The amounts of protein in the preparation were determined by Lowry's method in Hartree's modification [10].

The pH dependence of lytic activity was determined by changing the pH of the incubation medium from 5 to 8.

The thermal stability of the lytic activity was determined after incubation of the preparations at 55°C and pH 6.0 for 5-90 min. The inactivation constants were calculated by means of the equation of a first-order reaction.

LITERATURE CITED

1. B. G. Libman, K. A. Kagramanova, and Z. V. Ermolaeva, Sov. Med., No. 11, 34 (1971).
2. I. S. Kulaev, A. I. Severin, and G. V. Abramochkin, Vestn. Akad. Med. Nauk SSSR, No. 8, 64 (1984).
3. I. A. Cherkasov, N. A. Kravchenko, P. E. Pavlovskii, and L. P. Bragina, Bioorg. Khim., 1, 50 (1975).
4. I. A. Cherkasov, N. A. Kravchenko, P. E. Pavlovskii, V. V. Grabova, and V. Ya. Mokeev, Bioorg. Khim., 2, 1422 (1976).
5. L. G. Blasov, P. I. Tolstykh, T. E. Ignatyuk, and O. N. Razzakov, Antibio. Chemother., 33, 848 (1988).
6. Kazuo Kumira and Kazuo Uno, Japanese Patent 55-42629 (1980).
7. E. Galas and T. Z. Antezak, Polish Patent 125,942 (1984).
8. I. S. Petrova and T. T. Vinugonaite, Prikl. Biokhim. Mikrobiol., 2, 322 (1969).
9. T. I. Bogacheva, O. A. Mirgorodskaya, G. E. Grinberg, G. A. Mikhailets, B. V. Moskvichev, and I. I. Tereshin, Biokhimiya, 46, 863 (1981).
10. E. E. Hartree, Anal. Biochem., 48, 422 (1972).

GLUTAMATE RECEPTOR BLOCKERS FROM VENOMS OF THE SPIDERS Argiope lobata AND Araneus tartaricus

B. U. Atakuziev, K. Nasyrov, D. Kalikulov, and P. B. Usmanov

UDC 577.352.5:615.919

The presence of glutamate receptor blockers with molecular masses below 1000 Da and pI ≥ 9 has been detected in the venoms of the spiders Argiope lobata and Araneus tartaricus.

It has been found previously that the venom of the spider Argiope lobata blocks nerve-muscle transmission in insects and vertebrates by interacting with the structures of the postsynaptic membranes [1], while the effect on the glutamatergic synapses of the locust (Locusta migratoria) had an irreversible nature [1]. Kawai et al. [2] have reported that a low-molecular-mass toxin (JSTX) isolated from the venom of the spider Nephila clavata irreversibly blocks synaptic transmission in a nerve-muscle junction of the lobster. These investigations have served as a kind of impulse to the extension of the search for new glutamate receptor blockers. Thus, in recent years highly specific glutamate receptor blockers have been detected in an isolated from the venoms of various orb-weaving spiders (family Araneidae) [2-13]. It was found that they all — and at the present time they number more than ten — are, as a rule, low-molecular-mass components (<1000 Da) and have great structural homology [8, 12]. The basic structure of these molecules includes the amino acid arginine linked through a peptide bond with a polyamine which, in its turn, is linked to the carboxy group of asparagine the alpha-amino group of which is attached to a phenolic moiety

Institute of Physiology, Academy of Sciences of the Uzbek SSR, Tashkent.

Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 532-537, July-August, 1990. Original article submitted September 28, 1989.

0009-3130/90/2604-0450$12.50

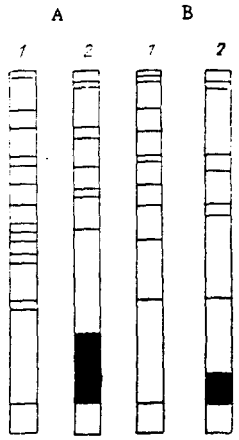

Fig. 1. Electrophoresis in PAG of the venoms of the spiders Argiope lobata (1) and Araneus tartaricus (2): A) Tris-glycine buffer, pH 8.3; from (−) to (+); B) α-alanine buffer pH 4.3; from (+) to (−). Current strength 7 mA/tube; time of electrophoresis 90 min.

