

SWEET BRIAR COLLEGE



3 2449 0407160 /



Digitized by the Internet Archive
in 2010 with funding from
Lyrasis Members and Sloan Foundation

<http://www.archive.org/details/characterization00unge>

Characterization of the Venom of *Glycera dibranchiata*

A Senior Honors Thesis in the Department of Biology, Sweet Briar College

by

Natasha K. Ungerer

Defended and Approved 08 April 2002

Prof. Simpson

date

Prof. Rosinski

date

Prof. XXX

date

ABSTRACT

Venom extract from *Glycera dibranchiata* was characterized using size-exclusion gel chromatography. Select fractions were assayed on fiddler crabs for the presence of lethal components. The results were compared to previous work with the venom of *G. convoluta* in an attempt to clarify the relationship between the venoms of these two species. Two protein peaks were evident in *G. dibranchiata* venom, with average molecular weights of 660,00 and <1,000, while two points of high lethality were observed. The first lethality peak, from molecular weights of 11,000-43,000 is independent of either protein peak, while the second lethality peak, from 2,500-6,000, roughly corresponded to the beginning of the second protein peak. There is the suggestion of another protein peak between the first and second peaks, but its molecular weight cannot be estimated because its actual peak is not evident. It can be concluded that the venoms of *G. dibranchiata* and *G. convoluta* have similar elution profiles but the molecular weights of their protein and toxic constituents differ. In addition, *G. convoluta* venom has four distinct protein peaks, while only two were evident for *G. dibranchiata* venom.

INTRODUCTION

Background

Glycera dibranchiata (Fig. 1) is a member of the phylum Annelida and the family Glyceridae. *G. dibranchiata* is commonly known as the “bloodworm” due to the hemoglobin contained in its coelomic cavity that gives its body a red color (Klawe & Dickie 1957). Bloodworms are most commonly found in the soft muds of intertidal

regions of the Atlantic and Pacific coasts (Klawe & Dickie 1957), but may also be found below the low-water mark in some areas (Verrill 1873, cited by Klawe & Dickie 1957). Although they are considered errant polychaetes, it is well-documented that bloodworms create burrows for themselves and spend the large majority of their time in the substratum (Klawe & Dickie 1957).

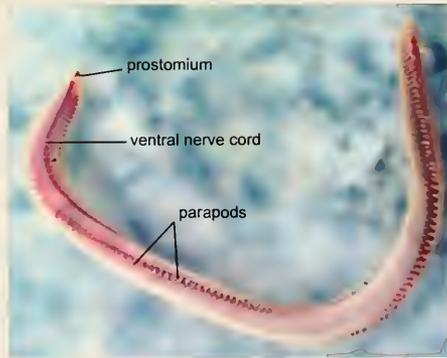


Figure 1. *Glycera dibranchiata*, proboscis retracted.

The anatomy of *G. dibranchiata* was first described by Ehlers (1868), and later by Klawe & Dickie (1957). According to the latter report, *G. dibranchiata* can reach lengths of up to 37 cm, though is more commonly about 15 cm in length, with approximately 300 segments in a 20 cm specimen. They are circular in cross-section and both anterior and posterior ends taper to a point. The anterior end, called the prostomium, is distinguishable from the posterior end by presence of a small brain, visible as a small

black dot beneath the body wall. Four small antennae are present at the anterior tip. The posterior end terminates at the pygidium with two caudal cirri. It is questionable whether *G. dibranchiata* has an anus and, if so, whether it serves a function or not (Simpson, personal communication). Lacking a true vascular or blood system, glycerids accomplish circulation by body wall contractions and ciliated tracts in the parapodial gills and on coelomic surfaces .

The maximum life span of *G. dibranchiata* is about five years, with the average probably between three to four years (Klawe & Dickie 1957). The sexes are separate and mature worms undergo either spermatogenesis or oogenesis (Simpson 1962b). As gametogenesis takes place, the adult worm undergoes epitoky, morphological changes including a decrease in thickness of the body wall and gut diameter along with an increase in coelomic volume due to the release of gametes into the coelomic cavity (Simpson 1962b). This is accompanied by the presence of irregular black masses up to 2 mm in diameter found in the coelomic cavity, thought to be a breakdown product of the digestive tract or other tissues (Klawe & Dickie, 1957; Simpson 1962b).

Simpson (1962a) reported that as gametogenesis nears completion, masses of sexually mature bloodworms abandon their muddy burrows and swim to the surface to release their gametes, a process called swarming. After release of gametes, it is unlikely that the adult worm will survive, due to the extensive breakdown of tissues (Simpson 1962b). Simpson was able to rear larvae to the trochopore stage, but due to difficulties in maintaining the larvae past this stage, little is known about the development and maturation of *G. dibranchiata*.

Because *G. dibranchiata* is commonly found in organically rich, muddy intertidal zones, it has long been considered a detritus feeder (Klawe & Dickie 1957). However, Ehlers (1868), the first to characterize *G. dibranchiata*, thought that the species could be carnivorous due to the presence of jaws and associated venom glands. In addition, Ockelmann & Vahl (1970) found that *G. alba*, a close relative of *G. dibranchiata*, fed on other errant polychaetes as well as amphipods. An unpublished student report (Cates, 2001) describes finding acicula and bundles of setae in the gut of *G. dibranchiata*, as well as an observation of a bloodworm ingesting a small nereid.

Glycera species are unique for possessing a very large, eversible proboscis, used primarily for burrowing and likely for feeding (Klawe & Dickie 1957, Ockelmann & Vahl 1970). Heacox (1974) describes in great detail the structure of the proboscis. According to Heacox (1974), Wells (1937) was the first to divide the proboscis into three regions: an eversible buccal portion, a pharynx with jaws and associated venom glands, and the esophagus, which is folded into an S-shape in the inverted position (Fig. 2).

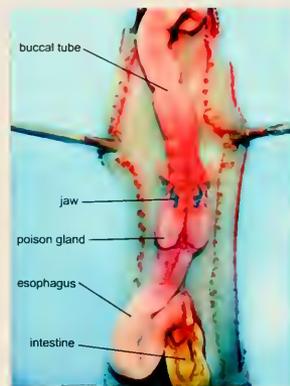


Figure 2. The dissected anterior third of the digestive tract of *G. dibranchiata*.

