Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 213-220

Regulatory Mediators in the Venom of *Chelonus* sp.: Their Biosynthesis and Subsequent Processing in Homologous and Heterologous Systems

Davy Jones* and Mietek Wozniak

Department of Entomology, University of Kentucky, Lexington, KY 40546

Received May 18, 1991

Following titration of the contents of the venom gland reservoir, the rate of biosynthesis of venom proteins was sufficiently rapid over the next 6-24 hrs to restore their titer to the level initially synthesized during early adulthood. There was no evidence of processing of smaller molecular weight components from much larger forms. Although most proteins were stable in young host embryos, two specific processing products of a 32.5 kDa venom protein were found in such hosts. The natural injection of venom proteins into either very old embryos or young embryos subsequently held at 4°C for six days resulted in rapid degradation to biologically inactive forms. These data are the first report of direct examination of the biosynthesis of wasp venom proteins and the first analysis of the processing of specific hymenopteran venom proteins in target tissues.

Biochemical interactions between parasitic wasps and their host may result in the release of material into the host by the parasite which alters host biochemical pathways (20). Such regulatory molecules can be effective probes of both homologous and heterologous systems. A paralyzing venom component from one wasp was used to probe the mechanism of action of scorpion toxin (26). In an analogous way, scorpion toxins have been extremely useful in characterizing and purifying sodium channel proteins (27). Nonparalyzing venoms of parasitic wasps contain components which have been shown to block apolysis (5), and to promote uncoating of polydnavirus at nuclear pores (23). In our own studies of the venoms and regulatory mediators of parasitic wasps (4,9,10), we have identified a specific 32.5 kDa venom protein from *Chelonus* spp. (8), the injection of which is necessary for survival of the endoparasite (24). Immunological and other studies have shown that the protein, while present in small amounts in the 'synthesis' regions of the venom gland, accumulates to the highest levels in the 'reservoir' portion of the gland (24). The synthesis and processing of proteins in nonparalyzing venoms has not been addressed, other than the data described above for *Chelonus* venom. Additional questions remain, such as the ontogeny of venom protein synthesis, the biosynthetic capacity of venom glands, and additional questions on processing within the wasp and within the wasp's (embryonic) host.

*To whom correspondence should be addressed.

0006-291X/91 $1.50

MATERIALS AND METHODS

Insects. The endoparasitic wasp *Chelonus* near *curvimaculatus* (Braconidae) was reared on host *Trichoplusia ni* (12). Usually, "naive" wasps (which had not stung any host eggs), 24-36 hours old, were used, except for an ontogenic study.

Dissection of venom glands. The venom glands of *C.* near *curvimaculatus* were dissected in 7% sucrose containing 150 mM NaCl and 20 mM Tris-HCl buffer, pH 7.6. Glands were homogenized manually, or, prior to separate homogenization, the venom apparatus was divided into synthesis region (plus a major part of the transport filament) and the venom gland reservoir (connected to a small remaining part of the filament).

Immunological methods. Rabbit polyclonal antibodies raised against all soluble proteins from the venom gland were used (16). A specific polyclonal serum against 32.5 kDa venom protein was developed by injection of rabbit each time with homogenized acrylamide gel pieces containing 40-50μg of pure 32.5 kDa protein isolated by the method described by Taylor and Jones (24). This antibody interacts with both 32.5 kDa protein (9) and its primary translation product (Jones D. and Sawicki G., unpubl. data).

Gel electrophoresis and immunobloting. Sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransfer of proteins from the gel to nitrocellulose followed by immunoblotting was performed (3,14). The proteins were transferred to nitrocellulose in 25 mM Tris, 192 mM glycine containing 20% methanol. Then the nitrocellulose was blocked with a solution containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 20% horse serum and 5% BSA followed by washing with a solution containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% milk powder, 0.5% Triton X-100, 0.2% SDS. The antigens were probed with 1:200 diluted antiserum against total venom proteins or with 1:1,000 diluted antiserum against the 32.5 kDa protein. The antigen-antibody complex was detected with ^{125}I goat-anti-rabbit IgG by autoradiography.

Comparison of the relative quantity of abundant venom proteins or intensity of autoradiographic signals was carried out by scanning of major bands on the gel or film using an LKB Ultroscan XL enhanced laser densitometer.

RESULTS

The ontogenic analysis showed that during pupal development the venom gland contained little of the proteins later found within the gland (Fig.1), while newly ecdysed females contained detectably more. Within 24 hours after eclosion, the venom reservoir appeared to contain a full compliment of each of the identified venom proteins.