(2,4-dihydroxyphenylacetic acid) or with an indole moiety (4-hydroxyindolylacetic acid) [8, 12]. The fact that each venom, as a rule, contains several toxins leads to the possibility of their use as sources of molecular tools that may prove useful in the study of the central and peripheral nervous systems.

In the present paper we consider the physicochemical characteristics, the isolation, and the elucidation of the mechanism of the action of the components of the venoms of two species of the Araneidae family – Argiope lobata and Araneus tartaricus. The UV spectra of the venoms of these spiders had the maxima at 276-278 nm and minima at 250-252 nm that are characteristic for the spectra of proteins. A determination of the amounts of proteins in these venoms showed that up to 60-75% of the total mass of the venom consisted of proteins, although it has been reported that for some spider venoms the proteins make up only 25% of the whole mass of the venom [13]. It may be assumed that the composition and amount of proteins in a venom are determined not only by the species affinity but also by the conditions of obtaining the venom.

The value of LD_{50} on intraperitoneal injection for the venom of Argiope lobata was 185 mg/kg, and for the venom of Araneus tartaricus 180 mg/kg, which show their comparatively weak lethal activity. Subcutaneous and intravenous injection did not give appreciable differences in the LD_{50} values of these venoms. The injection of these venoms into the brain in concentrations of 1-2 mg/kg caused the rapid death of the animals. The value of LD_{50} on intracerebral injection for both venoms was 0.75 mg/kg.

Electrophoretic analysis of the compositions of the two venoms showed that the best separation, into 15 and 11 components, respectively, for Argiope lobata and Araneus tartaricus, was achieved Tris-glycine buffer (pH 8.3); on the use of β-alanine buffer (pH 4.3) only 9 and 8 components, respectively, were detected. These results indicate appreciable variations in the composition of the electrophoretic components according to the species affinity of these spiders, as has been reported by other authors [13]. However, on the whole, in these venoms the anodic components predominated in number and mass. The substances migrating under certain conditions to the cathode can be regarded as basic. In the venoms of these spiders, such basic components were best revealed by the use of an acidic buffer medium (pH 4.3) (Fig. 1).

In spite of some differences in composition, which can be explained by species affinities the venoms of these spiders possessed similar capacities for blocking the synaptic transition both in vertebrates and in insects, acting predominantly postsynaptically. This may indicate that these venoms possibly contain components with similar properties. With the aim of identifying the active principles in these venoms, we made an attempt to isolate them.

Fractionation of the Venom of the Spider Argiope lobata. As the result of a number of analytical experiments, the following method was developed for isolating the synaptic-receptor blockers. A solution of the venom in 0.05 M ammonium bicarbonate buffer was first subjected to gel filtration on a column of Sephadex G-75 (Fig. 2A). In this way the venom was separated into five fractions which were designated in order of their issuance from the column as (I-V). Of the fractions shown in Fig. 2A, the first contained the high-molecular-

