 2. Analysis of Characteristic Infrared Absorption Bands Associated With Crude Venom From *N. vitripennis**

Frequency (cm^{-1})	Possible assignment
3,430	N–H stretching, secondary amines
2,930	Asymmetric C–H stretching of CH_2
2,362	CO_2
1,645, 1,513	C = O stretching, amides
1,456	C–N stretching
1,418	C–O–H in plane bending, carboxylic acids
1,340	O–H bending
1,058	P–O–H bending
996, 928	P–O stretching

*Interpretation of infrared absorption bands characteristic of crude venom as well as band assignments are based on 3 replicates of i.r. spectroscopic analysis using 50-µl lyopholized samples of crude venom reconstituted in isolation buffer (Fig. 1).

Infrared Spectroscopy

Infrared spectral analyses were performed to characterize the peptide and protein composition of wasp venom. The resulting infrared spectrum of crude venom from *N. vitripennis* is shown in Figure 2 with interpretation of characteristic absorption bands in Table 2. Absorption bands at 3,430, 1,645, and 1,513 cm^{-1} are consistent with the presence of secondary amine and amide groups, as would be expected for proteinaceous venoms (Stuart, 1997). The observed band at 2,930 cm^{-1} is characteristic of alkanes, while the vibration at 1,418 cm^{-1} reflects the acidic (carboxylic) nature of venom (Leonard, 1972). Absorption bands at 1,058, 996, and 928 cm^{-1} suggest that some venom components, possibly enzymes, contain phosphorous. The noticeable absence of bands at 3,600 (OH peak), 2,900 (C-H stretching), and 1,700 (C=O stretching) cm^{-1} (Fig.

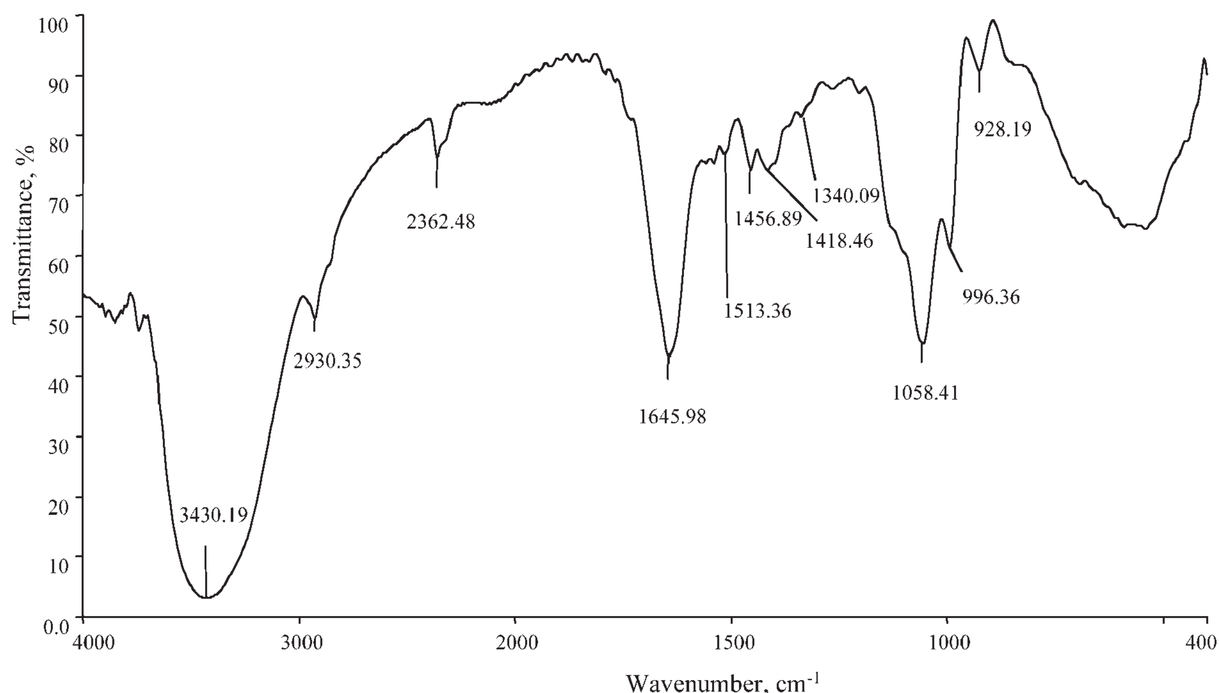

Fig. 2. Infrared radiation spectroscopic analysis of crude venom from *N. vitripennis*. Fifty microliters of crude venom in isolation buffer (pH 8.0) were used for i.r. analysis.

