The venom of the mud-dauber wasp Sceliphron caementarium

by William Rosenbrook

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University

Abstract:
The venom of the mud-dauber wasp (Sceliphron caementarium)obtained by electrical excitation of the wasp, has been investigated qualitatively and quantitatively in order that no major constituent might be overlooked. The protein fraction of the dried venom was found to be unexpectedly small and small peptides were not detected in any significant amounts. Seventeen non-protein constituents, among them three free amino acids and a lecithin-like compound, were separated by chromatographic techniques and . identified or characterized. Of particular importance was the discovery of what appears to be a series of non-nitrogenous compounds in the venom.

This is the first investigation of any wasp venom to deal with the pure venom as opposed to a venom apparatus extract. Examination of an extract of mud-dauber venom apparatuses has revealed a minimum of fifteen compounds not present in the venom itself, thus emphasizing the value of studies based on the natural venom.

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ABSTRACT

The venom of the mud-dauber wasp (Sceliphron caementarium), obtained by electrical excitation of the wasp, has been investigated qualitatively and quantitatively in order that no major constituent might be overlooked. The protein fraction of the dried venom was found to be unexpectedly small and small peptides were not detected in any significant amounts. Seventeen non-protein constituents, among them three free amino acids and a lecithin-like compound, were separated by chromatographic techniques and identified or characterized. Of particular importance was the discovery of what appears to be a series of non-nitrogenous compounds in the venom.

This is the first investigation of any wasp venom to deal with the pure venom as opposed to a venom apparatus extract. Examination of an extract of mud-dauber venom apparatuses has revealed a minimum of fifteen compounds not present in the venom itself, thus emphasizing the value of studies based on the natural venom.

INTRODUCTION

The members of the order Hymenoptera which are commonly referred to as "wasps" belong almost exclusively to the class Vespoidea, family Vespidae (social wasps), and to the class Sphecoidea (solitary or mud-dauber wasps).

Stings from wasps are so common that almost everyone is familiar with their effects. These stings are commonly considered to be harmless although quite painful. Information concerning these stings is rarely mentioned in the literature and investigations of the venom itself are even less common. During the last twenty years several papers have been prepared which survey a rather large number of deaths due to wasp and bee stings (e.g. 1,2). In the United States stings from wasps and bees cause more deaths than all other poisonous animals combined. In the 8 year period from 1950 through 1954 and 1957 through 1959 seventy-nine deaths have been directly attributed to wasps alone (2). Several of these deaths were caused by multiple stings and were probably the result of the direct action of the venom toxins. About 30 percent of the "single sting" deaths may be attributed to anaphylaxis, while most of the remaining deaths are not understood. The cause of death in the majority of the latter cases was stated simply as heart failure. Thus, death as inflicted by a wasp or bee sting is only poorly understood and the only satisfactory treatment at this time is the administration of some pressor amine such as epinephrine, phenylephrine, norepinephrine, aromine sulfate, isoproteranol, or triplennamine immediately during the shock episode.

This time limit makes it necessary to have a ready supply of one of these amines, which presupposes that the sting victim knows he is hyper-sensitive. The above survey (2) demonstrates conclusively that the majority of wasp sting victims had no prior knowledge of sensitivity to wasp sting.

Preventive medical treatment relies solely upon the method of allergic desensitization. This treatment is limited because anaphylactic shock does not appear to be the only cause of death from insect sting and because prior knowledge of sensitivity is required. Desensitization is only temporary and must be continued with a "booster shot" at least every two months. In addition, desensitization is now carried out with either whole body (3) or venom apparatus (4,5) extracts which may contain harmful antigens foreign to the venom itself. Of additional interest is the indication of common antigens among bees and wasps (3), which supports the idea of cross sensitization and suggests a definite similarity between the various venom proteins.

Bee venom has long been proposed as a therapeutic agent for such chronic ailments as arthritis and rheumatism (6), however, conclusive evidence of its effectiveness is not yet available. Statistics show that bee keepers have a very low incidence of cancer which may be due to their "bee sting immunity" (7) or to their diet of unpasteurized honey. Like bee venom wasp, venom may possess a therapeutic value.

The chemical nature of wasp venoms was first investigated in 1913 by Bertarelli and Tedeschi (8), who found that hornet venom

(Vespa crabo) resembled bee venom in its action on small animals, contained an unstable hemolysin and smelled of capryllic acid. All wasp venom investigations prior to the present research have been on crude preparations obtained by extracting the entire venom apparatus, i.e. the venom sac, acid glands and Dufour's alkaline gland. As a result, all of the constituents found to date can not necessarily be assumed to occur in the venom itself.