When the proboscis is inverted, the buccal tube is the most anterior portion of the proboscis, and extends posteriorly from the mouth for approximately 20 segments before joining with the pharynx (Gravier 1898, Wells 1937, cited by Heacox 1974). The junction of the buccal tube with the pharynx is marked on the coelomic surface by the presence of four lobed membranes called languettes, and on the luminal surface by four jaws. In the everted proboscis, the buccal tube turns inside out, the esophagus unfolds, and the pharynx becomes the most anterior part of the worm (Fig. 3).

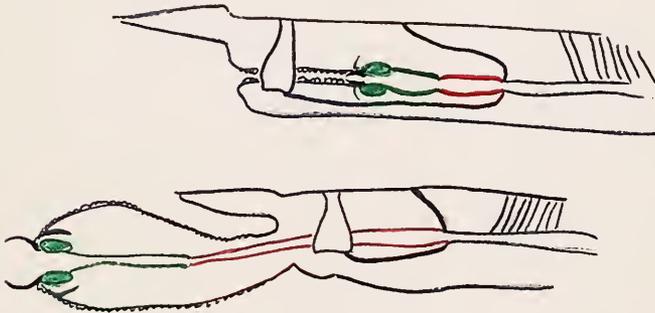


Figure 3. A schematic diagram of *G. dibranchiata* showing the inverted (top) and everted (bottom) proboscis. The pharynx is represented in green, and the esophagus in red.

The pharynx is the shortest of the three proboscis regions, approximately 10 mm long, and appears swollen due to the venom gland complex, consisting of four glands, ducts, and jaws surrounded by muscle. Muscular pads containing the venom glands appear as one dorsal and one ventral pair, each lying on a “corner” of the pharynx (Heacox 1974). The junction of the pharynx and esophagus is evident by a slight constriction where four broad, flat, longitudinal muscles, each extending from the posterior end of each poison gland, join together to become the beginning of the outer wall of the esophagus (Heacox 1974).

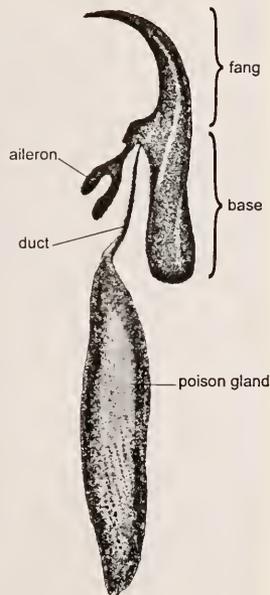


Fig. 4. The venom gland complex (diagram after Ehlers 1868).

The structure of the pharynx can be further broken down into three parts, a pharyngeal wall that forms part of the digestive tube, the poison-gland complexes attached to the pharynx, and delicate lobed membranes of unknown function protruding into the coelem (Heacox 1974). Each venom gland complex (Fig. 4) consists of a jaw and attached aileron, the venom gland and duct connecting gland and jaw, and the surrounding muscles (Heacox 1974).

Each jaw can be divided into two regions, the jaw proper and the attached aileron, a small y-shaped protrusion embedded in muscle that aids in the attachment of the jaw to the surrounding muscle (Heacox 1974). The jaw proper consists of a base and the fang. The base of the jaw is embedded in connective tissue of the anterior pharynx, while the fang protrudes from between the pharyngeal lips almost perpendicular to the associated gland complex.

Two openings are evident at the base of the jaw, one termed the myocoel, and the other the venom duct canal (Andolshek 1999). The duct is approximately one-fourth the length of the gland and tapers towards the anterior end (Heacox 1974). The myocoel has been found to contain a core of muscle and likely terminates two-thirds of the way up the jaw (Andolshek 1999). Though the myocoel was originally thought to be the venom duct canal by Michel (1970), recent studies have suggested otherwise. Based on scanning-electron microscope studies (Andolshek 1999, Alongi 2000), it has been confirmed that the duct enters the jaw through a small opening on the aileron side of the jaw. These SEM studies show that the duct has many regularly-spaced pores on the outer curvature of the jaw. These pores appear plugged with tissue, with the exception of those near the tip of the fang (Fig. 5). Agreeing with Wolf (1977) and Charletta & Boyer (1974),

Andolshek (1999) states that it is likely that the duct terminates before the open pores, while the pores that appear plugged are bound to the duct. Based on this observation, it seems probable that the jaw has several sites for venom release.

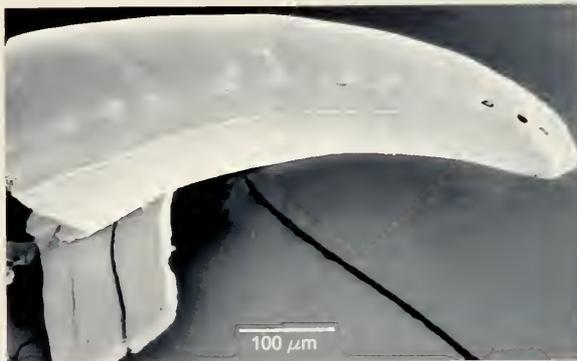


Figure 5. A SEM photograph of a fang of *G. dibranchiata*, demonstrating the plugged pores along the outer curvature of the jaw, and the open pores near the tip of the fang.

The venom gland (Fig. 6) itself is cylindrical in nature, with a diameter of 0.75-1.0 mm and length of 2.0-2.6 mm. The wall of the gland is made up of long, narrow cells which taper slightly towards the lumen (Heacox 1974). These cells are composed almost entirely of an apical vacuole, with the remaining cellular constituents pushed into the

base of the cell (Heacox 1974). There appear to be three types of secretory cells, but there is some debate on whether they are actually three distinct types (Michel 1966, cited by Heacox 1974), or rather one cell type in different stages of the secretory cycle.