The amount of the major 32.5 kDa protein inside the venom reservoir of unemerged wasps had a level similar to the emerged adults although the steady state amount of this protein in the synthesis region was still less than that for emerged wasps. Other major venom proteins such as the 53 kDa reached their final titer in the reservoir by the time of adult emergence, although their titer in the synthesis region was still submaximal.

The steady state concentration of venom reservoir proteins showed a progressive titration of venom proteins in the reservoir during *ad lib.* stinging of the host (Fig. 2). Females which refused further stinging after over 100 stings usually had been essentially completely titrated for venom reservoir contents. Upon dissection, these reservoirs appeared flat, instead of with their normal round, bulbous shape, although the synthesis region still contain detectable proteins which are normally found stored in the venom reservoir. Immunoblotting results showed the titer of the 32.5 kDa protein in the reservoir during *ad lib.* stinging had been reduced after 50 ovipositions to 63% of its previous level and to 6.4% after 130 ovipositions. However, the amount in the synthesis region after 130 ovipositions was still 53% of the amount before stinging.

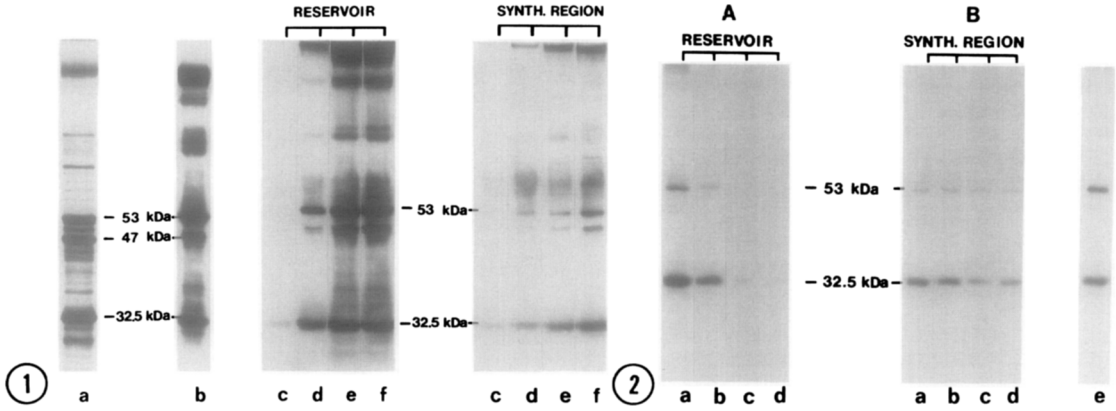


Fig.1. Biosynthesis of major venom proteins during wasp development analyzed by immunoblot. a - Major venom proteins after SDS-PAGE and silver staining. The extract from five total venom glands was loaded on the gel; b - Immunoblot of extract from one venom gland probed with antibody against all venom proteins: (c-f) Immunoblot of extract from reservoir or synthesis region obtained from the following stages of wasp development: c - black-pigmented pupa; d - adult wasp not emerged from cocoon; e - adult wasp, about 12 hrs after emergence from the cocoon; f - adult wasp 24-36 hrs after emergence from the cocoon.

Fig.2. Titration of major venom proteins inside venom glands during multiple oviposi-tions. A - Extract from venom gland reservoirs; B - extract from venom gland synthesis regions; (a-d) - Immunoblot following SDS-PAGE of extract from venom gland reservoirs or synthesis regions obtained from the following steps of multiple oviposition; a - before stinging, from "naive" wasp 12-16 hrs after emergence from the cocoon ; b - after 10 ovipositions; c - after 50 ovipositions; d - after 120 ovipositions: e - immunoblot of extract from one venom gland of wasp 12-16 hrs after emergence from the cocoon. All samples were probed with antibody developed against all venom proteins. The X-ray film was exposed for a short time so that signals for the major proteins would not to be overexposed, facilitating densitometric analysis.

In general, the amounts detected in host eggs early during the oviposition sequence (e.g. ovipositions 10-20, 21-30, etc.) were very similar to the amounts injected right up the time the female stopped stinging (e.g. ovipositions 101-110, 111-120), except the last few ovipositions (Fig.3). At the beginning of oviposition sequence (1-10), there was a tendency to inject slightly more venom proteins.

