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Mulberry Silkworm, *Bombyx mori* L., as a Host for Neurotoxic Braconidae I. Insect-toxic Properties of Bracon Venom Gland Extract and Its Fractions

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The silkworm *Bombyx mori* L. was studied as the potential "host" of popular in Uzbekistan biocontrol ectoparasite, entomophagous *Bracon hebetor* Say. Being one of representatives of economic-beneficial insects, the silkworm (larvae, pupae and imago) can be used as highly sensitive test organism for revealing of neuro toxic effects of insects venom as well as of their purified components in screening assays. In comparative aspect, except a mulberry silkworm, representatives of Uzbekistan pests cotton-boll worm *Helycoverpa armigera* Hbn., lesser mulberry pyralid *Glyphodes pyloalis* Wlk., codling moth *Corpocapsa pomonella* L., malaria mosquito *Anopheles claviger* and Colorado potato beetle *Leptinotarsa decemlineata* Say have been subjected to insect toxic test of bracon venom gland extract (VGE) and its fractions which were obtained by gel-chromatography on Sephadex G-100. The paralyzing effect of the VGE and its fractions was shown in a various degree on the pests.

Key words: Silkworm, *Bombyx mori* L., Host, *Bracon hebetor* Say, Neurotoxin, Pests, Biocontrol.

Introduction

Mulberry silkworm *Bombyx mori* L. does not considered in entomological scientific literature as a host for braconids and other entomophagous parasites apparently due to disappearance of its wild species and inclusion it in number of completely man-cultivated animal. As against a honey bee, another important economic insect, mulberry silkworm is considered as more "conservative" by way of natural contacts to other representatives of insects due to the strictly designed recommendation to sericulture, barring such contacts. On the other hand, mulberry silkworm is quoted among the scientist as one of the most studied, accessible and simple in breeding insect with outstanding parameters of development, productivity and reproduction. With creation of artificial diets for silkworm this insect can be used all over the year not only in sericulture (Horie and Watanabe, 1980; Madyarov, 1988) but also in scientific researches including production of recombinant proteins (Maeda. *et al.*, 1985), species specific baculoviruses (Maeda, *et al.*1989) and even in extreme conditions of space experiments (Madyarov *et al.*, 1995). As the glaring representative of invertebrates world with the above listed advantages, mulberry silkworm is a fine test-object in numerous researches of effect of the environments factors and for a long time is utilized in fundamental developments on physiology, biochemistry, ecology, toxicology, epizootology and other scientific fields.

In the present work mulberry silkworm *Bombyx mori* L. is considered as a "host" of beneficial and extremely toxic entomophagous ectoparasite *Bracon hebetor* Say (Mirzaeva *et al.*, 2001). The paralyzing effect of insects venom have been studied both at natural sting and in effect of injection of VGE and its fractions obtained by gel-filtration. Silkworm larvae of different instars, pupae and but-

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terflies were subjected to effect of the VGE and its active fractions. The imago as the most energy weak stage of insects development is the most sensitive to the effect of active fraction of bracon VGE. Possible use of mulberry silkworm as excellent test-organism during creation of modern bioinsecticide is discussed. Experimental results confirming known data on high molecular weight (> 40 kDa) of neurotoxins *Bracon hebetor* Say (Visser *et al.*, 1983; Quistad *et al.*, 1994) are also represented in this work.

Preliminary investigations of insect toxic effect of natural bracon venom, VGE and its fractions on larvae of some Uzbekistan pests including cotton-boll worm, lesser mulberry pyralid, codling moth, malaria mosquito and Colorado potato beetle have been carried out.