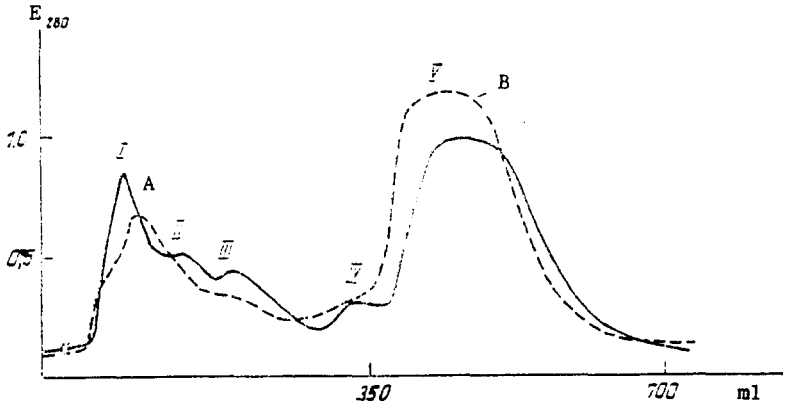

Fig. 2. Separation of the venoms of the spiders Argiope lobata (A) and Araneus tartaricus (B) on Sephadex G-75 (column 2.5 × 80 cm; 0.05 M ammonium bicarbonate buffer, pH 8.4; rate of elution 20 ml/h).

mass components of the venom. According to the theory of gel filtration [16], fractions (II-IV) should contain components with molecular masses in the range of 30,000-5000 Da. None of these fractions (I-IV) possessed the desired activity. The greatest interest was presented by the fifth fraction, containing practically all the activity of the whole venom. Fraction (V) consisted of a mixture of comparatively small peptides, free amono acids, and other components with molecular masses of less than 5000 Da, which corresponds to literature information on the molecular dimensions of the glutamate blockers of the venoms of spiders of the family Araneidae [8, 12].

The results of comparative electrophoretic investigations of the venom and its fractions obtained after separation on Sephadex G-75 revealed their heterogeneity. In the following stage of purification, the low-molecular-mass fraction (V) was subjected to ion-exchange chromatography. A solution of 50-100 mg of fraction (V) in 10 ml of 0.05 M ammonium bicarbonate buffer, pH 8.4, was deposited on a column of DEAE-Toyopearl 650M equilibrated with the same buffer. Part of the fraction [component (V^b)] was not absorbed under these conditions and issued in the starting buffer. The subsequent desorption of the protein components, designated as (V^a) was effected by feeding 0.5 M ammonium bicarbonate buffer solution to the column. In this way, fraction (V) was separated into only two subfractions: the main subfraction (V^b), containing positively charged components, and (V^a) containing acidic components. A determination of biological activity showed that it was present only in fraction (Vb). The active fraction (V^b) obtained was then again subjected to gel filtration on a column of Toyopearl HW-40 F (Fig. 3A).

In view of the unsatisfactory separation of the individual protein peaks from one another in the course of separation, we collected twelve fractions designated in the order of their issuance from the column as (V^b-1)–(V^b-12). Some of these fractions possessed the desired biological activity, but the most effective in this respect was fraction (V^b-7). In the concluding stage of purification, this fraction was subjected to chromatography on a column of Ultragel AcA 202 (Fig. 4A), as a result of which a glutamate receptor blocker which we have called argilobatin (fraction 7) was isolated with a yield of 1.4% of the weight of the initial venom.

The biological activities of the whole venom, of the fractions, and of argilobatin were investigated in experiments on the glutamatergic synapses of the locust and on intracerebral injection into white mice. The addition of the venom and of the fractions to a solution bathing a locust nerve-muscle preparation showed that they suppressed the synaptic potentials (MEPSPs and EPSPs). Fractions (V^b-4), (V^b-9), and (V^b-10) had a reversible nature of their action to different degrees, while fraction (V^b-7) and the argilobatin isolated from it blocked synaptic transmission irreversibly.

As mentioned above, the intracerebral injection of the venom to white mice in concentrations of 1-2 mg/kg caused almost instantaneous death, while on the injection of lower concentrations (0.2-0.5 mg/kg) the following symptoms of poisoning were observed; first, pronounced excitation, sudden runs, and rotation about an axis appeared, and then paresis of