Within 1 hr, after titration of the female, the venom reservoir had begun to accumulate a qualitative profile of proteins within the reservoir essentially identical with that observed under the 'fully stocked' condition (Fig. 4). No evidence was discernible of processing from larger precursors within the reservoir. The detectable proteins within the synthesis region of the gland were identical in size with those accumulating in the reservoir.

The complete regeneration of some major venom proteins inside the venom glands after their titration required 6 to 24 hours (Fig.4). The lowest amount of 32.5 and 53 kDa proteins in the reservoir was detected just after titration and it required between 3-6 hours for 32.5 kDa protein and between 6-24 hours for 53 kDa protein regeneration within the reservoir of a final level actually greater than that which occurred before sting-ing. The amount of these proteins in the synthesis region decreased for 3 hours after titration while the titer in the reservoir increased during this time. Later, the amount of

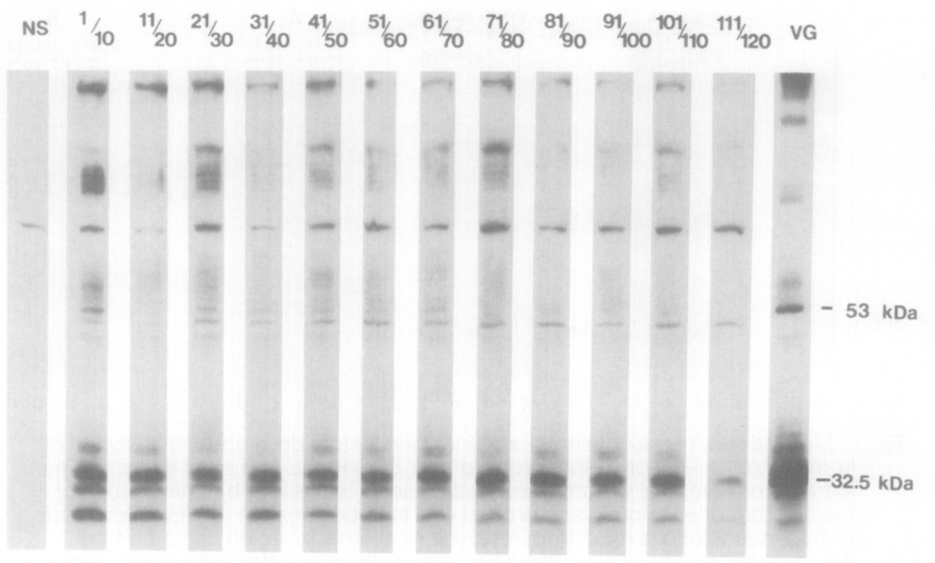


Fig.3. The amount of venom proteins inside host eggs injected during successive ovipositions by the female wasp. NS - Extract from ten non-stung eggs. The following are singly stung eggs collected during the oviposition sequence and combined into groups of 10 eggs: 1/10 - eggs stung during oviposition 1 to 10; 11/20 - eggs stung during oviposition 11 to 20; etc.; VG - extract from one venom gland 12-16 hrs after emergence from the cocoon. All samples were probed with antibody raised against all venom proteins.

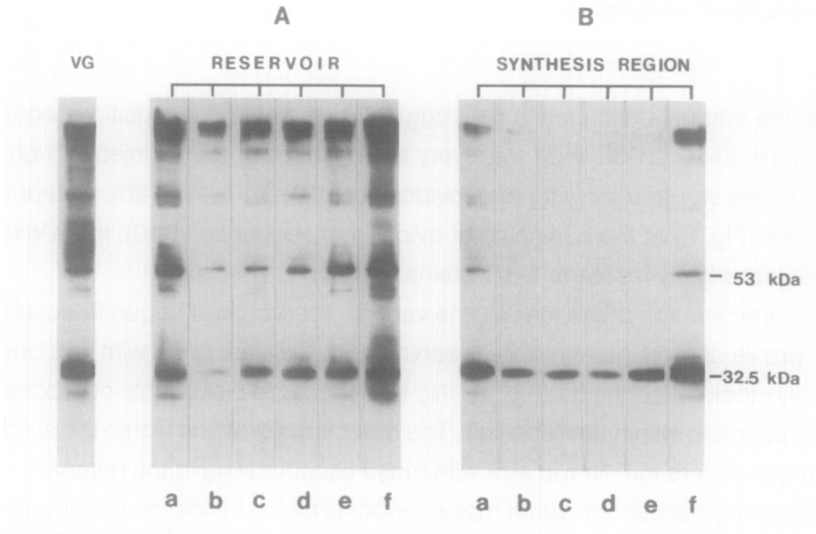
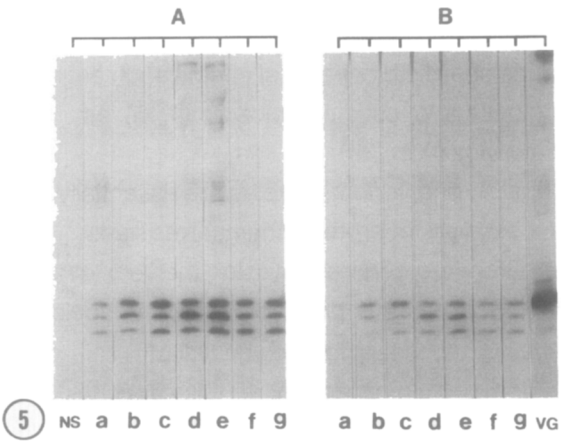


