Chemistry of Natural Compounds, Vol. 37, No. 6, 2001

COMPARATIVE BIOCHEMISTRY OF THE PHYSIOLOGICALLY ACTIVE COMPONENTS OF VENOM, HEMOLYMPH, AND EGGS OF THE KARAKURT SPIDER (*Latrodectus tredecimguttatus*)*

A. A. Akhunov,[1] Z. Golubenko,[1] N. A. Abdurashidova,[1] E. Ch. Mustakimova,[1] F. A. Ibragimov,[1] and S. Mackessy[2]

UDC 577.993

The physicochemical and functional properties of the venom, hemolymph, and egg extract of the karakurt spider Latrodectus tredecimguttatus *were studied. A highly purified toxin that differs from the neurotoxins found previously in the venom was isolated from the hemolymph and characterized.*

Key words: whole venom, hemolymph, karakurt spider egg extract.

The use of biologically active natural products to solve scientific and technical problems of modern biology and medicine is a promising approach. Therefore, further searches for new physiologically active components in natural substances involve their identification, isolation, overall characteristics, and the determination of the areas and methods of application. These searches are important not only for fundamental science, which expands and deepens understanding of the environment, but also for practical application.

Extracts of the venom glands of *Latrodectus* spiders have been used as starting materials to study the venom and latrotoxins in them [1]. Information about the toxic properties of hemolymph and egg extract was also obtained [2]. However, these latter studies were incomplete. Therefore, many issues are unresolved. The main ones concern the nature and properties of toxins in these materials and the possibility that they are identical or similar to toxins in the venomous secretions of these spiders.

We present results from research on the physicochemical and functional properties of the venom, hemolymph, and egg extract of *Latrodectus tredecimguttatus*. As a rule, preparations obtained from the venom glands of the spiders contain a trace impurity of hemolymph components. Therefore, the study of these preparations using a commercial antiserum against karakurt venom that was prepared in Tashkent at a Scientific-Research Institute of the Ministry of Health of the Republic of Uzbekistan and monospecific antisera against α- and β-latrotoxins is of interest. Thus, antiserum with a 1:64 titer against the venom extracted from spider venom glands gives three precipitation lines upon binary radial immunodiffusion against spider hemolymph. Monospecific antisera against α-latrotoxin purified until homogeneous with a 1:64 titer and β-latrotoxin with a 1:32 titer gave no precipitation lines upon Ouchterlony reaction against hemolymph and egg extract. Therefore, hemolymph and egg extract contain toxins different from those of spider venom glands.

A determination of the toxicity is the first step in the study of the biological activity of any natural substance. The toxicity (LD_{50}) is known to depend on the physiological state of the specimens from which the venom is obtained, the time of year, and the methods of preparation, drying, and storage, in addition to the geographical distribution of the population of venomous subjects. Furthermore, the sensitivity of experimental animal species to the sample is important [1]. We used venom and hemolymph of spiders collected in summer (July-August) in Dzhizak District of Uzbekistan. Spider egg silk was obtained in September from the insectarium of the Zoology Institute of the Academy of Sciences of the Republic of Uzbekistan.

*Present at the IVth International Symposium on the Chemistry of Natural Compounds (Isparta, Turkey, June 6-8, 2001).

1) A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, 700143, Tashkent, ul. Kh. Abdullaeva, 83, fax (371) 162 70 71, e-mail: root@ibc.edu.uz; 2) University of Northern Colorado, 501 20 Street, Greeley, CO 80639, USA, e-mail: spmacke@bentley.unco.edu. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 482-485, November-December, 2001. Original article submitted July 24, 2001.