Materials and Methods

Mulberry silkworm larvae of "Orzu" strain were bred in laboratory conditions in spring and summer breeding season with use of "Balhi-tut" mulberry leaves. Mulberry silkworm larvae of different instars (II-V) and also *Bracon hebetor* Say individuals in active imago stage of development (being purchased in Republic Entomophagous Breeding Laboratory) have been subjected to interaction host - parasite. Fixed amount of adult female bracon individuals and mulberry silkworm larvae of different instars were exposed for interaction during 2 days in Petri dishes with mulberry leaves. Separate stages of interaction and the further behavior (sting process, behavior of silkworm larvae during effect of the venom, laying of eggs by bracons, hatching of bracon larvae and their development) were observed with the help of a binocular magnifier MBC-2. Obtaining of crude bracon venom was performed by modified method described minutely in the former work (Mirzaeva *et al.*, 2001). Fixed amount of bracons imago was treated by chloroform vapor to stop movement of individuals. The venom gland together with the sting was isolated from the body of female bracon and inserted on the surface of overcooled ice to prevent denaturation and extraction of venom from the gland. Glands of bracon females being collected and frozen in such a manner were extracted by distilled water for homogenization of tissue in precooled porcelain mortar. The extract after separation of insoluble part by centrifugation (5,000 rpm, 10 min) was dried in stream of cooled air. The VGE obtained in such a manner was kept before using at temperature 0 – 4°C. Fixed amount of this venom was dissolved in isotonic solution (0.9% NaCl) containing 10,000 units of penicillin and 10 mg streptomycin in 1 ml for study of neuro-toxic properties by injection of different dozes of crude venom being injected in different places of larvae bodies in amount of 1 – 15 µl. Part of VGE after dissolution in isotonic solution and centrifugation (15,000 rpm, 20 min) was fractionated on Sephadex G-100 chromatographic column (0.6 × 75 cm) previously equilibrated and calibrated in same solution using LKB Chromatographic System (Sweden). Fractions (0.5 ml/30 min) were collected and tested for toxicity being injected to insects (5 – 10 µl of fractions). Mature pupae (7^{th} day cocooning) and silkworm imago after exit from the cocoon, mating and eggs-laying have been used as examinees except for larvae. The value reverse to paralyzing time of insect under effect of injected fraction, *i.e.*, 1/t, min^{-1}, was selected as a measure of relative toxicity.

The larvae of some Uzbekistans pests - cotton-boll worm *Helicoverpa armigera* Hbn., lesser mulberry pyralid *Glyphodes pyloalis* Wlk., codling moth *Carpocapsa pomonella* L., malaria mosquito *Anopheles claviger* and Colorado beetle *Leptinotarsa decemlineata* Say were tested in preliminary assay. Test conditions were the same as in case of mulberry silkworm except for tests of mosquito larvae. In this case VGE fractions (0.2 ml) added in 2 ml larvae breeding water media.

Results and Discussion

Host parasite interaction

The evidences testifying that mulberry silkworm *Bombyx mori* L. is a host of insects- entomophagous have not been found in available literature. In the same time, comparison of mulberry silkworm larvae with other larvae of a classic hosts of parasitic entomophagous testifies that mulberry silkworm could become a laboratory "host" (test organism) for many entomophagous species on study of host-parasite interaction as well as probe for toxic and pathogenic factors of insects control (both synthetic, recombinant and natural). Particularly at the present time it is important to have accessible and easily reproducible test-objects with clear response reactions for mass screening of new synthesized chemical and biological preparations for evaluation of their insecticide activity and also in search of specific high effective natural insect toxins for the task of creation of new effective bioinsecticides (Inceoglu *et al.*, 2001). Creation and use of high specific baculovirus based insecticides is especially important for agrobiocenoses of Central Asian region due to traditional territorial vicinity of two main commercial crops (cotton plantations and linear mulberry planting around them) as well as residing of farmers families excluding the use of plants protection by chemical means during seasonal rearing of silkworm. In connection with above mention the