Various animal and insect venoms (including wasp and bee) have been found to contain a thermostable phospholipase A(9, 10), phospholipase B(9), proteases (9, 10), thrombokinases (9), lipases (9) and carbohydrases (9). The facts suggested that the toxic action of the venoms was produced by the action of the various enzymes on the tissues of the victim. Wasp "poison" (undefined) was also shown to possess a factor which saponified sodium or calcium glycerophosphates, but had no effect on plant phosphatides or natural fats (11). Riboflavin was reported in the venom glands of hymenoptera (12), but the method of analysis leaves this finding unsubstantiated. Another group of investigators (13) determined that wasp and bee venom did not contain sulfhydryl or disulfide derivatives. However, Kaiser and Michl (14) report 7.45% cystine and/or cysteine in the protein hydrolysate of bee venom. In addition 5-hydroxytryptamine and free amino acids (undefined) were found in wasp venom and acetylcholine in hornet venom (14).

Recently, the dried venom apparatus of the common wasp (Vespa vulgaris) was found to contain 1.6 to 2.0% histamine and about 0.03% 5-hydroxytryptamine (15). In the same article Jaques and Schachter made the first report of a "slow contracting substance", which was later identified (16, 17, 18, 19) as a kinin. Wasp venom kinin bears a striking resemblance in its physiological properties to the nonapeptide from human blood (20), bradykinin (kallidin), but is not identical with it. Kinin is believed to be the major pain producing factor of wasp venom (21) and its contracting effect on smooth muscle probably contributes to the toxicity of the venom. Jaques has also reported the presence of hyaluronidase (a spreading factor) (22, 23), cholinesterase and lecithinase (22) in Vespa vulgaris venom.

Michl (24) discovered the first pipecolic acid derived from animals in the protein hydrolysate of snake and wasp (Vespa germanica) venom in 1957.

Previous investigations of wasp venoms, based on qualitative analysis of venom apparatus extracts, have revealed only a fraction of the complex character of these venoms. Since the percentages of the various components found thus far is unknown, many unidentified substances may be present in wasp venoms. In addition, no attempt has yet been made to exhaustively analyse the venom of one particular species of insect. As a result, the protein fraction of these venoms has been emphasized and the possibility of other physiologically active venom

constituents has been largely ignored.

The venom of the mud-dauber wasps is of special interest because of its paralytic action on spiders (25) and its unusually mild immediate reaction on humans, i.e. negligible pain and swelling. The natural history of the paralyzing sting of various wasps gives little information concerning the physiology involved except possibly the speed of onset, duration, or completeness of the paralytic state. However, wide variations in these effects suggest that paralyzing venoms differ in type as well as in potency (26). Other evidence indicates that paralysis induced by sphecoid wasps may not result from a direct neural lesion but from a generalized "poisoning" of the blood (27). It was at first believed that the mud-dauber sting could not produce death in humans, but an authenticated case of a mud-dauber sting death has since been discovered (2), and it now appears likely that the rarity of severe sting reactions may be due to the mild temper of the wasp, rather than to the composition of its venom. Since the mud-dauber is a solitary wasp (the first of the sphecoid wasps to have the chemistry of their venom investigated) its venom might very well differ from that of the vespidae already investigated, e.g. the venom of the former paralyzes rather than kills the prey and its venom apparatus lacks Dufour's alkaline gland (28).

This research was intended to obtain a quantitative characterization of pure Sceliphron caementarium venom and to develop techniques which might facilitate the ellucidation of other complex insect venoms. The

mud-dauber wasp was particularly cooperative and their use eliminated most of the problems encountered in working with more vicious insects.

EXPERIMENTAL

Collection and Maintenance of Wasps

The mud-dauber wasp is most commonly found in areas of high humidity, although at least one species is native to every region of North America. Most of the mud-dauber wasps for this research were obtained from southeast Missouri and others of the same species were collected in the San Joaquin Valley of California. Initially, the wasps were maintained in cages under conditions similar to those of their natural habitat and fed on a diet of honey and spiders as described by Shafer (25). In the later stages of this research the venom was extracted from the wasps in the field and their venom apparatuses (with lancet attached) were immediately removed since only a fraction of the venom was released by the extraction technique. The venom apparatuses (with lancet attached) were removed from the insects by the method of Jaques and Schachter (15) and stored over phosphorous pentoxide at 5° C. The species of mud-dauber was identified by Dr. K. V. Krombein, Insect Identification and Parasite Research Branch, Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

Extraction of Venom

The methods which have been described for obtaining bee venom by electrical excitation (29, 30, 31) are not applicable to mud-dauber wasps or to certain other wasps and hornets because of insufficient

excitation voltage or because of the danger of fighting among these insects.