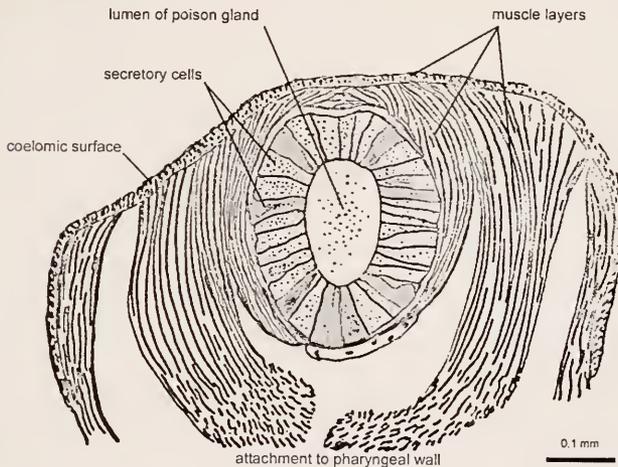


Figure 6. A cross section of a venom gland of *G. dibranchiata*, demonstrating the secretory cells surrounding the lumen.

Toxins are relatively common among marine invertebrates. Poisons and venoms are distinguishable based on the mode of delivery; poisonous animals typically lack a distinct device for delivery, though their tissues are either partially or entirely toxic to predators, while venomous animals have a special apparatus that synthesizes and delivers

the venom (Edstrom 1992). Venoms can be used to capture prey, or can be defensive and serve to deter predators (Edstrom 1992).

Venoms often contain a variety of components that in conjunction cause a toxic reaction in other animals; among the most common components are neurotoxins, toxins that act on the nervous system and affect synaptic transmission, often at the neuromuscular junction (Edstrom 1992). Neurotoxins can act presynaptically, postsynaptically, or by a variety of other mechanisms. A presynaptic neurotoxin alters the terminal axon and affects the release of acetylcholine (ACh), while postsynaptic neurotoxins bind to ACh receptors, or nearby receptors, on the muscle end plate and block the arriving signal (Edstrom 1992). Both presynaptic and postsynaptic neurotoxins cause eventual paralysis by disrupting neuromuscular transmission.

Michel (1966) gives the earliest published account of the toxic effects of *G. convoluta* venom, reporting it to be lethal for crustaceans. Several studies of *Glycera* venom since 1966 have focused on the mechanism of action, especially that of *G. convoluta*, a European relative of *G. dibranchiata*. The venom of *G. convoluta* has been shown to trigger Ca^{++} -dependent acetylcholine (ACh) release in *Torpedo* electric organ preparations (Morel *et al.* 1983, Manaranche *et al.* 1980, Thieffry *et al.* 1982) and rat brain synaptosomes (Maddedu *et al.* 1984). Additionally, *G. convoluta* venom increases spontaneous quantal transmitter release at cholinergic and non-cholinergic nerve terminals (Bon *et al.* 1985, Manaranche *et al.* 1980, Thieffry *et al.* 1982). These reactions suggest the presence of a presynaptic neurotoxin.

The overall action of *Glycera* venom has been shown to cause immobilization and a slow paralysis of the prey, along with mild disintegration of its collagenous and protein

structure when administered intramuscularly to crustaceans (Michel & Keil 1975). This effect is likely due to the existence of at least one neurotoxin, as well as proteolytic enzymes that aid in tissue hydrolysis.

Michel & Keil (1975) fractionated *G. convoluta* venom using Sephadex G-75 (fractionation range 3,000-70,000) and Sephadex G-200 (5,000-2.5 x 10⁵). Using the Sephadex G-75, they found two large protein peaks, one emerging with the void volume and one of very low molecular weight. Fractionating with Sephadex G-200, they found four protein peaks, and assaying select fractions for toxicity, they found a major peak of toxicity between 110,000-120,000. Again, a protein peak of high molecular weight (>100,000) and a peak of low molecular weight were found, with two very small peaks in between these larger peaks. It is also evident that the venom of *G. convoluta* contains protease and phospholipase activity that is distinct from the toxic effects of other venom components (Bon *et al.* 1985, Michel & Keil 1975, Thieffry *et al.* 1982).

Much of the research on *G. convoluta* venom has focused on α -glycerotoxin, a proteinaceous presynaptic neurotoxin of molecular weight $300,000 \pm 20,000$, that is distinct for its ability to cause an increase in quantal transmitter release at frog or crayfish neuromuscular junctions (Bon *et al.* 1985). Using gel-filtration chromatography with Bio-Gel A 1.5m, Bon *et al.* (1985) separated *G. convoluta* venom into six distinct fractions (A-F, Fig. 7). Fraction C, containing glycerotoxin, was concentrated using ion exchange chromatography and α -glycerotoxin was partially purified from the crude venom extract. Fraction C was toxic for both crustaceans and mice by causing progressive paralysis. Fraction D contained proteins ranging in molecular weights from 60,000-150,000 and induced sudden convulsions prior to death when injected into

crustaceans, but was not toxic for mice. This suggested the presence of two discrete toxins, one contained in fraction C that was toxic for both mice and crustaceans, and another contained in fraction D, which was toxic only for crustaceans.

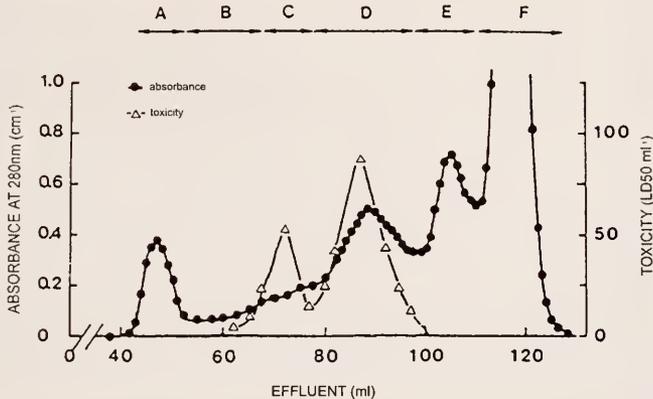


Figure 7. Bon *et al.*'s (1985) results from fractionation of *G. convoluta* crude venom extract on Bio-Gel A1.5m gel. Selected fractions were assayed for toxicity in shrimp.

Although *G. convoluta* is a close relative of *G. dibranchiata*, several researchers have suggested that these two venoms act in different manners and may not contain the same toxin(s) (Bon *et al.* 1985, Morel *et al.* 1983, Maddedu *et al.* 1984). Based on personal communication with Thieffry and Manaranche (unpublished), Bon *et al.* (1985) report that *G. dibranchiata* venom is devoid of α -glycerotoxin because it does not cause an increase in quantal transmitter release at neuromuscular junctions. In addition, Bon *et al.* (1985), Kagan *et al.* (1982), and Maddedu *et al.* (1984) have suggested that one of the

active components in the venom of *G. dibranchiata* may be closely related to that of α -latrotoxin (a component of black widow spider venom).