 0009-3130/01/3706-0562$25.00

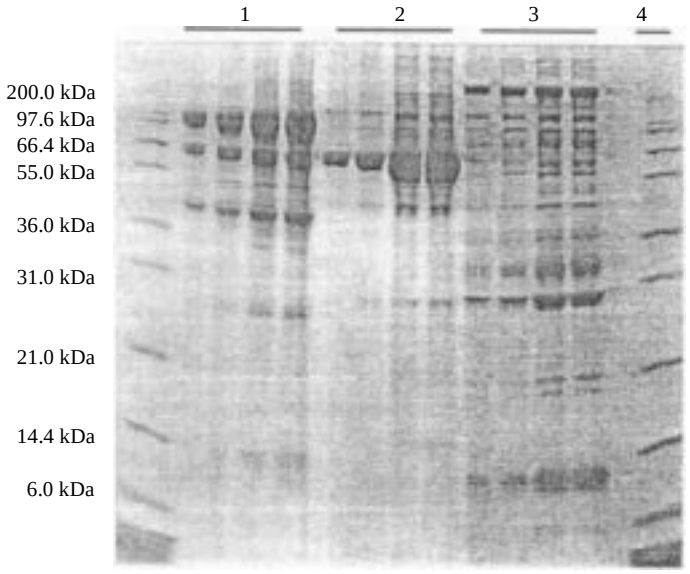

Fig. 1. SDS-electrophoresis in PAAG (14%) of proteins of whole venom (1), hemolymph (2), and egg extract (3) of *Latrodectus tredecimguttatus*, markers (4).

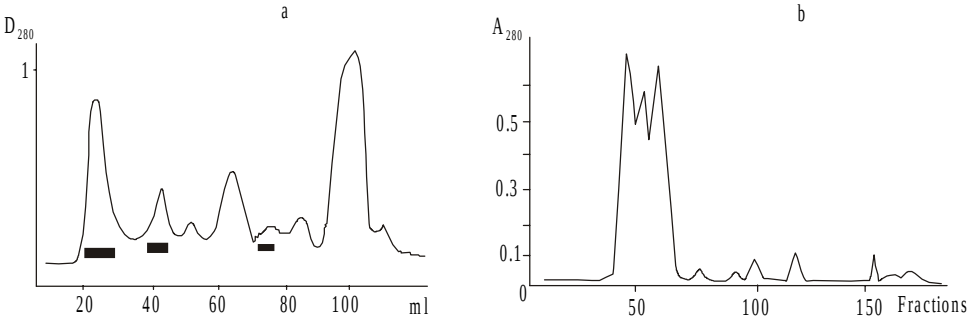

Fig. 2. Gel filtration of karakurt spider egg extract on Sephadex G-25 (1.5×150); eluent, ammonium acetate buffer (0.005 M, pH 7.8). Fractions possessing toxicity are marked (a); karakurt spider hemolymphs over a BioGel P-100 column equilibrated with HEPES buffer (10 mM, 0.1 M NaCl) (b).

We now present data for the toxicity of venom, hemolymph, and egg extract of karakurt spider towards mice (for i.p. administration).

	LD_{50}, mg/kg
Whole spider venom	8.5 (7.00÷9/37)
Spider hemolymph	53.0 (50.70÷58.20)
Egg extract	36.0 (24.00÷39.20)

It can be seen that venom obtained by extraction of the venom glands is the most lethal. Egg extract is slightly less toxic. Spider hemolymph is also toxic towards mammals. Administration (i.p.) to mice of ~10 μL of hemolymph leads to the death of the animals (20-25 g) within a day. Animals perish after 2-3 h if the dose is increased to 50 μL. These results agree with those in the literature in that newborn spiders, eggs, and egg sacs with eggs are lethal [2].

Detailed data on the composition of hemolymph and egg extract in addition to the physicochemical properties of the toxic proteins in them were obtained during the purification of the isolated proteins. Electrophoresis under denaturing conditions has shown that whole venom and hemolymph contain components of molecular weight from 8 to 300 kDa (Fig. 1) whereas egg extract also contains low-molecular-weight proteins (4-5 kDa).