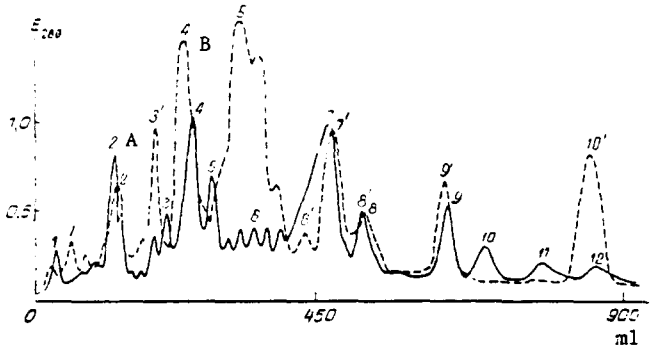

Fig. 3. Gel filtration of fractions (V^b) of the venom of Argiope lobata (A) and Araneus tartaricus (B), on TSK gel Toyopearl HW-40 F (column 1.5 × 180 cm, 0.15 M ammonium bicarbonate buffer, pH 8.4, rate of elution 15 ml/h).

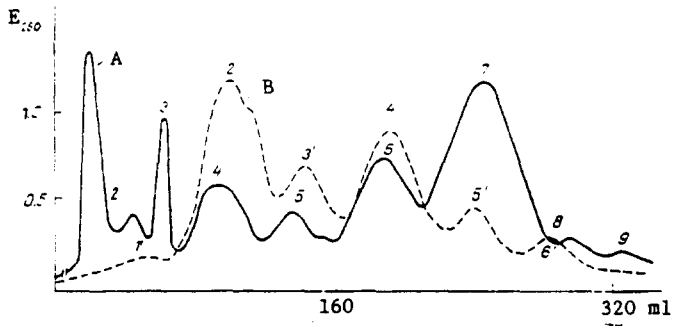

Fig. 4. Gel filtration of fractions (V^b-7) of the venom of Argiope lobata (A) and Araneus tartaricus (B), on Ultragel AcA 202 (column 1.5 × 90 cm; 0.05 M ammonium bicarbonate buffer, pH 8.5; rate of elution 5 ml/h).

the extremities set in, the mice fell now to one side and now to the other, and then they lay on their sides and were incapable of moving. The effects lasted 1.5-2 h, after which slow recovery of the motor function set in, and after 10-12 h, the animals had returned to normal.

The active fractions (V^b-4), (V^b-9), and (V^b-10) in concentrations of 0.2-1 mg/kg also caused the symptoms of poisoning characteristic for the whole venom but did not lead to the death of the animals. Argilobatin in a concentration of 0.2 mg/kg caused the death of the animals and at lower concentrations the symptoms of poisoning characteristic for the venom were observed where the mice survived. The value of LD_{50} on intracerebral injection for argilobatin was 0.15-0.17 mg/kg.

Fractionation of the Venom of the Spider Araneus tartaricus. As already mentioned, the venom of this spider, in spite of some different physicochemical parameters, had a biological action similar to that of the venom of Agriope lobata, and therefore to identify the active principles of this venom we used the same method of isolation as in the case of the Argiope lobata venom. While in the first stage of purification no particular differences were observed in the profile of the elution curve (Fig. 2B), when fraction (V^b) was chromatographed on a column of Toyopearl HW-40 there were considerable divergences (Fig. 3B). Thus, a total of 10 fractions were obtained, and the profile of the elution curve of the venom differed from that in the case of the separation of fraction (V^b) of the Argiope lobata venom. A determination of biological activity showed that the most active fraction in this respect was (V^b-5), from which, by chromatography on a column of Ultragel AcA 202 a glutamate receptor blocker – fraction 4 – was isolated with a yield of 1.1% (Fig. 4B). The results of an investigation of the physicochemical characteristics and mechanism of the action of this component and of the other chromatographic fractions obtained will be published separately.

Thus, from the venoms of the spiders Argiope lobata and Araneus tartaricus glutamate receptor blockers have been isolated by column chromatography with yields of 1.4 and 1.1%, respectively. According to preliminary results, the molecular masses of these components are 657 and 624 Da, respectively, and pI ≥ 9. In addition to these two blockers, a number of blockers of glutamatergic synapses were obtained from these venoms, but their action was reversible to different degrees.