Fig.4. Regeneration of the venom proteins in the venom glands after multiple oviposition, analyzed by immunoblot following SDS-PAGE (probed with antibody raised against all venom proteins). A - Extract from venom gland reservoirs; B - extract from synthesis region of venom glands; (a-f) - immunoblot of extract from 3 reservoirs or synthesis regions of venom glands obtained after successive ovipositions: a - before stinging; b - immediately after all multiple oviposition when the female refused further oviposition ; c - (1 hr), d - (3 hrs), e - (6 hrs), f - (24 hrs) after time point in b; VG - extract from 2 venom glands 12-16 hrs after emergence from the cocoon.

both proteins in the synthesis region increased (while their titer in the reservoir also increased) and after 24 hours their titer was even higher (especially for 32.5 kDa) than before titration.

The processing of venom proteins in a heterologous system, the host embryo, was examined in hosts of various physiological conditions. When < 24 hrs old embryos were stung, the venom was not detectably degraded 4 hrs later (Fig. 5). However, when venom proteins were injected into essentially pharate first instar larvae (within several hrs of hatching) venom proteins were reduced to an average of only 20-50% of their original abundance. The venom proteins were also processed to inactive products in stung, young embryos which were then held at 4°C for 6 days (Fig.5).

When young embryos were multiply stung it led to a proportional increase in the amount venom proteins detected in young hosts several hours later. However, when multiple levels of venom proteins were introduced into old host embryos, they were highly degraded almost immediately, i.e. that processing system was not saturable at the levels of venom proteins introduced (Fig.5). For example, the amount of 32.5 kDa protein



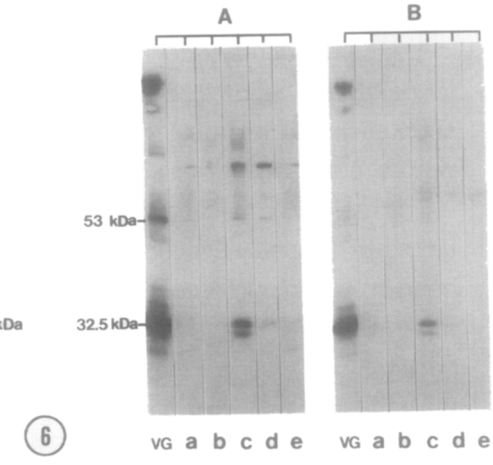


Fig.5. Venom protein survival inside the host embryo after multiple oviposition, analyzed by immunoblot following SDS-PAGE. A - Samples probed with antibody raised against all venom proteins; B - samples probed with antibody developed against major venom 32.5 kDa protein; NS - extract from five non-stung eggs ; VG - Extract from one venom gland 12-16 hrs after emergence from the cocoon; (a-e) - extract from the following, one day old embryos of *T. ni*: a - one time stung embryo; b - two times stung embryo; c - three times stung embryo; d - four times stung embryo; e - five times stung embryo; f - extract from three days old, five times stung embryo (mature embryo); g - extract from three days old, five times stung embryo (1st instar larva, 2-4 hrs old).

Fig.6. Venom proteins survival inside the host embryo analyzed by immunoblot following SDS-PAGE. A - Samples probed with antibody raised against all venom proteins; B - samples probed with antibody developed against the major venom 32.5 kDa protein; VG - extract from two venom glands of wasp 12-16 hrs after emergence from the cocoon; (a-e) - extract from *T. ni* embryos: a - non-stung, one day old embryo; b - non-stung, three days old embryo (1st instar larvae 2-4 hrs old); c - one time stung, one day old embryo; d - one time stung, one day old embryo later incubated six days at 4°C after stinging; e - one time stung, three days old embryo (1st instar larvae 2-4 hrs old).

inside the three days old embryo stung five times was reduced after 4 hours to the level of this protein which occurred in a one day old embryo stung only one or two times.

