Earthworms produce a collagen-like substance detected by the garter snake vomeronasal system

(Lumbricus/Thamnophis/glycoprotein/chemoattractant)

DONALD M. KIRSCHENBAUM\*†, NANCY SCHULMAN‡, AND MIMI HALPERN‡

Departments of *Biochemistry and †Anatomy and Cell Biology, Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203

Communicated by Chandler McC. Brooks, October 15, 1985

ABSTRACT Earthworms (Lumbricus terestris) are a favorite prey of garter snakes of the genus Thamnophis and form a major component of their natural diet (1, 2). Garter snakes respond to objects coated with warm-water washings from earthworms by rapid tongue-flicking and an attack (1–3), thus suggesting that these washings contain a chemoattractant for the snake. In addition, garter snakes can be trained to follow trails of earthworm washings to receive bits of worm as rewards (4, 5). Garter snakes use their vomeronasal systems, but apparently do not require a functional olfactory system, to detect and appropriately respond to this chemoattractant contained in earthworm washings (3, 6–8). Stimulation of the vomeronasal organs is typically naturally occurring complex large molecules that have been difficult to characterize chemically (9–14).

Earthworm washings (EWW), prepared as described by Wilde (3) and Burghardt (1), have been found to contain a chemoattractant for garter snakes that is water-soluble, absorbs at 280 nm, contains carbohydrate, and gives a positive reaction with ninhydrin and positive Lowry and Bradford tests for protein (15–17).

A solution of EWW that had been dialyzed, lyophilized, and reconstituted in 0.15 M NaCl to 5 times its original concentration was separated on various calibrated gel-exclusion columns (16). When 0.15 M NaCl was used as eluent, two peaks were detected by 280 nm absorption (16). The first peak, F1, comprising components of Mf > 65,000, contained all the chemoattractant. This peak also contained neutral carbohydrate and ninhydrin-positive material and gave positive tests for protein. The second peak, F2, comprising components of much lower molecular weight, contained neutral carbohydrate but was devoid of chemoattractant (16).

Snakes were able to discriminate the chemoattractant in EWW (pH 5.8) from solvent after the EWW was heated for up to 120 min in a boiling water bath, but not after the EWW was heated for 240 min in a boiling water bath. When a 0.1 M HCl solution of EWW was heated in a boiling water bath, chemoattractant activity was lost within 15 min. However, even after a 0.1 M HCl solution of EWW had been heated for 3 hr in a boiling water bath, an F2 peak could still be detected by absorption at 280 nm, carbohydrate assay, and reaction with ninhydrin, although the amount of material detected in the F2 fraction was much less than originally present at zero time (17). When a 0.1 M NaOH solution of EWW was heated in a boiling water bath, chemoattractant power was lost in <15 min (17). Concomitant with the loss of the chemoattractant was the disappearance of the F2 peak, as shown by the absence of 280 nm absorption, carbohydrate, and ninhydrin-positive material (17). The F2 material was thus very much more labile in alkali than in acid. After alkali treatment, the F2 peak increased, as measured by 280 nm absorption, carbohydrate content, and ninhydrin-positive material.

This paper presents evidence relative to the structure of this vomeronasally detected chemoattractant for garter snakes.

MATERIALS AND METHODS

Sources. Dialysis tubing was purchased from Thomas. Prior to use, it was boiled in doubly glass-distilled water (ddH2O) for 30 min and stored in ddH2O with 0.02% sodium azide. Sephadex G-75 and Blue Dextran were purchased from Pharmacia. Aca 34 and AcA 44 were purchased from LKB. Bovine serum albumin, ovalbumin, myoglobin, and phenylmethylsulfonyl fluoride were purchased from Sigma. 2,4-Dinitrophenylalanine was prepared in this laboratory. Fractions were eluted with the aid of an ISCO model 312 pump and collected with an ISCO Retriever III fraction collector.

Preparation of a Solution of Chemoattractant from EWW. EWW was prepared (1, 3) from 6 g of earthworms (L. terrestris; purchased from Connecticut Valley Biological Supply, South Hampton, MA) suspended in 20 ml of ddH2O at 60°C and centrifuged at ~550 X g for 20 min at room temperature. The EWW was dialyzed (Mf, 3500 cutoff) overnight against ddH2O at room temperature. The dialyzed solution of EWW was lyophilized, and 15 mg of the resulting powder dissolved in 2 ml of 0.15 M NaCl to ~5 times its original concentration. This sample was placed on a 1.6 X 42.5 cm column of AcA 44 that had been calibrated with Blue Dextran, bovine serum albumin, ovalbumin, myoglobin, and dinitrophenylalanine. Fractions (2 ml) were collected and assayed for A280 and carbohydrates (18).

Abbreviations: EWW, earthworm wash; ddH2O, doubly glass-distilled water.