The only published report of study of *G. dibranchiata* venom is a highly detailed account of the effect of the venom on lipid bilayers, which found that the venom causes formation of ion-permeable channels in the membrane (Kagan *et al.* 1982). In a Senior Honors Thesis project, Goracci (1979) worked on a characterization of *G. dibranchiata* venom, using gel-filtration chromatography and various protein assays. Comparing her results to those obtained by Michel & Keil (1975) for *G. convoluta*, Goracci concluded that the protein content of the venoms of the two species was very similar, if not the same. Goracci (1979) found four protein peaks, at molecular weights of >232,000, 160,000, 121,000, and 31,000, as well as two trypsin-active peaks, at 200,000 and 128,000. However, she did not assay the fractions for their effects on fiddler crabs, so no conclusion could be made regarding the molecular weight of the toxic components.

A requirement for any investigation on a venom is a way to measure its toxicity. Unpublished student research at Sweet Briar College has previously used *Uca* (fiddler crabs) as the assay organism when working with the venom of *G. dibranchiata*. Because fiddler crabs are rather expensive and require relatively large doses of the crude venom extract to elicit lethal effects, several student projects attempted to find a more suitable assay organism. Ungerer (2001), Hager (1998), and Beck (1995) have previously attempted to find another suitable assay organism, testing a variety of insects and crustaceans. Ungerer (2001) attempted to assay the venom on crayfish, crickets, beetles, and isopods (land-dwelling crustaceans), as well as fiddler crabs. It was determined that

fiddler crabs remained the most logical assay organism and would be used for further studies.

Based on Goracci's (1979) results indicating a similarity between the venoms of *G. dibranchiata* and *G. convoluta*, and in light of the debate regarding the presence or absence of α -glycerotoxin in the venom of *G. dibranchiata*, this study was undertaken for the purpose of characterizing the protein and toxic components of the venom of *G. dibranchiata* using gel-filtration chromatography.

MATERIALS AND METHODS

Specimens

Bloodworms were obtained by overnight shipment from Maine Bait Company (Newcastle, Maine). Upon arrival, the worms were placed in groups of ten in Nalgene, 12" x 9" containers with an approximately 2cm depth of Instant Ocean. The containers were stacked and placed in the cold room (10°C). The Instant Ocean was changed every two days and the worms were dissected within two weeks of arrival.

Fiddler crabs (*Uca pugilator*) were obtained from Gulf Specimen Marine Laboratories (Panacea, Florida) and were stored in groups of approximately 15 in 12" x 9" Nalgene containers with a 2cm depth of Instant Ocean and plant material they had been shipped in. The crabs were fed a crustacean food from Carolina Biological every other day. Several small crumbs of the food were placed in the Nalgene containers for several hours, after which the containers were rinsed and new Instant Ocean added. Because the crabs were never observed eating the crustacean food, small pieces of dried cat food were used in place of the crustacean food; however, they were not observed

eating this either. The crabs were kept in the cold room (10°C) and were used for assay within three weeks of arrival.

GENERAL METHODS

Preparation of Extracts

In obtaining the control and venom extracts of *G. dibranchiata*, the basic methods of Goracci (1979) were followed with the following changes:

1. The tissues were frozen in the vapor phase of a liquid nitrogen tank for 1-5 days prior to sonification because freezing helps to fracture the tissues and therefore facilitates the sonification process.
2. An amount of Instant Ocean approximately equal to the amount of tissue was added prior to sonification to ease the sonifying process and lessen the amount of heat buildup created by sonification (i.e. 0.5mL Instant Ocean added to 0.5g tissue)
3. Instead of crushing the samples with a mortar and pestle and adding abrasive sand as needed, the tissues were brought to fluid consistency using a Model 250 Branson Sonifier. Glass vials approximately 2" tall and with a mouth diameter of approximately 1/2" were used to sonify the tissues.
4. 160 venom glands required sonification for approximately 8 minutes at 10% duty control and "2" output control. The comparable amount of muscle required 5 minutes at 20% duty control and "2" output control followed by approximately 5 minutes at 10% duty control and "4" output control. The glass container was kept in the beaker, on ice, for approximately the first four minutes of sonification. Then, to ensure that all the tissue was liquified, the sonifier was turned off and the bottle removed from

the beaker of ice. It was then held manually, and the sonifier tip inserted into the bottle, almost touching the bottom of the bottle. In this way, the tip could be pressed against specific pieces of tissue that had not been liquified previously.

5. Samples were centrifuged for approximately 1-1/2 hours at 20,000 rpm with an SS-34 rotor at approximately 7°C.

Gel-filtration Chromatography

Size-exclusion gel chromatography was used to separate the components of the venom extract. A Bio-Rad column 50 cm in length and 1.5 cm in diameter was filled to approximately 10 cm from the top of the column with Bio-Gel A 1.5m fine grade, the chromatography gel used by Bon *et al.* (1985), which has a fractionation range of <10,000-1,500,000. A 0.050 M sodium phosphate/potassium phosphate buffer of pH 7 was used to elute the samples. To determine the molecular weight of the venom fractions, protein standards, consisting of thyroglobin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1,350), obtained from Bio-Rad were used to standardize the column. An aliquot of 0.25 mL was applied to the column and eluted at a flow rate of 0.15 mL/. Two runs, collected in 1.18 mL and 1.8 mL fractions, respectively, were averaged and used to calibrate the column. Blue dextran, a polysaccharide of molecular weight 2,000,000, was used to determine the void volume (the elution volume of a molecule entirely excluded from the gel) of the column. Based on the void volume of blue dextran, (V_o), and the elution volumes, (V_e), for the five protein standards, a graph was created using V_e/V_o versus log molecular weight

(Table 1 and Fig. 5), and was used to determine the molecular weights of any unknown molecules eluted through the column.

Table 1. Proteins used to calibrate the column.