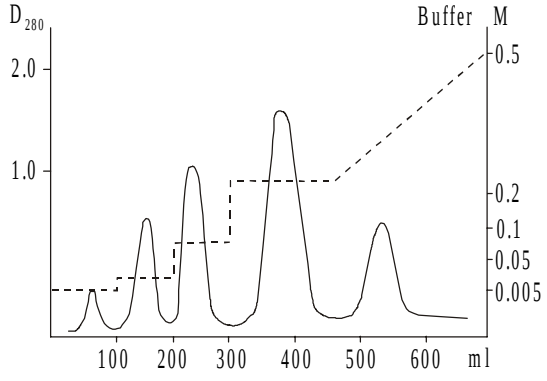

Fig. 3

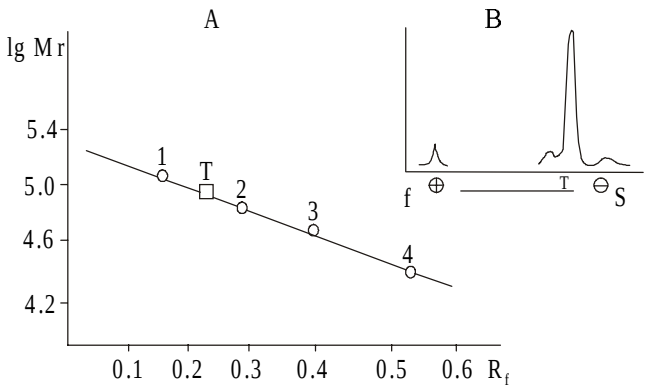

Fig. 4

Fig. 3. Ion-exchange chromatography of fraction GL-1-1 over DEAE Sephadex A-25.

Fig. 4. Relative molecular weight of the toxic fraction (GL-1-1-4) from hemolymph in SDS-PAAG. Marker proteins: bovine serum albumin dimer (134 kDa) (1), toxic hemolymph fraction (T), catalase (60 kDa) (2), ovalbumin (45 kDa) (3), chymotrypsinogen (25 kDa) (4); plot of R_f values as a function of relative logarithm of protein molecular weight (A) and densitogram (B).

The fractions resulting from purification of the egg extract by fractionation over Sephadex G-25 (Fig. 2a) with subsequent ion-exchange chromatography over DEAE-Sephadex lost toxicity.

A toxic high-molecular-weight fraction was isolated by gel-filtration of hemolymph over BioGel P-100 (Fig. 2b). Subsequent ion-exchange chromatography over DEAE-Sephadex A-50 (Fig. 3) produced a highly purified toxin with LD_{50} = 0.83 mg/kg that retained presynaptic activity in experiments on frog neuromuscular preparation but elicited acetylcholine release from rat brain in in vitro experiments. The yield of the fraction was 5-7% of the total hemolymph mass. It should be noted that this fraction is highly labile and tends to aggregate.

According to electrophoresis with SDS, the hemolymph toxin is a protein of molecular weight 89 kDa (Fig. 4). The isoelectric point of the isolated protein in PAAG plates with ampholin was pH 4.7 in a pH gradient of 3.5-10.0.

Differences in the components and properties of venom, hemolymph, and spider egg extract were noted when their toxicities and physicochemical properties were compared. The inability of antiserum against karakurt venom to neutralize hemolymph toxin further proves that the isolated toxin is structurally different from latrotoxins.

EXPERIMENTAL

We studied karakurt spiders *Latrodectus tredecimguttatus* collected during an expedition.

We used Sephadex G-25, BioGel P-100, G-50, G-100, G-200, DEAE-Sephadex A-25 (Pharmacia), DEAE-cellulose (Reanal), reagents for disk electrophoresis and isofocusing (LKB), marker proteins from an MSA set (Serva), etc.

Preparation and Characterization of Studied Materials. Whole venom of *Latrodectus tredecimguttatus* was obtained from adult males. The homogenate from glands was centrifuged (Beckmann) at 18,000 rpm for 30 min. The supernatant was separated, lyophilized, and stored at 0-4°C. The yield of lyophilized material was 0.25-0.1 mg from a single specimen.

Karakurt spider hemolymph was obtained by separating the hemolymph from the cephalothorax with a Pasteur pipette. The hemolymph is a yellowish-brown viscous and odorless liquid. No precipitate forms in distilled water and salt solutions. The hemolymph was clarified by centrifugation at 18,000 rpm for 20 min and lyophilized. A light, dry, white precipitate appeared during lyophilization. The resulting material was stored at 4°C.

Egg extract was isolated from 2-week egg silk before hatching of newborns but after sufficient development. Eggs were homogenized in neutral buffer of weak ionic strength. The extract was clarified by centrifugation at 18,000 rpm for 20 min.

Toxicity (LD_{50}) was determined by the method of Litchfield and Wilkson [3]. The studied material was administered

i.p. to white mice (20 g).