bracon which is widespread in the nature and being used by man practically in pest control and mulberry silkworm were chosen as parasite and its prospective host for this study. Experiments on exposition of this insects in small space (Petri dish) have shown that mulberry silkworm larvae of 2nd 5th instars represent excellent example of host for paralyzing type of the entomophagous. The larvae have felt a presence of the enemy at once that was expressed in termination of movement (as though they have hidden, have stood), but in 15 min have renewed the moving and eating of feed. In this experiment, we were not witness for the first active contacts in the day time, but, having them left for the night and next day we have found out one of paralyzed larvae (in area of the third segment) with 5 newly-laid out eggs. Later, for the 4th day after laying out of eggs, 4 bracon larvae hatched. Then they developed on the paralyzed silkworm larvae. In the other experiment (2 bracon female + 1 larva of the 3rd instar in each Petri dish) we observed an active host-parasite interaction. In this experiment after some period of their enclosing in Petri dishes was observed one of the females having sat on the silkworm larva and began to be tried on for sting. After two unsuccessful attempts bracon has all the same stung a larva in preliminary chosen place. The sting remained in a larva body for 2 min and 10 sec. Then bracon began to suck hemolymph (1 min and 15 sec.) Later, not flying away, it has repeatedly stung and has sucked away more hemolymph. We have not seen process of eggs laying out in this experiment. These and other experiments carried out spring and summer time 2001 with different amount and ratio of parasite and host (2nd – 5th instars) allow us to conclude: a) the parasite and the host sharply felt presence of each other; b) the bracon reaches necessary fast paralyzing effect by numerous sting of the host; c) young and adult individuals of silkworm larvae (average weight of larvae from 100 mg up to 5.0 – 6.0 g) significantly surpassing on mass a bracon female, they can actively defend from an attack of bracon forcing down by their head or sharp movements of body; d) bracon can lay out eggs not only on back of the host, but also on lateral parts of body, the number of eggs in a laying on one larvae varied from 3 up to 12 pieces, bracon larvae are hatched not from all eggs; e) bracon is capable to paralyze and use the paralyzed silkworm larvae for the feed, laying out of eggs, for hatching of larvae and their subsequent development.

Obtaining of VGE and its fractions

An attempt to fractionate VGE and to isolate its toxic fraction in the present work with the purpose to study its neuro toxic effects on insects, including on mulberry silkworm is undertaken. Till now molecular characteristics of toxic components of the bracon venom are not determined precisely. The value of its molecular mass varied over a wide range from 18 up to 73 kDa (Visser *et al.*, 1983; Kosovskii *et al.*, 1986; Quistad *et al.*, 1994). The native insect venom which was injected into a host in the sting time was not used for any of the works. The methods used in these works for isolation of toxic components included homogenization of whole insects or their toxic apparatus with associated tissues that leads to lysis and denaturation of neuro toxic polypeptides of native venom during its isolation and purification that was reflected, apparently, in such a wide difference of molecular weights of the studied toxins.

The following simple methods were preventing intensive lysis of biological material. Fast narcosation of bracon individuals in chlorophorm vapors, separation of stings with venom gland at females, accumulation of this material on the surface of overcooled ice and keeping it till homogenezation at 15°C were used in this work for obtaining of VGE. Fast homogenezation of collected biomaterial was performed in preliminary cooled mortar by cooled distilled water (0 – 4°C). The whole operation on extraction was finished within 10 – 15 min, the extract was centrifugated (5,000 rpm, 5 min) and dried in stream of the cooled air (for intensive evaporation). Brown ointment-like substance was obtained and it was kept at 0 – 4°C before use. Preparation of VGE in isotonic-antibiotic solution with protein concentration 0.5 mg/ml (by Lowry method) was used for insects injection.

The obtained VGE of 14 mg were dissolved in 0.5 ml of same solution for fractionation of bracon VGE by of gel-chromatography on Sephadex G-100 as described in Materials and Methods. As it has been shown on chromatogram (Fig. 1B), most part of substances eluted in "low molecular" fractions. The obtained fractions were kept at 0 – 4°C before test on insects.

Effect of VGE and its fractions on mulberry silkworm and other insects

The first doze depended experiment of VGE was carried out on mulberry silkworm larvae of 5th instar as was described in Material and Methods. The corresponding amount of isotonic-antibiotic solution was injected to control larvae. Any discomfort of control insects was not observed. The paralysing effect of venom gland extract was demonstrated in accordance to the injected dozes (Fig. 1A). When injection of 10 µl was made in head segments of larvae the larvae paralysis started faster within the 5 min. More intensive secretion of excrements in a comparison with control larvae was the other peculiarity of effect of VGE. The paralyzing effect was short at small dozes of VGE after which larva began start to move and