Processing products of about 30 and 27.5 kDa appeared within several hours in young host embryos. These proteins were only marginally detectable in hosts immediately homogenized in buffer containing SDS (loading buffer) after stinging. The product reacts intensely with the anti 32.5 kDa protein antibodies, strongly suggesting that this protein is processed in host embryos to these specific products (Fig.5 and 6).

DISCUSSION

The venom proteins of higher, social or predatory Hymenoptera have been studied in detail for their allergenic and biochemical properties. For example, the honey bee venom component mellitin has been studied extensively for its primary sequence and processing (14). Its tertiary structure has been analyzed and with this information its mechanism of action on its target (cell membrane lipids) has been proposed (17). The size and charge properties of such paralysis-inducing proteins in several parasitic wasp venoms have been estimated (21). By indirect methods, the amount of paralyzing venom injected into hosts has been estimated, as well as some aspects of biosynthetic appearance of paralysis-inducing activity (1).

The components of nonparalyzing venoms have been addressed even less than those for paralyzing venoms. Such venoms can induce specific biochemical effects such as suppression of the host immune response or blockage of cuticular apolysis (4,15), as well as more nonspecific effects, such as on rate of growth (25).

The molecular mechanisms of action of nonparalyzing venom proteins are essentially unknown, primarily because the structures of these venom proteins themselves have been unknown until recently. Parasitic Hymenoptera are generally missing the very low molecular weight proteins (e.g. melittin, 3 kDa) found in higher predatory wasps or social bees (15). The venom of *C. near curvimaculatus* is necessary for the survival of the egg-larval endoparasite (17). A specific venom protein necessary for this effect has been isolated and microsequenced for its N-terminal residues (24). Recently, the sequence of the protein as inferred from cloned cDNA was reported (7).

In the present study on venom biosynthesis, we have found that the proteins are nearly absent from the venom glands of old pupae and are still lower than the normal titer in very young adults, but are rapidly accumulated in the synthesis region and reservoir of the venom gland within a day after adult emergence. Subsequently, the female puts nearly the same amount of venom into each successive host, of over 100 hosts, until finally no more hosts are stung. All but the last few hosts which are stung receive an amount of venom similar to that injected into proceedings hosts. These results suggest that the female can precisely measure the amount of venom being injected, even though the shape of the venom reservoir may change from nearly spherical to essentially flat. After the reservoir is emptied, the female refuses to sting anymore hosts. This the behavior of the female would seem to ensure that the host into which the endoparasite will be

injected receives an adequate amount of venom to assure the survival of the endoparasite. These results explain the difficulty reported previously in generating pseudoparasitized individuals by ovipositionally titrating the females (6).

Rapid biosynthesis of *all* of the venom proteins after titration implies either a massive induction of transcription of new mRNAs for the proteins, or that a high level of mRNA is maintained in the gland, without concomitant translation, until the proper regulatory signals induce biosynthesis of the proteins. Molecular studies with the cloned venom proteins (5) will permit determination of the resolution of such rapid biosynthesis.

When the steady state level of protein in the venom reservoir is reduced essentially to zero, the kinetics of subsequent biosynthesis of each venom protein does not reveal any evidence of processing of smaller venom proteins from much larger ones. Most of the proteins in the venom gland region itself are indistinguishable in size from those in the reservoir (24). These results suggests that if the smaller venom proteins are processed from larger ones, the dynamics of biosynthesis and processing steps causes these primary venom proteins to be, at steady state, an undetectably small fraction of the proteins present. We observed that the venom became quickly degraded when injected into old embryos. As was reported previously, this permutation leads to death of the endoparasite and occurrence of 'pseudoparasitism' (11,13,16). Those results led to the proposition that degradation of the venom before it can act on the host to promote parasite survival results in death of the parasite and pseudoparasitism. The present study has provided an independent test of that hypothesis by examining the stability of the venom under other conditions ($4^{o}C$ for 6 days) that cause pseudoparasitism. The result was that the venom was degraded in the parasitized host embryo prior to replacement of the host (and internal parasite) at a temperature normally conducive to host and parasite development. These results further support the importance of the action of the venom for survival of the endoparasite and successful parasitism.