2) indicates that the contents of venom reservoirs lack a carbohydrate moiety (Fritz and Schenk, 1979; Stuart, 1997).

HPLC Analysis

Crude venom isolated from venom reservoirs was fractioned using a reversed phase C_{18} column. Based on the absorption peaks monitored at 280 nm, venom appears to be a complex mixture of peptides and proteins (Fig. 3), which was further supported by SDS-PAGE profiles of crude venom (Fig. 4). Comparisons made to the retention times of several internal standards suggest that apamin (Fig. 3, t = 9.90 min, peak 4) and histamine (Fig. 3, t = 25.38 min, peak 7) are major components of crude venom. However, by comparison to crude venom alone, confluent monolayers of BTI-TN-5B1-4 cells displayed very little sensitivity to pure apamin, and this toxin did not augment venom activity when co-incubated with *T. ni* cells (Table 3). Similarly, incubation of crude venom with anti-histamine or anti-phenoloxidase did not lower the toxicity of venom toward cultured cells,

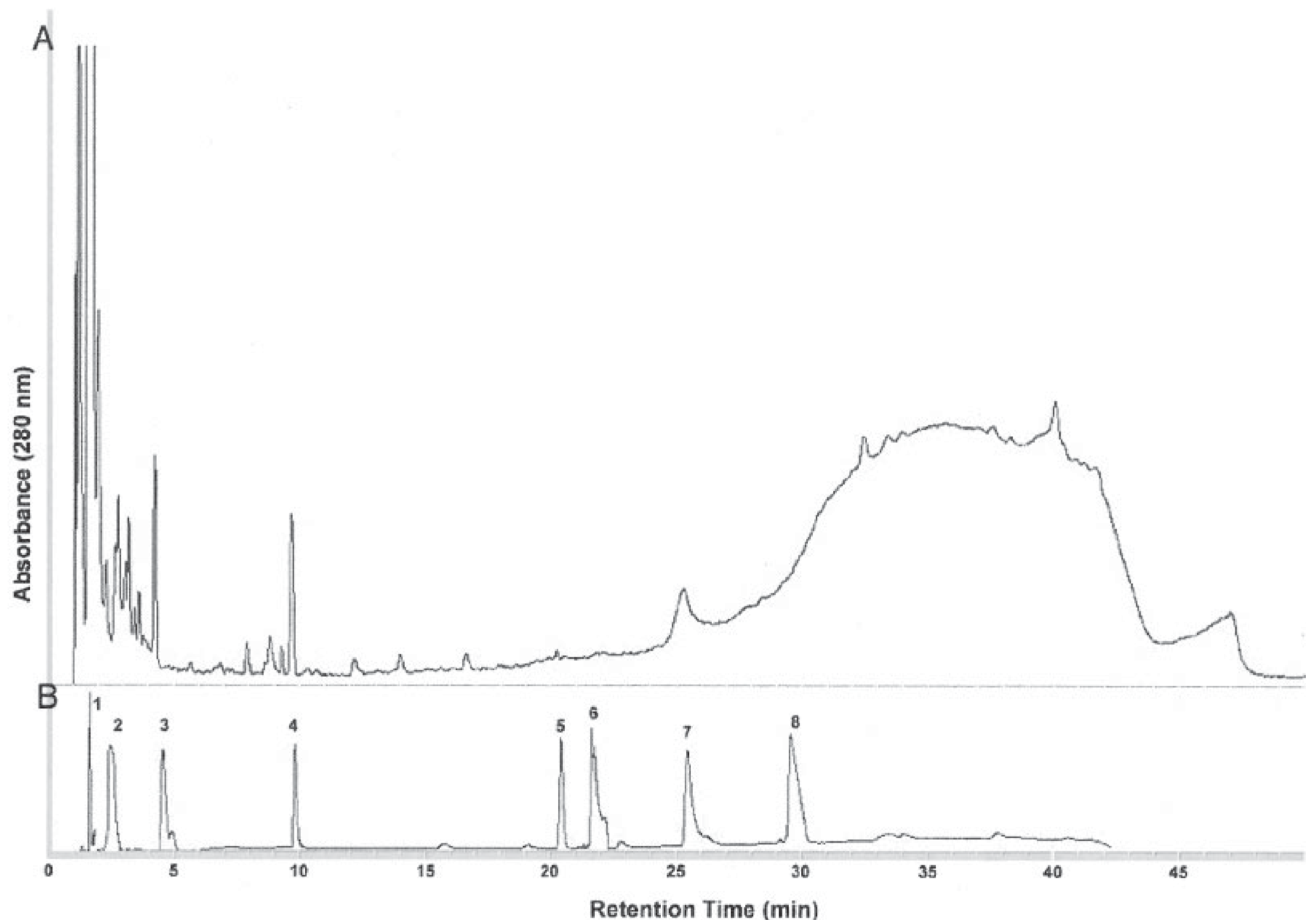

Fig. 3. Fractionation of crude venom (150 venom reservoir equivalents of lyophilized venom dissolved in 150 µl isolation buffer) from *N. vitripennis* by reversed phase HPLC (*Ace*lll C_{18} column, 12.5 cm × 4.0 mm). Eluent A: 0.1% TFA in acetonitrile: water (80:20); eluent B: 0.1% TFA in water. Fractionation was performed using a linear gradient of 5–80% A at 40 min with a flow rate of 1.0 ml/min and an injection volume of 20 µl. Absorbance was monitored at 280 nm for (A) crude venom and (B) standards (1) noradrenaline, 0.7 mg/ml; (2) dopamine, 10.3 mg/ml; (3) serotonin, 11.2 mg/ml; (4) apamin, 1 µg/µl; (5) phospholipase B, 0.5 µg/µl; (6) phospholipase A_2, 1 µg/µl; (7) histamine, 22.8 mg/ml; and (8) melittin, 1 µg/µl.