†Deceased October 4, 1985.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Amino Acid and Carbohydrate Analyses. The high molecular weight fractions in the first major peak that was eluted from the AcA 44 column, the only fractions that contain chemotaxtractant, were pooled, dialyzed, lyophilized, and hydrolyzed in 6 M HCl for 24 hr at 110°C. The amino acid analysis of the hydrolysate was done using a Beckman model 120C. The hydroxyproline assays (19) were done on hydrolysates prepared by heating at 130°C for 4 hr. The hydrolysis and analysis of EWW and F2 for carbohydrates was done by J. R. Clamp of the University of Bristol (Bristol, England).

Preparation of Earthworm Cuticle. Earthworm cuticle collagen was prepared as described by Murray et al. (20), in the presence of the protease inhibitor phenylmethylsulfonyl fluoride. Another preparation of earthworm cuticle collagen was made by the method of Watson (21). The homogeneity of each preparation was demonstrated by the constancy of the hydroxyproline/protein ratio (20, 22) after two separate reprecipitations for each preparation.

Preparation of Decuticilized Earthworms. Earthworms were placed in anhydrous ether for 15 min (21). The worms were dried and their cuticles were removed, much like peeling off a sausage casing. The decuticelized earthworms were then placed in ddH2O at 60°C and a solution of EWW was prepared as described above for intact worms.

Behavioral Testing. The testing procedure has been described in detail (15). A trial, 2 min in duration, consisted of the simultaneous presentation of an EWW sample and a ddH2O or another control sample on Coplin jar covers placed...
RESULTS

Amino acid analysis of dialyzed, lyophilized chemoattractant isolated by gel-filtration chromatography from EWW (Fig. 1) revealed hydroxyproline in a quantity ~16-fold greater than proline, when compared on a residues per 1000 residues basis. Serine and threonine were present in large amounts. No hydroxylysine and no cysteine were present. More than one-third of the residues were glycine. These results suggested that the chemoattractant activity resided in an invertebrate collagen-like molecule. The results from the amino acid analysis were compared with previously reported analyses of earthworm cuticle collagen (22), earthworm cuticle gelatin (21), and an α(1) chain of human placental collagen (23). The star diagrams in Fig. 1 illustrate these comparisons. A strong similarity between earthworm cuticle collagen, earthworm cuticle gelatin, and dialyzed, lyophilized F2 (F2-DL) is seen (Fig. 1 A and B). In contrast, comparison of amino acid profiles for the α(1) chain of a vertebrate collagen and for F2-DL (Fig. 1C) reveals little similarity.

An analysis of dialyzed, lyophilized EWW and a similarly prepared F2 fraction showed that the former contained 12.3% galactose, equivalent to 82% of the total carbohydrate present, while the latter had 11.1% galactose, equivalent to 74% of the total carbohydrate present. Earthworm cuticle collagen has been reported to have 10–14% galactose as the major carbohydrate (24, 25), equivalent to ~90% of the total neutral sugar present (25).

Gel-exclusion chromatography of earthworm cuticle collagen on an AcA 44 column produced an elution profile (Fig. 2) very similar to the one obtained in our previous studies of EWW (16). High molecular weight material (F2) was eluted close to the Blue Dextran marker and ahead of the bovine serum albumin marker, and lower molecular weight material (F4) was eluted between the myoglobin and dinitrophenylalanine markers. A 4-fold higher galactose concentration (38 μg/ml) was found in F2 than in F4 (10 μg/ml).

A saline solution of purified earthworm cuticle collagen was a strong chemoattractant for garter snakes (Fig. 3). All the chemoattractant in the cuticle collagen solution was found in F2; F4 had no chemoattractant activity as determined by our testing procedures. EWW obtained from decuticlitezed earthworms contained no chemoattractant (Fig. 3). Earthworms used to prepare EWW could not be used to prepare more chemoattractant solution after two 1-min washes at 60°C (Fig. 3).

DISCUSSION

Our results show that a chemoattractant for snakes in EWW is similar in amino acid composition and carbohydrate content to earthworm cuticle collagen (22, 24, 25) and gelatin (21). Further support for the idea that a chemoattractant in

FIG. 2. A solution of 15 mg of lyophilized cuticle, prepared by the modified technique of Murray et al. (20), in 2 ml of 0.15 M NaCl was fractionated on a 1.6 × 42.5 cm column of AcA 44, using 0.15 M NaCl as eluent. Elution positions of markers are shown by arrows. DB, Blue Dextran; ALB, bovine serum albumin; OVA, ovalbumin; MYO, myoglobin; DNPA, dinitrophenylalanine.