In summary, the present study has provided the first data for the nonparalyzing venom of parasitic wasps on the ontogeny of venom protein biosynthesis, its ovipositional titration and its subsequent biosynthetic replenishment. We have also provided the first data on the control of female wasps over the amount of nonparalyzing venom injected into successive hosts. We have also substantiated through independent tests the hypothesis that there is a 'developmental window' during host embryogenesis in which the venom must act if it is to ensure parasite survival. With these data, and with antiserum and cloned cDNAs for the venom proteins in hand, future studies will be able to address the molecular basis for mechanisms of regulation of expression of genes for venom proteins that promote the ready availability of venom toward its function in parasite survival.

ACKNOWLEDGMENTS

This research was supported, in part, by NIH grant GM33995. Published with the approval of the Director of the Kentucky Agricultural Experiment Station.

REFERENCES

1. Beard, R. I. (1978) Venoms of Braconidae. *in* Handbook for Experimental Pharmacology, (Bettini, S. ed.), Springer-Verlag, N.Y. pp. 773-800.
2. Beckage, N. E., Templeton, T. J., Nielsen, B. D., and Nesbit, D.J. (1987). Insect Biochem. **17**, 439-455.
3. Burnette, M.W. (1981) Analyt. Biochem.**112**, 195-203.
4. Chelliah, J. and Jones, D. (1990) J. Gen. Virol. **71**, 2353-2359.
5. Coudron, T. (1991) Arch. Insect Biochem. Physiol. 13:83-94.
6. Jones, D. (1987) J. Insect Physiol. **33**, 129-134.
7. Jones, D. (1990) 38th Ann. Meeting, Entomol. Soc. Amer., Prog. Book, pg. 136
8. Jones, D. and Leluk, J. (1990) Arch. Insect Biochem. Physiol. **13**, 95-106.
9. Jones, D., Soldevila, A., Chelliah, J., Taylor, T, and O'Neill, A. (1990) Vth Internat. Colloq. Invert. Pathol. Microb. Contr., Adelaide, Australia.
10. Jones, D., Taylor, T. Farkas, R., Chelliah, J. Haene, B., Brown, W., and Reed-Larsen,D. (1990) *in* Advances in Invertebrate Reproduction 5 (M.Hoshi and O.Yamashita eds) pp.157-162, Elsevier Sci. Publ., B.V. (Biomed. Div.)
11. Jones, D. (1985) J. Comp. Physiol. **155**, 583-590.
12. Jones, D. (1986) Exper. Parasitol. **61**, 10-17.
13. Jones, D., Jones, G., Sreekrishna, S. and Click, A. (1984) XVII Intern. Cong. Entomol., Hamburg, Abstr. Vol., pg. 204.
14. Laemmli, E.K. (1970) Nature, **227**, 680-685.
15. Leluk, J., Schmidt, J. and Jones, D. (1989) Toxicon **27**, 105-114.
16. Leluk, J. and Jones, D. (1989) Arch. Insect Biochem. Physiol. **10**, 1-12.
17. Leluk, J., Schmidt, J., and Jones, D. (1988) *in* Endocrinological Frontiers in Physiological Insect Ecology (F. Sehnal, A. Zabza, and D.L. Dehlinger, Eds) Vol.1, pp.457-460, Wroclaw Tech. Univ. Press, Wroclaw, Poland.
18. Kreil, G., Haiml, L. and Suchanek G. (1980) Eur. J. Biochem. 111, 49-58.
19. Kitano, J. (1986) J. Insect. Physiol. **32**, 369-375.
20. Lawrence, P. O. (1986) J. Insect Physiol. **32**, 295-298.
21. Piek, T., and Spanjer, W. (1986) *in* Venom of the Hymenoptera (T. Piek Ed), pp.261-308, Academic Press, Orlando, FL.
22. Posch, M., Rabusch, U., Mollary, C. and Laggner, P. (1983) J. Biol. Chem. **258**, 1761-1766
23. Stoltz, D. B., Belland, E. R., Lucarotti, C. J. and Mackinnon, E. A. (1988) J. Gen. Virol. **69**, 903-907.
24. Taylor, T. and Jones, D. (1990) Biochim. Biophys. Acta **1035**, 37-43
25. Tanaka, T. and Vinson, S. B. (1991) Ann. Entomol. Soc. Am. **84**, 87-92.
26. Walter, C., Zlotkin, E., and Rathmayer, W. (1976) J. Insect Physiol. *22*, 1187-1194
27. Vijverberg, H. P. M. and Lazdunski, M. (1984) J. Physiol., Paris, **79**, 275-279.