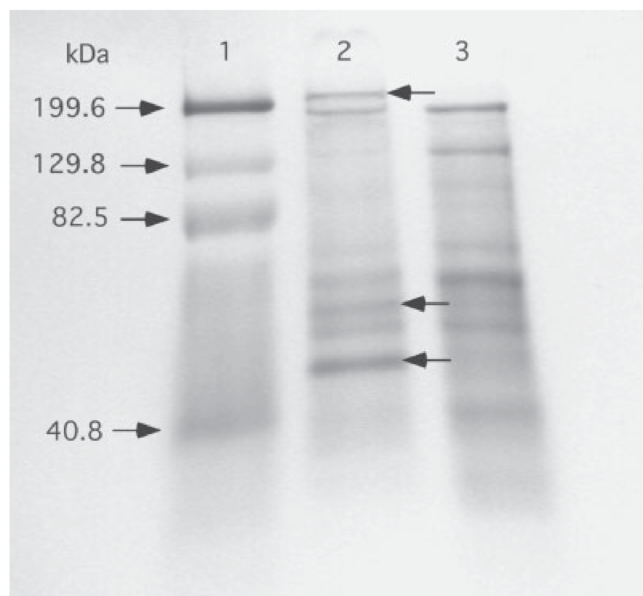

Fig. 4. SDS-PAGE analysis of crude venom from *N. vitripennis* using 10% Tris-HCl polyacrylamide gels (Bio-Rad). **Lane 1:** Molecular weight standards (10 μl), mysosin (199.6 kDa), β-galactosidase (129.8 kDa), bovine serum albumin (82.5 kDa), and carbonic anhydrase (40.8 kDa); **Lane 2:** Crude venom (30 μg) in isolation buffer; Lane 3: Crude venom (30 μg) in isolation buffer lacking sucrose. Proteins were visualized using 0.1% (w/v) Coomassie brilliant blue G-250 staining (Garfin, 1990).

even at antibody levels (1:10) that should have saturated binding sites on any venom proteins (Table 3).

TABLE 3. In Vitro Evaluation of Apamin, Histamine, and Phenoloxidase Activity in Crude Venom From *N. vitripennis**

Treatment	N	Cell death (%)
Untreated	9,708	1.2 ± 0.8^a
Saline (isolation buffer)	11,417	2.9 ± 1.3^a
Venom	10,046	100^b
Apamin (10 μg)	8,643	1.7 ± 0.9^a
Venom + apamin	12,479	100^b
Anti-histamine (1:10)	11,580	2.6 ± 0.5^a
Venom + anti-histamine	10,989	100^b
Anti-phenoloxidase (1:10)	9,056	3.8 ± 2.1^a
Venom + anti-phenoloxidase	10,047	100^b

*The possible presence of apamin, histamine, and phenoloxidase in crude venom was assessed in vitro by evaluating the insecticidal activity in the presence of apamin, anti-histamine, and anti-phenoloxidase. Assays used confluent monolayers of BTI-TN-5B1-4 cells and mortality was assessed at 24 and 48 h post-incubation using vital staining.