Protein	MW	V_e/V_o
Thyroglobin	670,000	1.02
Gamma globulin	158,000	1.50
Ovalbumin	44,000	2.08
Myoglobin	17,000	2.28
Vitamin B-12	1,350	2.63

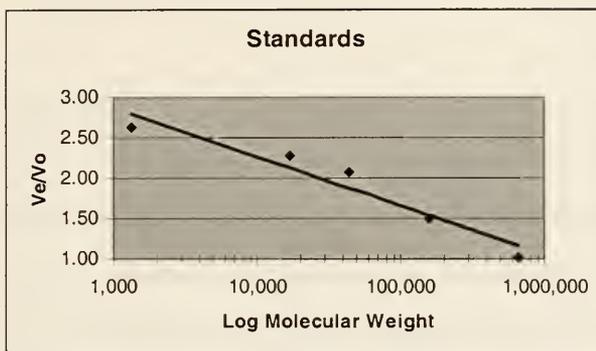


Figure 8. The average of two runs with five proteins ranging in molecular weight from 1,350 to 670,000 used to standardize the chromatography column, with trendline.

The venom extract sample was then applied to the column and fractions were collected at a flow rate of approximately 0.15 mL/min. The amount of sample applied and the fraction amount varied with the runs depending on the purpose of the run, as detailed below.

A UV monitor connected to the chromatography apparatus recorded the absorbency of each fraction as it was eluted. However, the UV monitor was recording with a delay when compared to readings taken by the Beckman DU 640

Spectrophotometer set at 280 nm, the absorbance for proteins, so protein content was determined spectrophotometrically from this point on. It seems likely that as the effluent traveled through the UV monitor its absorbance was recorded, but this same volume of effluent had to then travel approximately 15 cm to the fraction collector, causing the absorbency and fraction recordings to not correlate. It would likely be possible to arrange the tubing and UV monitor in a position so that the effluent would travel a shorter distance to the fraction collector, allowing for a more accurate printout from the UV recorder.

Concentration of Fractions

Following chromatography and absorbency determination, fractions were selected for assay based on the methods of Bon *et al.* (1985), who assayed fractions beginning before the first protein peak, and through the final protein peak. Fractions directly preceding, following, and in between the two protein peaks were concentrated and assayed. Concentration of the fractions was necessary to remove buffer that had been used to “push” the venom components through the column; this was accomplished with Millipore Centriprep YM-30 centrifugal filter devices.

Assay of Toxicity

This procedure was based on methods employed in previous venom studies (Goracci 1979, Hager 1998, Page 1994). Each crab was positioned on its dorsal surface with its legs gently spread out and held to the side. A Tuberculin syringe with a 28-1/2 gauge needle was used to inject the crabs at the ventral joint membrane at the base of the

second walking leg on the right side (Page 1994). Following injection, the crabs were placed in a large culture dish containing a depth of approximately 1cm Instant Ocean for observation for approximately 10 minutes and then placed in individual finger bowls and returned to the cold room. Symptoms of toxicity have been described in detail by Matheson (1997) and include the drooping and falling of eyestalks, the flexion up or rotation out of walking legs, and overall lethargy. The effect of the injected sample was determined to be lethal if the crab was dead after 24 hours.

CONTROL

Several *Uca* were initially injected with a dose of 6 mg venom gland/g crab of the crude venom extract prior to chromatography to ensure the toxicity and effectiveness of the preparation. All control crabs exhibited typical symptoms of envenomation and died within 24 hours. Several crabs were injected with 0.1 mL of buffer solution to ensure that it caused no side effects. Aside from mild bubbling or foaming from the mouth, which is a typical sign of injection, these crabs exhibited no signs of envenomation.

EXPERIMENTAL PROCEDURES

Table 2 summarizes the six experiments performed.

Table 2. Characteristics of venom runs.

Run	Sample Size (mL)	Fraction Size (mL)	Concentrated	Assay		
				Fraction #s	# crabs/fraction	Dose (mL)
1	1.1	3.6	No	19-23	2-3	0.1
2	1	3.6	No	-	-	-
3	1	3.6	Yes	8-11, 14-23	3	0.05
4	1	3.6	Yes	9-24	5	0.05
5	0.15	2.7	No	-	-	-
6	1	2.7	Yes	9-27	3-4	0.05-0.06

Run 1

The purpose of this run was to use a large sample of crude venom extract so that there would be sufficient amounts of venom components present after fractionation. A volume of 1.1 mL of crude venom extract was eluted through the column bed and 3.6 mL fractions were collected. Protein content was determined spectrophotometrically and fractions 19-23 were assayed by injection into two or three crabs per fraction, with a standard dose of approximately 0.1 mL per crab. The fractions were not concentrated because Centriprep tubes had not arrived yet.

Run 2

A volume of 1 mL of crude venom extract was eluted through the column and collected in 3.6 mL fractions and protein content was determined. The intended purpose of this run was the same as run 1, but due to difficulty in sonifying the venom glands, the sample was very thick and clogged the flow adaptor on the chromatography apparatus; it was decided not to concentrate or assay the fractions because of possible loss of too much of the sample.

Run 3

This was the first run in which fractions were concentrated and assayed. A volume of 1 mL of crude venom extract was eluted through the column and collected in 3.6 mL fractions. Protein content was determined and fractions 8-11 and 14-20 were concentrated; (a limited number of Centriprep tubes were available, so fractions with the lowest protein content between the two peaks were not injected). The concentrated

fractions were then assayed on three crabs per fraction with a standard dose of 0.05 mL/crab.

Run 4

Run 4 was essentially a repetition of run 3, with a larger number of crabs assayed per fraction. A volume of 1 mL of crude venom extract was eluted through the column and collected in 3.6 mL fractions. Protein content was determined and fractions 9-24 were concentrated and injected into five crabs per fraction with a standard dose of 0.05 mL/crab.

Run 5

Run 5 was used to determine if greater separation could be accomplished when a smaller sample was initially applied to the column bed. A volume of 0.15 mL of crude venom extract was eluted through the column and collected in fractions of 2.7 mL. Protein content was then determined.

Run 6

Run 6 was similar to runs 4 and 5, with the exception of fraction size. A volume of 1 mL of crude venom extract was run through the column and 2.7 mL fractions were collected. Fractions were concentrated and a weight-dependent dose was administered to three or four crabs for fractions 9-27. A dose of 0.05 mL/crab was given to crabs weighing less than or equal to 2.0 g, while a dose of 0.06 mL/crab was given to those weighing more than 2 g.