The method described here (32) has been found adequate for obtaining pure venom from one to several hundred individual bees, wasps or hornets. The insect suffered no apparent damage and many were often used for repeated venom extractions.

The apparatus (Figure 1) consisted of a small (about 1/4-inch diameter) half-cylinder of fine brass mesh about 1/2-inch long soldered to the end of a 2-inch length of rigid iron wire which was bent for insertion into a rubber stopper. The insect was anesthetized with carbon dioxide or immobilized in the cold, placed in the half-cylinder, and bound in place with a 1/4-inch ribbon of aluminum foil which was twisted behind the half-cylinder. The mounted insect was supported by a clamp directly beneath a nichrome wire lead from a spark coil (about 10,000 volts). A microscope well-slide was placed under the insect in such a manner that only the sting lancet reached into the well. When the insect revived, it was excited by a brief high-voltage shock, controlled by a key switch, until venom was excreted.

After the venom was deposited on the slide it was dried over phosphorus pentoxide and stored at 5°C. With this apparatus, two or three mud-dauber wasps could be "milked" each minute with no apparent effect on the insect other than pronounced hunger and thirst.

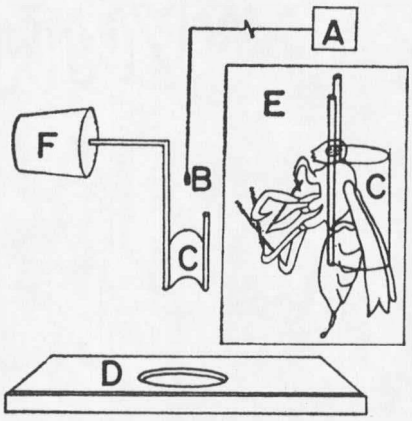

Figure 1. Procuring venom from a wasp. A, Spark coil with 6-volt d-c power supply; B, nichrome wire; C, brass mesh half-cylinder; D, microscope well-slide; E, insert showing insect mounted in half-cylinder before being wrapped with aluminum ribbon; F, rubber stopper.

It was found that the abdominal segment of the mud-dauber wasp could be removed and would remain "alive" for as long as 24 hours in this isolated state. In addition, this isolated segment made the same response to the "milking" procedure as the whole insect. Because of the increased efficiency in handling the abdominal segments as opposed to the whole insect, venom extracted from these segments was used exclusively in the later stages of this research.

Identification and Determination of Free Amino Acids (33)

The mud-dauber venom was examined for free amino acids since such compounds had been reported in other wasp venoms (14). All paper and thin-layer chromatography throughout this investigation utilized the ascending technique. The identity of one of the venom constituents as

histidine was determined by paper chromatography on Whatman No. 3 MM paper. Comparison of venom samples and known histidine spots was made using (a) 80% phenol (R_f-value of known histidine =0.65; R_f-value of venom spot =0.68). (b) 2,6-lutidine(55 ml):ethanol(25 ml):water(20 ml): diethylamine(2 ml) (R_f-value of known =0.35; R_f-value of venom spot =0.32), (c) 1-butanol:acetic acid;water(34) (R_f-value of known =0.16; R_f-value of venom spot =0.19 with detection by Pauly's reagent (35), as well as by a 2% solution of ninhydrin in acetone. Additional comparison using silica gel thin-layer chromatography developed with abs. ethanol:water (63:37 v/v) (36) gave known histidine an R_f-value of 0.44 and the venom spot an identical R_f-value. Cochromatography of venom and known histidine revealed no new spots and the ninhydrin color of the venom histidine spot was identical with that of the known histidine.

Quantitative estimation of histidine was made by chromatographing 1.4 mg of venom on a silica gel G thin-layer plate using the ethanol: water developing system. The region of R_f-value 0.42 to 0.46, previously shown to contain only histidine, was scraped from the plate and triturated in a centrifuge tupe with 1.00 ml of deionized distilled water. Residual silica gel was removed by centrifugation and 0.250 ml aliquots of supernatant solution were removed and used for the spectrophotometric determination method of Stegemann and Bernhard (37) using a histidine standard curve; a histidine content of 13 ± 2 mg was obtained corresponding to 0.8 to 1.1% of the dried venom.