Salt Precipitation

Ammonium sulfate precipitation was used as a means to differentially isolate the venom proteins responsible for developmental arrest and death. Ammonium sulfate was added stepwise to crude venom (in phosphate isolation buffer) until 100% saturation was achieved, and then each fraction or cut was assayed for the ability to induce developmental arrest and death in young pharate adults of *S. bullata*, and also trigger cell death in vitro. Under these conditions, three salt fractions (5, 80, and 100%) displayed biological activity toward *S. bullata* in terms of halting fly development and evoking death, with the 80% cut most closely resembling crude venom (Table 4). Likewise, when the fractions was assayed in vitro using *T. ni* cells, all 3 fractions were cytotoxic, but the 80% cut was most lethal toward the cultured cells (Table 4).

SDS-PAGE analysis of the salt fractions indicated that the 5% cut had a nearly identical protein profile as crude venom (Fig. 5, Lanes 2–3). Protein bands with estimated molecular weights ranging from 13 to 146 kilodaltons (kDa) were detected in both crude venom and the 5% fraction (Fig. 5). However, two high molecular weight proteins (188.1 and 200.5 kDa) observed in crude venom were absent from the 5% cut, and the 200.5-kDa band was also not present when crude venom had been prepared in phosphate isolation buffer minus sucrose (Fig. 4).

The protein profile of the 80% ammonium sulfate fraction revealed partial purification of 4 proteins with apparent molecular masses of 125, 116.9, 100.3, and 67.7 kDa (Fig. 5). Two of these bands (67.7 and 100.3 kDa) were observed in the 100% cut, although the biological activity of this fraction was significantly less than that of the 80% cut in terms of inducing developmental arrest ($F = 132.5$, df = 8, 300, $P < 0.001$) and triggering death in vivo ($F = 99.2$, df = 8, 300, $P < 0.001$) and in vitro ($F = 92.4$, df = 8, 300, $P < 0.001$) (Table 4).

Membrane Separations

Fractionation of crude venom proteins was also attempted using centrifuge filters containing mem-

TABLE 4. Insecticidal Activity in Venom Fractions Separated by Ammonium Sulfate Precipitation*

Fraction	% Response				
	In vivo			In vitro	
	N	Developmental arrest	Death	N	Death
Saline	30	0^a	0^a	8,976	0^a
Crude venom	30	95.1 ± 6.1^b	100^b	7,760	100^b
Salt fraction					
5% AS	30	54.2 ± 5.0^c	80.4 ± 3.7^c	10,056	91.4 ± 4.5^b
15% AS	30	0^a	0^a	9,004	1.5 ± 0.8^c
25% AS	30	0^a	0^a	11,307	0^a
40% AS	30	0^a	0^a	8,925	1.1 ± 0.4^c
65% AS	30	0^a	0^a	8,458	0.8 ± 0.6^c
80% AS	30	93.2 ± 2.7^b	100^b	9,350	100^b
90% AS	30	0^a	0^a	10,336	2.3 ± 1.0^c
100% AS	30	63.4 ± 3.8^c	56.7 ± 4.8^d	11,974	64.8 ± 6.7^d

*Salt was added stepwise to crude venom from *N. vitripennis* until 100% saturation, and then the precipitate that formed with each fraction was assayed for biological activity. In vivo activity was assessed by injecting 1 µl of test sample into the dorsal surface of adult *S. bullata*, whereas in vitro activity was measured by incubating BTI-TN-5B1-4 cells with 1 µl of each salt fraction. Mortality was observed 24 h later.