RESULTS

Gel-filtration Chromatography

Figure 9 summarizes the results of gel-filtration chromatography with Bio-Gel A1.5m gel for the six venom runs and Table 3 gives the molecular weight estimation for protein peaks of each of the six runs when compared to the standards.

Two recognizable protein peaks, one of a high molecular weight, and one of a very low molecular weight are evident in the six runs. Run 2 (Fig. 9) appears to have three peaks, but it is likely that the first and second peaks are one peak, because the protein content drops in only one fraction and then increases again. In addition, the beginning of another peak is observable between the first and second peaks, and appears as a “shoulder” to the second peak in runs 1, 2, 5, and 6. No molecular weight estimation can be made for this “middle” peak because it is unclear where the upper level of this peak is. When the elution volumes of the two recognizable protein peaks for the six runs are averaged together, molecular weights of approximately <1,000 and 600,000 are found.

The elution volumes of the protein peaks differ from run to run; this is likely due to differences in fraction sizes collected, differences in the volume of sample eluted through the column, as well as fluctuations in the column height. Because of this, runs 5 and 6 are likely the most accurate because they were carried out within days of the standard runs. Based on the average of runs 5 and 6 alone, the molecular weights of the two protein peaks are 670,000 and 1,800.

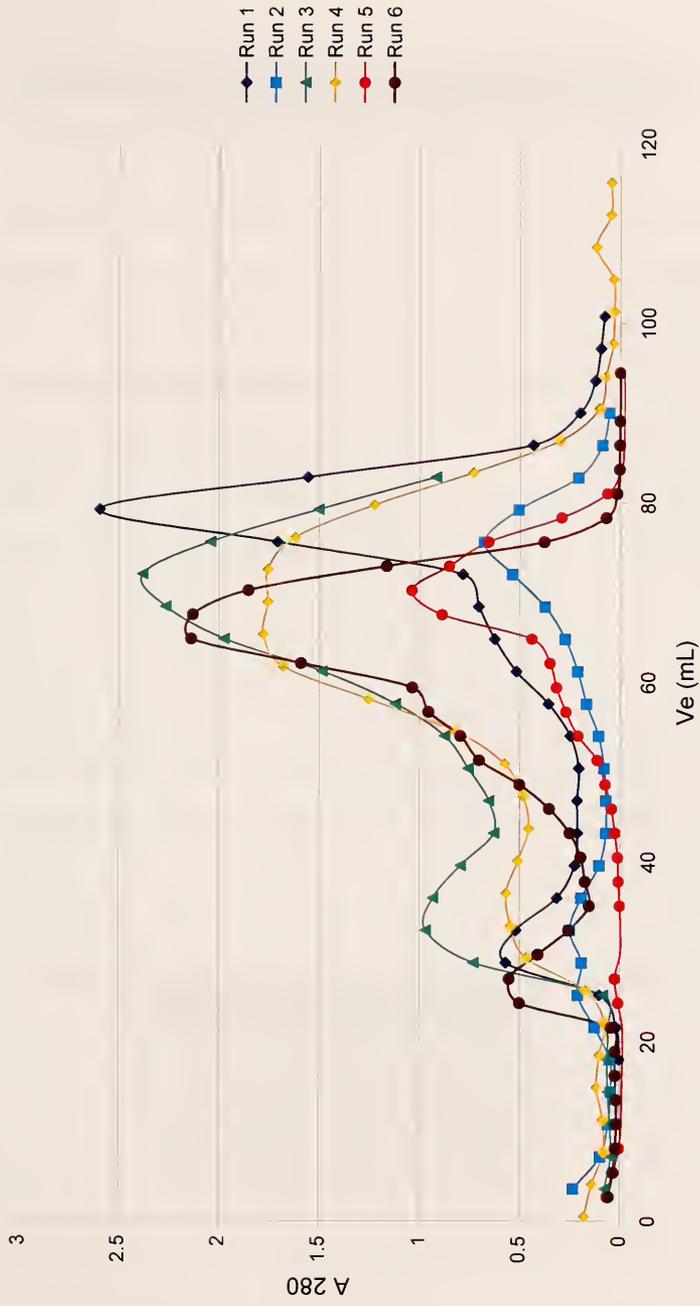


Figure 9. Protein elution profiles for venom runs 1 - 6

Table 3. Molecular weight estimations for protein peaks in venom runs 1-6.

Venom Peak	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
1	630,000	400,000	400,000	230,000	670,000	650,000
2	<1,350	<1,350	<1,350	4,000	1,800	4,000

As is evident in figure 8, the protein content, measured spectrophotometrically at 280 nm, differs between the runs, though the overall trends are the same. Absorbances greater than 1, as in runs 1, 3, 4, and 6, are not considered entirely accurate; the protein content in those fractions with an absorbance greater than 1 is likely higher than illustrated on the graphs. Differences in the amount of venom extract applied to the columns (Table 2) could be responsible for the differences in protein content observed among the runs, as well as differences in the health of the worms at the time of gland extraction.

Assay

Selected fractions from runs 3, 4, and 6 were assayed for toxicity. Table 4 summarizes the molecular weight at the points of highest lethality. Figures 9-11 illustrate the percentage of crabs killed by the assayed fractions in relation to the position of the protein peaks.

Table 4. Molecular weights of toxic fractions for runs 3, 4, and 6.

Run	Fraction #	% Killed	MW
3	11	100	200,000
	14-16	100	32,000-12,000
	18-19	100	5,000-1,700
4	15	100	32,000
6	18-20	100	90,000-44,000
	23	100	10,000

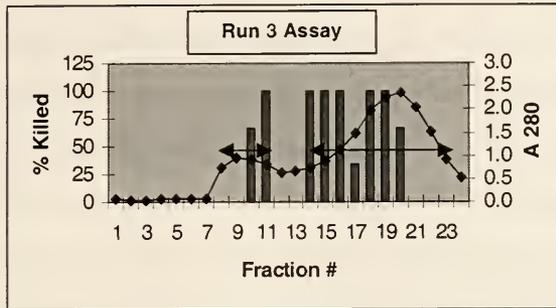


Figure 10. Percentage of fiddler crabs killed by venom fractions 8-11 and 14-23. Black arrows represent the assayed fractions.