Methionine was detected in the same manner as histidine. Using the 80% phenol, R_f-values for known methionine and the corresponding venom spot were 0.75 and 0.74 respectively and with the 2,6-lutindine:ethanol: water:diethylamine the known sample and venom spot gave R_f-values of 0.52 and 0.50 respectively. On silica gel thin-layer chromatography using the ethanol:water system, the known and venom spot R_f-values were both 0.64.

Quantitative estimation was made by the method employed for histidine, except that a methionine standard curve was used. A methionine content of 0.9 to 1.1% was determined.

Pipecolic acid was detected in the same manner as histidine and methionine. Using 80% phenol, R_f-values of authentic pipecolic acid (Biochemical Research Laboratories, Los Angeles, Calif.) and the corresponding venom spot were 0.86 and 0.84 respectively. With the 1-butanol: acetic acid:water system, R_f-values of the known and venom spot were 0.43 and 0.44 respectively. On silica gel thin-layer chromatography, with ethanol:water, R_f-values of both known and venom spots were 0.50. Cochromatography revealed no new spots.

Quantitative estimation was made by the method used for histidine and methionine except that elution from the silica gel was made with 1 ml of glacial acetic acid on a vortex mixer at 57°C for 15 minutes. Only 50% of the pipecolic acid was eluted by this method so that standard knowns had to be run simultaneously with the unknown, using identical

procedures. The spectrophotometric analytical method described by Schweet (38) was used and revealed a pipecolic acid content corresponding to 0.12 to 0.16% of the dried venom. An independent assay based on elution from a paper chromatograph (Whatman No. 3 MM) developed with 80% phenol gave identical results.

Protein Fraction

The paper chromatogram of a natural venom sample showed positive Azocarmine G and Amidoschwartz 10B tests only at the origin, so the protein fraction of the venom is not moved by 80% phenol.

Dried natural venom (4.41 mg) was dissolved in 1 ml of triple distilled water and applied in a series of closely spaced points along the short edge of a 23 X 50 cm sheet of Whatman No. 3 MM paper. The sheet was developed for twelve hours with 80% phenol, dried in a stream of air, and washed with ether to remove residual phenol. The dried paper was cut on a line 2 cm above the origin and parallel to it, a region previously shown to be free from the ninhydrin-positive compounds separable by 80% phenol. The residue on this strip was eluted with distilled water, lyophilyzed, and determined gravimetrically. A weight corresponding to 31% of the original dried venom was obtained. Since compounds other than proteins also remained at the origin, this figure represents a maximum protein content of the venom.

The venom from 35 insects was dissolved in 0.25 ml of distilled water and applied at a single point 2.5 cm from the bottom of a 4 X 25 cm

strip of Whatman No. 1 paper. This strip was developed for six hours with 1-butanol:acetic acid:water (100 ml of 1-butanol combined with 10 ml of glacial acetic acid and saturated with water) and dried in a stream of air. The chromatogram was then stained with Amidoschwartz 10B which revealed that the venom contains at least four different proteins. Three of the proteins showed R_f-values of 0.039, 0.080 and 0.113 while the bulk of this fraction remained at the origin.

An attempt was made to estimate the protein content of the venom from its optical density at 280 mμ. Approximately 4 mg of dried venom was dissolved in 5 ml of distilled water and filtered through a 0.22 μ Millipore membrane filter (Millipore Filter Corp., Bedford, Mass.). A spectrum of this solution was obtained on the Beckman DK-2 recording spectrophotometer. Strong absorption (O.D.=0.96) at 270 mμ by other venom constituents unfortunately obscured the protein spectrum.

A reliable estimate of the protein content was finally obtained by a gel-filtration fractionation of the venom. The various fractions were determined gravimetrically on a Mettler M5 micro balance. Approximately 3 gm of Sephadex G-25 medium (Pharmacia, Uppsala, Sweden) was allowed to swell for 8 hours in 20 ml of triple distilled water, and then it was deaerated in a suction flask.

Figure 2 shows the result of a gel-filtration of 4.0 mg of mud-dauber venom on a 7.9 ml column (1 X 10 cm) of dextran gel. The venom had been dissolved in 0.2 ml triple distilled water, and after applying the