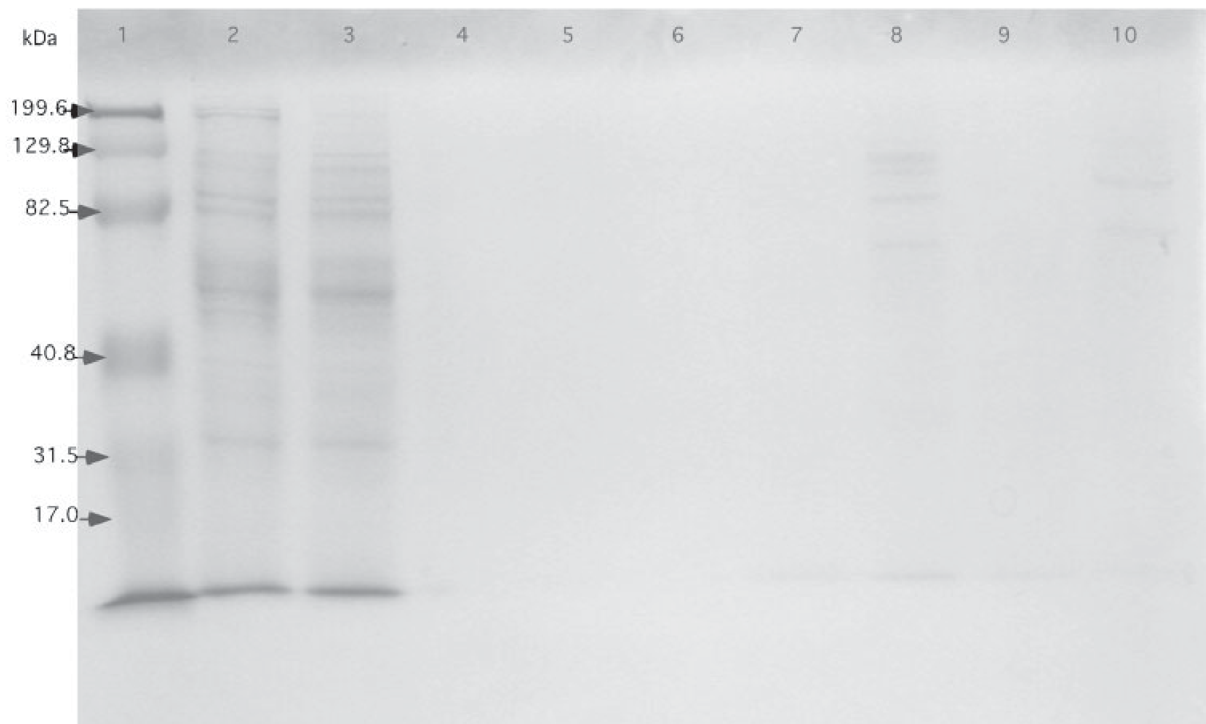

Fig. 5. SDS-PAGE analysis of ammonium sulfate fractionation of crude venom from *N. vitripennis* using 4–15% Tris-HCl polyacrylamide gels (BioRad). **Lane 1:** Molecular weight standards (10 µl), mysosin (199.6 kDa), β-galactosidase (129.8 kDa), bovine serum albumin (82.5 kDa), carbonic anhydrase (40.8 kDa), soybean trypsin inhibitor (31.5 kDa), and lysozyme aprotinin (17.0 kDa); **Lane 2:** Crude venom (30 µg) in isolation buffer; **Lane 3:** 5% ammonium sulfate (AS) cut (precipitate); **Lane 4:** 15% AS cut; **Lane 5:** 25% AS cut; **Lane 6:** 40% AS cut; **Lane 7:** 65% AS cut; **Lane 8:** 80% AS cut; **Lane 9:** 90% AS cut; **Lane 10:** 100% AS cut. Proteins were visualized using 0.1% (w/v) Coomassie brilliant blue G-250 staining (Garfin, 1990).

branes with specific size exclusion limits (10,000 to 100,000 MWCO). Retentates of all centrifuge membranes with MWCOs between 10,000–75,000 were highly toxic when injected into adult flies or exposed to confluent monolayers of BTI-TN-5B1-4 cells (Table 5). Only the 100-K MWCO retentate failed to display any lethal activity. By contrast, in vitro and in vivo toxicity were observed with filtrates from membrane filters with MWCOs ranging from 30,000–100,000 (Table 5).

When samples were assayed for the ability to induce developmental arrest in young pharate adults of *S. bullata*, retentates from all filters except the 100K MWCO membrane elicited a halt in fly development comparable to crude venom (Table 5). In contrast, only filtrates derived from 75- and 100-K MWCO membranes were capable of stimulating developmental arrest (Table 5). As a control, phosphate isolation buffer alone was loaded onto each type of membrane filter, and retentates and filtrates assayed. In all cases, no biological activity was observed.