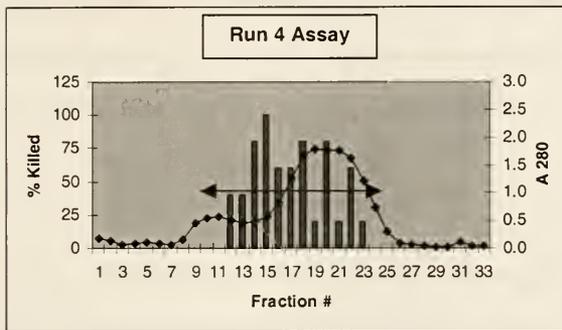


Figure 11. Percentage of fiddler crabs killed by venom fractions 9-24. Assayed fractions are represented by a black arrow.

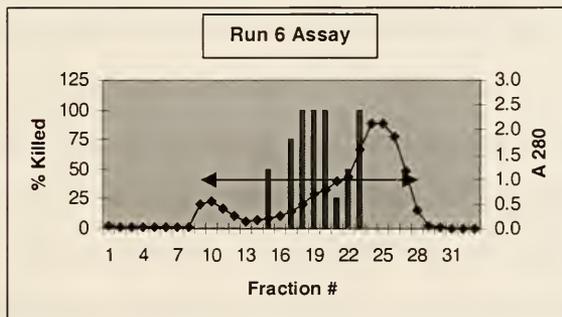


Figure 12. Percentage of fiddler crabs killed by venom fractions 9-27. A black arrow represents assayed fractions.

Because two fractions from run 3 were not assayed, and only three crabs were used per fraction, the results of this run are not entirely reliable. Run 4, though a larger number of crabs was assayed, is not entirely accurate because a weight-dependent dosage was not given. Run 6 is the most accurate because doses were given based on weight, though there is still room for error within the dosage. In addition, run 6 was carried out within days of the standard run and void volume determination, making the molecular weight estimations based on the standard runs more exact. Therefore, the results of run 6, will be used in further discussion of the molecular weights of the toxic peaks. It appears that there is a definite peak of toxicity at molecular weights between 20,000 and 43,000, while another toxic fraction is evident at a molecular weight of 6,000.

DISCUSSION

The original intent of this project was to characterize the protein and toxic components of *G. dibranchiata* venom using gel-filtration chromatography and to compare the results to new information obtained for the venom of *G. convoluta* in an attempt to ascertain the similarity between these two venoms. It was hypothesized that the venom of *G. dibranchiata* would have similar characteristics to that of *G. convoluta*, based on their close evolutionary relationship.

One problem in interpreting these results is that results from previous studies (Bon *et al.* 1985, Goracci 1979, and Michel & Keil 1975) do not agree entirely, though aspects of their results are similar. Michel & Keil (1975) observed four protein peaks in the venom of *G. convoluta*, as did Goracci (1979) for *G. dibranchiata* venom. It is difficult to interpret Goracci's (1979) results, as she used Sephacryl S-200 gel, which has

an fractionation range from 1,000-100,000, but found that three of the protein peaks were of a molecular weight greater than 100,000 (121,000, 160,000, and 232,000). Therefore, her conclusions can only be regarded as questionable. Michel & Keil (1975) used Sephadex G-75 and Sephadex G-200, and found a large protein peak of high molecular weight, a large peak of low molecular weight proteins, as well as two additional smaller peaks between these two large peaks. Bon *et al.* (1985) also found four protein peaks, but the overall elution profile is different from Michel & Keil (1975) in that protein peaks two and three are as large or larger than the peak one, whereas Michel & Keil (19875) found that the peak one was larger than peaks two and three (Fig. 7).

Based on a comparison with researchers mentioned above, the results of this study most closely resemble results of Bon *et al.* (1985) for *G. convoluta* venom. The elution profiles for the venoms of *G. dibranchiata* (Fig. 7) and for *G. convoluta* (Fig. 9) are similar, with the exception of the third and fourth protein peaks that are found for *G. convoluta* venom, but are not seen in the elution profile for *G. dibranchiata* venom. The two additional protein peaks found for *G. convoluta* venom that were not found for *G. dibranchiata* venom are of very low molecular weights and have no biological activity (Bon *et al.* 1985. Similarities between the protein content of these two venoms are that both contain a high molecular weight fraction (> 500,000) and a low molecular weight fraction.

Based on the results of run 6, two toxic peaks are found at molecular weights of 22,000-43,000 and 6,000. Bon *et al.* (1985) also found two toxic peaks (Fig. 7), the first of which was between the first two protein peaks, and the second of which corresponded

to the second protein peak. Bon's most toxic peak for crustaceans, found in fraction D, is in between 60,000 and 150,000 in molecular weight.

A conclusion regarding the presence of α -glycerotoxin cannot be made based on these results. Though the points of highest toxicity for *G. dibranchiata* venom were not at molecular weights of 300,000, the effects of this protein have been studied only on an electrophysiological level, and its effects at an organismal level, if any, are not known. The most toxic peak (fraction D) found by Bon *et al.* (1985) for *G. convoluta* venom did not correspond to the presence of α -glycerotoxin. Therefore, it is possible that *G. dibranchiata* venom could contain α -glycerotoxin, but that the highest toxicity points would not correspond directly to this molecular weight; there are other components more responsible for the lethal effects.

There are several possible explanations for the differences in protein peaks and toxic peaks found in runs 1-6. First is the possibility that the different molecular weights of the protein peaks found for each run are due to the differences in the size of the fractions collected. Initially, after the delay in the UV monitor recorder was discovered, 3.6 mL fractions were collected in order to cut down on the amount of time for spectrophotometry readings. When larger fractions are collected, the true elution volume of the protein peaks could be masked.

In addition to fraction size, fluctuations in the column throughout the semester could be responsible for differences in molecular weights found between runs. Because the runs took place over the course of three months, it is likely that the amount of gel in the column decreased very slightly with each run with the addition of samples, causing the elution volumes to differ with each run. This would also explain the overall decrease

in elution volumes for both the first and second protein peaks. The standard runs were both carried out later in the semester, in between runs 4 and 5, and so having used these for determination of molecular weights of venom runs carried out earlier in the semester may lead to some inaccuracy.