Immunological Methods. Antibody titer was determined by binary radial diffusion on agar plates by the Ouchterlony method [4]. Agar (3%) gel containing antibodies was placed on a level surface as an even layer (both ingredients were mixed at 56°C). Holes were cut in the gel and filled with antigen solution (1 mg/mL, 15 µL each). Diffusion lasted for 48 h. Antigen molecules diffused radially from the holes and formed a precipitation ring upon encountering the antibodies. Amido Black 10B was used as a dye.

Fractionation of Protein Mixtures. Gel filtration was carried out on BioGel P-100 over standard columns (LKB) equilibrated for one day with HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 10 mM) buffer and NaCl solution (0.1 M) at pH 6.8. The sample was placed on the gel column under a layer of liquid and eluted with equilibrated buffer at 30 mL/h.

Ion-exchange chromatography was carried out over DEAE-Sephadex A-50 anion exchanger. Standard columns were packed with activated ion-exchanger and equilibrated with ammonium acetate buffer (0.005 M, pH 7.6). Material was absorbed from equilibrated buffer of low ionic strength and eluted by ammonium acetate buffer of gradually increasing concentration (0.05, 0.1, 0.2 M) and then with a linear gradient (0.2-0.5 M). The resulting fractions were desalted over Sephadex G-25 and G-50 and by dialysis. They were lyophilized and stored cold. The optical density of the eluate was monitored during gel filtration and ion-exchange chromatography using a Unicord P spectrophotometer (LKB). Fractions of 3-mL volume were collected using a fraction collector (LKB).

Electrophoresis was performed by the Ornstein method [5] on PAAG (7%) using acidic and alkaline buffers for 4 h at initial current 17 mA and potential 200 V/gel. Gel was dyed using coomassie R-250 (0.05%) mixed with acetic acid (10%) and ethanol (25%) for 2-3 h, washed with the same buffer without dye for one day, and dried. Densitograms were recorded if necessary. Electrophoresis in denaturing solutions was carried out in PAAG (15%) with SDS (0.1%).

Isoelectric focusing was performed on a Multiphore (LKB) instrument as follows: protein (30-50 mg) was placed on a PAAG (7%) plate containing the necessary ampholins and electrofocused at initial potential 100 V/gel. The working potential was 200 V/gel. Protein bands were dyed without preliminary rinsing of ampholins.

Molecular weights and the number of polypeptide chains in the molecule were determined using electrophoresis in SDS-PAAG (10%). Proteins were separated by electrophoresis before and after reducing the S–S bonds in them [6]. The molecular weight was determined as described by Andrews [7] using Sephadex G-100. The column sizes and elution conditions corresponded to those of Andrews [7]. The column was calibrated with cytochrome C, ribonuclease, trypsin, chymotrypsin, albumin, dextran blue, and myosin. The eluent was Tris-HCl (0.05 M) at pH 7.5 containing KCl (0.2 M). The elution rate was 30 mL/h. The volume of eluent was calculated gravimetrically. The molecular weight was determined from the volume of liquid passing through the column before the middle of the protein peak appeared by finding the molecular weight corresponding to this volume from a calibration curve.

ACKNOWLEDGMENT

The work was supported by a grant from the Civilian Research and Development Foundation (CRDF/ZB2-2002).

REFERENCES

1. A. S. Sadykov, A. A. Akhunov, and Sh. I. Salikhov, *Karakurt Venom* [in Russian], Fan, Tashkent (1985).
2. D. C. Buffkin, F. E. Russel, and A. Deshmukh, *Toxicon*, **19**, 393 (1971).
3. M. L. Belen′kii, *Quantitative Estimation of Pharmacological Effects* [in Russian], Leningrad (1963), p. 81.
4. O. Ouchterlony, "Gel-Diffusion Techniques," in: *Immunochemie. 15. Colloquium Ges. Physiol. Chem.*, Springer Verlag, Berlin, Heidelberg, New York (1965), Vol. 5, p. 13.
5. L. Ornstein, *Ann. N. Y. Acad. Sci.*, **121**, 321 (1968).
6. R. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
7. P. Andrews, *Biochem. J.*, **91**, No. 2, 222 (1964).