FIG. 3. Mean tongue-flick interest scores of snakes presented with earthworm washings (EWW), earthworm washings prepared from worms previously soaked in 60°C ddH2O for 1 min (EWW1), earthworm washings prepared from worms previously soaked twice (for 1 min each time) in 60°C ddH2O (EWW2), earthworm washings prepared from decuticlitezed earthworms (DECUT), and two different solutions of purified earthworm cuticle collagen (EWC1 and EWC2). Hatched bars represent scores for test samples (mean ± SEM, n = 6); open bars represent control (ddH2O) scores. Asterisks indicate test scores significantly different (P < 0.05) from control.
Table 1. Comparison of earthworm cuticle collagen and garter snake chemoattractant obtained from earthworm

<table>
<thead>
<tr>
<th>Earthworm cuticle collagen</th>
<th>Earthworm chemoattractant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitated with 20%-saturated ammonium sulfate (20)</td>
<td>Precipitated with 90%-100%-saturated ammonium sulfate (16)</td>
</tr>
<tr>
<td>Hydroxyproline/proline ratio ~10 (22)</td>
<td>Hydroxyproline/proline ratio ~15</td>
</tr>
<tr>
<td>High Thr + Ser content (22)</td>
<td>High Thr + Ser content</td>
</tr>
<tr>
<td>No hydroxylsine (22)</td>
<td>Little if any (0–1 residue) hydroxylsine</td>
</tr>
<tr>
<td>Carbohydrate present; galactose (10–14% by weight) the major carbohydrate (24, 25); small amounts of galactosamine and glucosamine (25)</td>
<td>Carbohydrate present; galactose (11%) the major carbohydrate; small amounts of fucose, mannose, glucose, galactosamine, glucosamine, N-acetylgalactosamine, and N-acetylgalactosamine</td>
</tr>
<tr>
<td>β-Elimination of carbohydrate (25)</td>
<td>β-Elimination of carbohydrate (proposed)</td>
</tr>
<tr>
<td>Small amount of ninhydrin-reactive material</td>
<td>Small amount of ninhydrin-reactive material (16)</td>
</tr>
<tr>
<td>Cuticle solution yields an F₂ fraction (Fig. 2)</td>
<td>F₂ fraction has all chemoattractant activity (16)</td>
</tr>
</tbody>
</table>

EWW is earthworm cuticle collagen-related or gelatin-related and is obtained from the observations that solutions of purified earthworm cuticle collagen contain a chemoattractant and that chemoattractant cannot be obtained from washes of earthworms whose cuticles have been removed.

We had previously observed a rapid loss of chemoattractant 280 nm absorption, carbohydrate content, and ninhydrin-positive material in EWW treated in hot alkali (17). Muir and Lee (25) and Spiro and Bhoyroo (26) have shown that earthworm cuticle collagen has at least 23 attachment sites per 1000 residues for O-glycosidically bound carbohydrate on serine and threonine residues. If these are distributed widely, as suggested by Maser and Rice (27), throughout the collagen structure, then the high molecular weight material F₂ when treated with hot 0.1 M NaOH, should form low molecular weight material (F₁) due to β-elimination and hydrolytic cleavage. The consequences of these cleavages should be the loss of 280 nm absorption in F₂ and an increase in F₁. The increase in ninhydrin-positive material in F₁, following hot alkali treatment of EWW may be due to generation of amino groups by peptide bond hydrolysis and to cleavage of the amide groups formed by β-elimination, to yield ammonia (17).

From our accumulated data and inferences from this information, we suggest that EWW contains a component of earthworm cuticle collagen/gelatin that acts as a chemoattractant for snakes. It is known that earthworm cuticle collagen is precipitated at a lower concentration of ammonium sulfate (20% saturation; ref. 20) than is our chemoattractant (90–100% saturation; ref. 16), and this is to be expected if earthworm chemoattractant is a fragment of cuticle collagen/gelatin. Also, the solution of chemoattractant is prepared at 60°C, much higher than the denaturing temperature of earthworm cuticle collagen (22°C; ref. 28). From the chemical characteristics mentioned, and additional ones listed in Table 1, we believe that the F₂ chemoattractant is chemically and structurally related to earthworm cuticle collagen/gelatin obtained by solubilizing earthworm cuticle at 60°C.

The earthworm produces a cuticle collagen/gelatin that contains an agent detectable by the vomeronasal apparatus of the garter snake. This agent is an attractant that enables snakes to locate their prey.

We thank Drs. L. Kesner, R. Carty, and J. Kang and Ms. Patti T. Yao for their assistance; Mr. T. Housley and Dr. M. Tanzer (University of Connecticut Health Science Center) for confirmatory amino acid analyses; and Prof. J. R. Clamp (Bristol Royal Infirmary) for the carbohydrate analyses. D.M.K. was a Faculty Exchange Scholar of the State University of New York. This research was supported by National Institutes of Health Grant NS11713.