The dosage of the concentrated fractions could explain differences in the molecular weights found for the toxic fractions. Under normal circumstances, without fractionation or re-concentration using Centriprep tubes, a dose of 6 mg venom/g crab weight is sufficient to kill fiddler crabs within one hour. Because it was nearly impossible to determine the concentration of venom in a fraction after it had been eluted through the column with buffer and then re-concentrated, a dose of approximately 0.05 mL was given to all crabs in runs 3 and 5. A volume of greater than 0.1 mL would likely negatively affect the fiddler crabs, regardless of toxicity. For this reason, it was decided to inject a dose of 0.05 mL per crab. However, it is possible that this dose could be lethal for a small crab (approximately 2 g), but allow for recovery in a larger crab (approximately 2.5 g).

In addition, the condition of the animals could affect the results. Bloodworms were shipped in early January, and then again every several weeks until late February. Therefore, their overall health may have varied from run to run. The fiddler crabs were sometimes kept for up to three weeks, and it is questionable whether they ate the food provided. Therefore, their condition of health could have made them more or less susceptible to the venom.

This experiment warrants further examination. A longer chromatography column could be used to obtain better separation of the proteins. With a correctly functioning UV

monitor and recorder, a more accurate determination of the elution volumes of proteins could be made. Assaying a larger number of crabs with a weight-dependent dosage would allow for clearer results regarding the peaks of toxicity. In addition, each of the two protein peaks, as well as the middle peak disguised by the second large peak, could be separated further using gel electrophoresis to determine the number of proteins present within each peak.

In conclusion, despite some of the technical problems encountered, the chromatography results were consistent throughout in indicating the presence of two protein peaks. The assayed run considered most accurate (run 6) indicates the presence of two toxic peaks. The elutions profiles for protein content and the presence of two toxic peaks agree with results presented by Bon *et al.* (1985) for the venom of *G. convoluta*. However, because of the presence of two additional protein peaks in *G. convoluta* venom, as well as differences in molecular weights of the protein and toxic peaks, it is concluded that the venoms of *G. dibranchiata* and *G. convoluta* differ in their protein and toxic component makeup.

LITERATURE CITED

- Alongi, A. 2000. A SEM study: A correlation of jaw characteristics in relation to age in *Glycera dibranchiata* [Senior Research Report]. Sweet Briar (VA): Sweet Briar College.
- Andolshek, K. 1999. A SEM study of the *Glycera dibranchiata* jaw and its relation to the poison glands [Senior Research Report]. Sweet Briar (VA): Sweet Briar College.
- Beck, J. 1995. The effects of *Glycera dibranchiata* venom on several different arthropods [Senior Research Report]. Sweet Briar (VA): Sweet Briar College.
- Bon, C., B. Saliou, M. Thieffry, and R. Manaranche. 1985. Partial purification of α -glycerotoxin, a presynaptic neurotoxin from the venom glands of the polychaete annelid *Glycera convoluta*. *Neurochem.* 7(1):63-75.
- Cates, M. 2001. What do *Glycera dibranchiata* eat? [Senior Research Report]. Sweet Briar (VA): Sweet Briar College.
- Edstrom, A. 1992. *Venomous and Poisonous Animals*. Malabar, FL: Kreiger Publishing Company.
- Ehlers, E. 1868. *Die Borstenwurm (Annelida Chaetopoda) nach Systematischen und Anatomischen Untersuchungen*. Wilhelm Engelmann, Leipzig.
- Goracci, G. 1979. A study of the venom of *Glycera dibranchiata* Ehlers [Senior Honors Thesis]. Sweet Briar (VA): Sweet Briar College.
- Hager, L.E. 1998. Effect of the venom of *Glycera dibranchiata* on *Nereis*, *Lumbricus*, and crickets and slugs of unknown species [Senior Research Report]. Sweet Briar (VA): Sweet Briar College.
- Heacox, A.E. 1974. The histology and histochemistry of the poison gland complex in the polychaete *Glycera dibranchiata* [Masters Thesis in Biology]. Garden City (NY): Adelphi University.
- Kagan, B., H.B. Pollard, and R.B. Hanna. 1982. Induction of ion-permeable channels by the venom of the fanged bloodworm *Glycera dibranchiata*. *Toxicon* 20(5):887-893.
- Klawe, W.L. and L.M. Dickie. 1957. Biology of the bloodworm, *Glycera dibranchiata* Ehlers, and its relation to the bloodworm of the Maritime Provinces. Bull. No. 115. Fisheries Research Board of Canada.
- Madeddu, L., J. Meldolesi, T. Pozzan, L.E. Cardona Sanclementes, and C. Bon. 1984. α -Latrotoxin and glycerotoxin differ in target specificity and in the mechanism of their neurotransmitter releasing action. *Neuroscience* 12(3):939-949.

- Manaranche, R., M. Thieffry, and M. Israel. 1980. Effect of the venom of *Glycera convoluta* on the spontaneous quantal release of neurotransmitter. *Journal of Cell Biology* 85:446-458.
- Michel, C. 1966. Machoires et glandes annexes de *Glycera convoluta*, annelide polychete Glyceridae. *Cah. Biol.* 8:367-373.
- Michel, C., and B. Keil. 1975. Biologically active proteins in the venomous glands of the polychaetous annelid, *Glycera convoluta* Keferstein. *Comp. Biochem. Physiol.* 50B:29-33.
- Morel, N., M. Thieffry, and R. Manaranche. 1983. Binding of a *Glycera convoluta* neurotoxin to cholinergic nerve terminal plasma membranes. *The Journal of Cell Biology* 97:1737-1744.
- Ockelmann, K.W., and O. Vahl. 1970. On the biology of the polychaete *Glycera alba*, especially its burrowing and feeding. *Ophelia* 8:275-294.
- Page, L. 1994 The effect of freeze-drying on the toxicity of *Glycera dibranchiata* venom [Senior Research Report]. Sweet Briar (VA): Sweet Briar College.
- Simpson, M. 1962. Reproduction of the polychaete *Glycera dibranchiata* at Solomons, Maryland. *Biological Bulletin* 123(2):396-411.
- Simpson, M. 1962. Gametogenesis and early development of the polychaete *Glycera dibranchiata*. *Biological Bulletin* 123(2):412-423.
- Ungerer, N. 2001. Sonification of *Glycera dibranchiata* tissues and the effects of venom on several arthropods [Junior Honors Report]. Sweet Briar (VA): Sweet Briar College.

3282 2638 5

07/30/02



MFB