SDS-PAGE analyses of the membrane fractions revealed that retentates from all membranes between 10–75-K MWCO had nearly identical protein profiles as crude venom (Fig. 6, only the 50- and 75-K MWCO membranes are shown as representations). The protein profile of the 50- and 75-K MWCO filtrate indicated partial purification of at least 3 proteins with apparent molecular masses of 125.7, 114.6, and 67.1 kDa (Fig. 6). This profile is nearly identical to that of the 80% ammonium sulfate fraction with the exception of a protein band of 100.3 kDa in the 80% cut. The latter protein may be particularly important for venom activity as filtrates from the 50- and 75-K MWCO membranes showed much lower biological activity than the 80% ammonium sulfate fraction (Tables 4 and 5).

DISCUSSION

During natural parasitism, *N. vitripennis* induces a developmental arrest in fly hosts that lasts until the fly is consumed by parasitoid larvae (Rivers and Denlinger, 1994a). Developmental arrest as well as death result from envenomation, and in the absence of feeding wasp larvae, the halt in fly development is sustained for an extended period of time (30–60 days at 25°C) (Rivers and Denlinger, 1995).

TABLE 5. Insecticidal Activity in Venom Fractions Separated by MWCO Membranes*

	% Response				
	In vivo			In vitro	
Fraction	N	Developmental arrest	Death	N	Death
Saline (sugar-free isolation buffer)	30	0[a]	0[a]	11,987	0[a]
Crude venom	30	97.1 ± 4.2[b]	100[b]	10,983	100[b]
MWCO					
10 K retentate	30	94.3 ± 6.7[b]	100[b]	12,348	100[b]
Filtrate	30	0[a]	0[a]	11,149	0[a]
30 K retentate	30	95.9 ± 3.4[b]	100[b]	10,806	100[a]
Filtrate	30	0[a]	56.1 ± 3.6[c]	8,359	71.9 ± 4.8[c]
50 K retentate	30	95.4 ± 5.5[b]	100[b]	12,007	100[b]
Filtrate	30	0[a]	47.8 ± 7.16[c]	9,939	65.9 ± 5.0[c]
75 K retentate	30	97.0 ± 3.7[b]	100[b]	10,035	100[b]
Filtrate	30	94.3 ± 4.2[b]	100[b]	11,972	100[b]
100 K retentate	30	0[a]	0[a]	8,365	0[a]
Filtrate	30	98.3 ± 6.8[b]	100[b]	12,894	100[b]

*Retentates and filtrates were assayed for insecticidal activity in vivo by injecting 1 μl of test sample into adult *S. bullata*, or examined in vitro by incubating BTI-TN-5B1-4 cells with 1 μl of each venom fraction. Mortality was observed 24 h later.

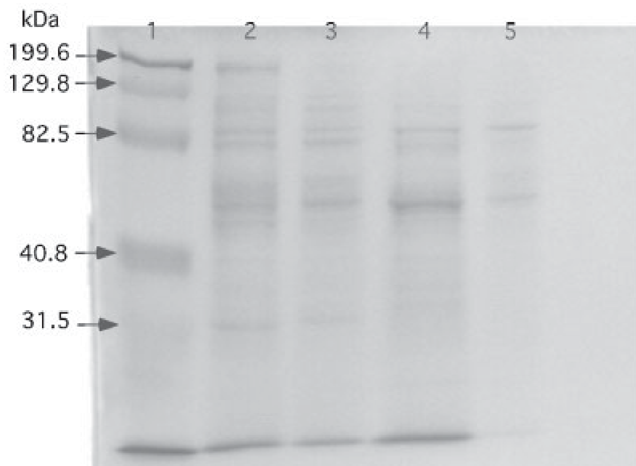

Fig. 6. SDS-PAGE analysis of MWCO (molecular weight cut-off) membrane fractionation of crude venom from *N. vitripennis* using 4–15% Tris-HCl polyacrylamide gels (BioRad). **Lane 1:** Molecular weight standards (10 µl), mysosin (199.6 kDa), β-galactosidase (129.8 kDa), bovine serum albumin (82.5 kDa), carbonic anhydrase (40.8 kDa), and soybean trypsin inhibitor (31.5 kDa); **Lane 2:** 75 K (MWCO) retentate; **Lane 3:** 50 K retentate; **Lane 4:** 75 K filtrate; **Lane 5:** 50 K filtrate. Proteins were visualized using 0.1% (w/v) Coommassie brilliant blue G-250 staining (Garfin, 1990).