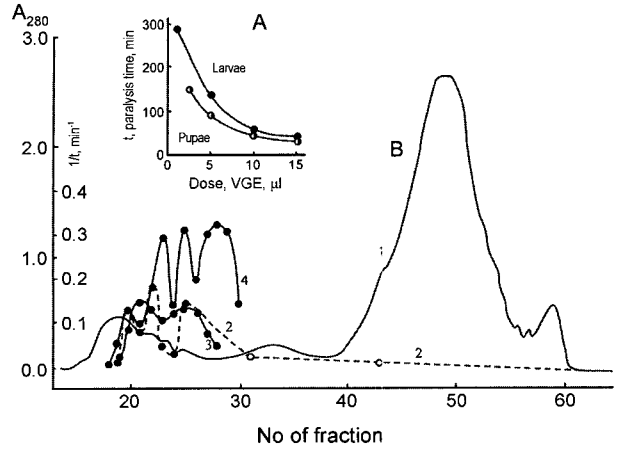

Fig. 1. Paralyzing effect of *Bracon hebetor* Say VGE (A) and its fractions obtained by gel-filtration on Sephadex G-100 (B) on silkworm *Bombyx mori* L larvae, pupae and imago. VGE venom gland extract (0.5 mg/ml); 1 A_{280}, optical density (280 nm), 2, 3, 4 paralyzing activity (1/t) of VGE fractions injected to silkworm larvae, pupae and moth, respectively (see Materials and Methods).

eat a feed. In the other experiment silkworm pupae were subjected to effect of VGE in different dozes (Fig. 1A). In the third experiment the fractions of VGE obtained by Sephadex G-100 gel-filtration were tested on larvae, pupae and imago of mulberry silkworm (Fig. 1B). In this experiment 5 – 10 µl of the taken for analysis fractions were injected to experimental insects in different stage of its development. The results show that VGE more effectively effects on pupae of mulberry silkworm (Fig. 1A). Pupae in this test are more preferable because they do not creep away and more sensitive to VGE effect. The same conclusion could be also done from result of Fig. 1B. Active fractions effect more quickly on silkworm pupae than on the adult larvae. Mulberry silkworm imago were most sensitive to effect on the fraction of VGE apparently as most weak stage of silkworm development. Important conclusion that neuro toxic components of natural bracon venom have molecular weight not less than 40 50 kDa and are represented not by one peptide follows from Fig. 1B.

Larvae of representatives of such pests of Uzbekistan as cotton-boll worm *Helicoverpa armigera* Hbn., lesser mulberry pyralid *Glyphodes pyloalis* Wlk., codling moth *Corpocapsa pomonella* L, malaria mosquito larvae *Anopheles claviger* and Colorado beetle *Leptinotarsa decemlineata* Say were also subjected to effect of VGE and of its fractions. If cotton-boll worm and codling moth are the "classic" hosts of bracon otherwise mulberry silkworm and lesser mulberry pyralid (new and dangerous pest of Uzbekistan) are unstudied as host of neurotoxic braconids. In the present work, mulberry pyralid have been preliminarily studied and characterized in parasitehost interaction with bracon. Active insects interaction and as result high defeating ability of bracon venom, bracon feeding, eggs laying, hatching and developments of its new generation on the paralyzed lesser mulberry pyralid have been marked. The larvae of cotton-ball worm, codling moth, mosquito larvae and Colorado beetle were the objects of paralyzing effect of VGE and its fractions. The larvae of cotton-ball worm and codling moth were affected most easily, but the larvae of mosquito and Colorado beetle less effectively, and in all cases observable effects were to similar effects in experiments with mulberry silkworm. The further study of species specificity and neurotoxic properties of native purified insect toxins of *Bracon hebetor* Say venom requires special detailed research on pests which is planned to be carry out.

Thus, it is shown in the present work that mulberry silkworm as the representative of the economic-important insects, can be considered and used as a high-sensitive test-organism from *Lepidoptera* order as the host of braconids and probably other species of poisonous insects. This is especially important that due to developed artificial diet for mulberry silkworm *Âombyx mori* (Madyarov *et al.*, 1988; Radzhabov *et al.*, 1991) and this insect can serve as experimental animal all over the year and even in extreme condition (Madyarov *et al.*,1995). On the other hand, the toxic fractions of studied VGE with outstanding insect toxic properties directed us to further work on isolation of individual components of *Bracon hebetor* Say native venom for their use in creation of bioinsecticides of new generation in the future.

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