Here, we show that the developmental arrestant and the agent(s) that induce host death are present in the venom gland and venom reservoir but not in other tissues associated with the female reproductive system. Psoralen treatment of crude venom preparations indicated that biological activity was associated with maternally derived secretions in the gland and reservoir and not attributed to products from symbiotic organisms (endosymbiotic viruses) as shown for some endoparasitic species (Quicke, 1997; Stolz and Whitfield, 1992) or ectoparasitoids harboring microsporidia (Geden, 1996). These observations are consistent with those of Ratcliffe and King (1967), who argued that contents of the venom gland (acid gland) alone are responsible for induction of death in adults of *M. domestica,* and that the venom reservoir stores venom in active form (Ratcliffe and King, 1969).

Despite this agreement, previous electrophoretic analysis of the wasp venom (Ratcliffe and King, 1967) revealed unique protein profiles for fluids from venom glands versus reservoirs, suggesting that venom is modified in the venom reservoir. Our study did not find any evidence to support the idea of venom modification after production in the acid gland. In fact, SDS-PAGE protein profiles were identical for crude venom prepared from venom glands and venom reservoirs. When venom was extracted from wasp tissues in buffer lacking a protein-stabilizing agent such as sucrose (at high concentrations), or enzyme inhibitors (Rivers, unpublished observations) of buffer pH was less than optima, biological activity of venom decreased. Based on SDS-PAGE analyses of crude venom prepared in sucrose-free buffer, the protein composition of venom was likely altered by these treatments. Venom analyses performed by Ratcliffe and King (1967) did not report controlling such factors when comparing the contents of venom reservoirs and glands. It is also important to note that these early investigations (Ratcliffe and King, 1967, 1969) failed to detect developmental arrest as a feature of envenomation, largely because non-natural host developmental stages (adult) were used in the biological assays. Previous characterization of the insecticidal properties of venom glands and reservoirs (Beard, 1964; Ratcliffe and King, 1967; Tiegs, 1922) must also be interpreted cautiously as the method (i.e., dipping an insect pin in venom prior to pricking the body) of venom injection was not necessarily quantifiable or consistently reproducible.

Biochemical and electrophoretic analyses of crude venom indicate that venom is predominantly composed of mid to high molecular weight proteins that are acidic in nature. This range of protein sizes as well as complexity of the protein profile in terms of total number of proteins is consistent with most parasitic wasp venoms that have been examined (Digilio et al., 2000; Leluk et al., 1989; Nakamatsu and Tanaka, 2003; Parkinson et al., 2002a; Uçkan et al., 2004). By contrast, low molecular weight proteins and peptides are typical of venoms of the aculeate Hymenoptera (Leluk et al., 1989; Schmidt, 1982), as are proteins abundant in neutral and basic amino acids (Piek and

Spanjier, 1986). Infrared spectral analyses suggest that venom proteins do not possess a carbohydrate moiety, a feature shared with venom from *P. turionella* (Uçkan et al., 2004) and many social hymenopterans (Leluk et al., 1989) but unique from other parasitic wasp venoms that appear to be rich in glycosylated proteins (Leluk et al., 1989; Piek and Spanjier, 1986). The infrared spectrum also revealed the presence of phosphorus-containing structures, typical of enzymes, in the venom. Though the presence of enzymatic activity in *N. vitripennis* venom has yet to be confirmed, venoms from several endoparasitic species and social Hymenoptera have been shown to contain multiple types of enzymes (Moreau et al., 2004; Parkinson et al., 2001; 2002a,b; 2003; Piek and Spanjier, 1986; Schmidt, 1982; Uçkan et al., 2004). Enzyme activity, however, does not likely account for the ability of venom from *N. vitripennis* to arrest host development or evoke death as crude venom preparations contained the protease inhibitor PMSF as well as the chelating agent EDTA, and high concentrations of anti-phenoloxidase antibodies had no influence on venom activity.

Venom stability at alkaline pH is in agreement with wasp toxins identified in venom from the ectoparasitoids *H. hebetor* (Quistad et al., 1994) and *E. comstockii* (Coudron and Brandt, 1996). *H. hebetor* produces a potent venom with at least two highly paralytic protein toxins (Brh-I and Brh-V, estimated molecular masses 71–73 kDa) (Quistad et al., 1994) and one smaller, less insecticidal toxin (ca. 20–40 kDa). All three proteins appear to be glycosylated. A 66-kDa venom protein has been isolated from *E. comstockii* that does not induce paralysis but does arrest host development by inhibiting larval–larval ecdysis (Coudron and Brandt, 1996). This arrestment protein is thought to be comprised of at least two subunits, suggesting that the native protein is possibly a dimer composed of two 31–33-kDa proteins (Coudron and Brandt, 1996). Size and activity estimates for venom proteins from these two parasitoids are in agreement with SDS-PAGE profiles for partially purified venom proteins from *N. vitripennis:* ammonium sulfate and MWCO membrane fractions of crude venom yielding biological activity (induction of developmental arrest and death) all contained a protein with an estimated molecular weight of 67–70 kDa. It seems highly improbable, however, that a single protein in venom from *N. vitripennis* is responsible for inducing both host arrestment and death. Indeed, though a protein band corresponding to approximately 67 kDa was observed in the 100% ammonium sulfate fraction, this salt cut induced developmental arrest in significantly fewer pharate adults of *S. bullata* than the 80% fraction that possessed two additional high molecular weight proteins (114.6 and 125.7 kDa). A similar reduction in toxicity, as well as ability to arrest fly development, was observed with venom fractions (filtrates from the 50- and 75-K MWCO) isolated by centrifugal membranes with correspondingly similar but not identical protein profiles to the 80% salt cut: a 100.3-kDa protein was detected in the salt fraction in addition to 3 proteins present in the membrane fractions. Collectively, these observations argue that multiple proteins are required for full venom activity. This certainly is consistent with the complex nature of parasitic wasp venoms that have been characterized and the milieu of host effects that they evoke (Quistad et al., 1994; Parkinson et al., 2002a,b, 2003).

Some lethal activity was also detected in filtrate collected from the 30-K MWCO membranes. However, this toxicity was significantly less by comparison to filtrates from membranes with molecular cutoffs higher than 75 K and fly development was not arrested by these samples. Surprisingly, no protein bands were observed by SDS-PAGE that corresponded to the filter size exclusion limits. Failure to detect proteins smaller than 30 kDa may reflect protein levels below the detection limits of the Coomassie staining (Garfin, 1990). It is also possible that the actual lethal protein is larger than the size cutoff limit of the membranes used since, like dialysis membranes, the listed size represents a median pore size, with both larger and smaller pores also present on the membranes (Pohl, 1990). Consequently, reduced venom activity may be attributed to a small amount of the protein passing through the membrane. SDS-PAGE analyses sup-

port the latter scenario for both the 30- and 50-K MWCO membranes.

HPLC separations of crude venom coupled with the use of internal standards suggested that low molecular peptides or proteins exist in venom in the form of apamin and histamine. It is not surprising, then, that neither were detected electrophoretically since their low molecular masses would allow them to migrate much more rapidly than other venom proteins in 10–12% acrylamide gels (Garfin, 1990). Though these paralytic agents are common in venoms from social Hymenoptera (Piek and Spanjier, 1986) and the endoparasitoid *P. turionella* (Uçkan et al., 2004), biological assays using cultured cells indicate that either apamin or histamine do not affect the cells tested or neither was present in active form within venom from *N. vitripennis*. This finding is not unexpected considering that apamin and histamine are typical of paralytic venoms used primarily for defense (Blum, 1981; Schmidt, 1982). In contrast, venom from *N. vitripennis* is non-paralytic (Rivers and Denlinger, 1994a), and is used to manipulate a non-mobile host (pupa) to maximize progeny production (Rivers et al., 1998). Clearly, additional studies are needed to further characterize and isolate venom proteins. The stability of venom at alkaline pH suggests that anion-exchange chromatography is a viable next step to purify individual venom proteins (Quistand et al., 1994).

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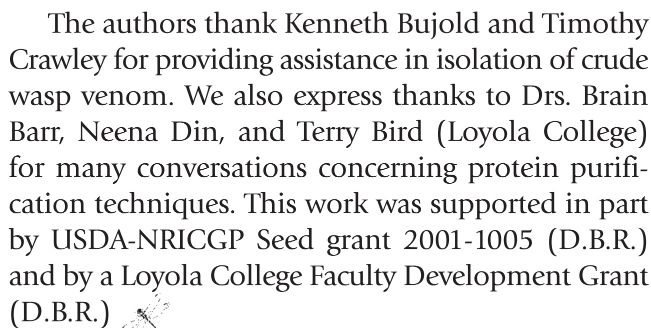

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