EXPERIMENTAL

The lyophilized venoms of the spiders Argiope lobata and Araneus tartaricus were obtained from the Central Asian Zonal Zoological Combine.

The absorption spectra of the venoms in the UV region were taken on a Specord spectrophotometer (GDR). Protein concentrations were determined by Lowry's method [17].

Toxicity was determined by intraperitoneal or intercerebral injection into white mice weighing ~20 g. For each dose, groups of six animals were used and the result obtained was expressed in milligrams of substance causing a lethal effect in 50% of the animals, calculated to 1 kg of the weight of the mice (LD_{50}, mg/kg). Intracerebral administration was carried out by the injection of 1 μl of the preparation with a microsyringe directly into the brain of the animal. Control groups received the same volume of distilled water or 0.1 M ammonium carbonate buffer solution.

Electrophoresis in polyacrylamide gel (PAG) was carried out by Reisfeld's method [18] using Reanal instruments and reagents (Hungary), and electrofocusing was performed on a Multiphor apparatus (LKB, Sweden) using standard plates.

Fractionation on Sephadex, TSK gel, and Ultragel and also chromatography on an ion-exchange residue were conducted in accordance with the recommendations of the manufacturers.

Molecular masses were determined by gel chromatography on TSK gel Toyopearl HW-40 F using appropriate marker proteins, and also by mass spectrometry.

The electrophysiological experiments were performed on the dorsoventral muscle M-120 of the locust Locust migratoria. The intracellular tap from the fibers was effected with the aid of glass microelectrodes filled with a 3 M solution of KCl.

LITERATURE CITED

1. B. A. Tashmukhamedov, P. B. Usmanov, D. Kalikulov, L. Ya. Yukelson, I. Kazakov, and B. U. Atakuziev, Toxins as Tools in Neurochemistry, Walter de Gruyter, Berlin (1983), p. 312.
2. N. Kawai, A. Niwa, and T. Abe, Brain Res., 247, No. 1, 169 (1982).
3. P. B. Usmanov, D. Kalikulov, N. Shadyeva, and B. A. Tashmukhamedov, Dokl. Akad. Nauk SSSR, 273, No. 4, 1017 (1983).
4. T. Abe, N. Kawai, and A. Niwa, J. Physiol., 339, 243 (1983).
5. P. N. R. Usherwood, I. R. Duce, and P. J. Boden, J. Physiol. (Paris), 79, No. 4, 241 (1984).
6. P. N. R. Usherwood and I. R. Duce, Neurotoxicology, 6, 239 (1985).
7. E. V. Grishin, T. M. Volkova, A. S. Arsen'ev, O. S. Reshetova, V. V. Onoprienko, L. G. Magazanik, S. M. Antonov, and I. M. Fedorova, Bioorg. Khim., 12, No. 8, 1121 (1986).
8. E. V. Grishin, T. M. Volkova, and A. S. Arsen'ev, Bioorg. Khim., 14, No. 7, 883 (1988).
9. A. Bateman, P. Boden, A. Dell, J. R. Duce, D. L. J. Quice, and P. N. R. Usherwood, Brain Res., 339, No. 2, 237 (1985).
10. H. Jackson, M. Urnes, W. Gray, and T. N. Parks, Soc. Neurosci. Abstr., 11, 107 (1985).
11. H. Jackson and T. N. Parks, Soc. Neurosci. Abstr., 13, 1078 (1987).
12. H. Jackson and P. N. R. Usherwood, Trend Neuroscience, 11, No. 6, 278 (1988).
13. H. Jackson and T. N. Parks, Ann. Rev. Neurosci., 12 (1989).
14. Y. Aramaki, T. Yashuhara, T. Higashijima, M. Yoshioka, A. Niwa, N. Kawai, and T. Nakajima, Proc. Jpn. Acad., 62 (B), No. 9, 359 (1986).
15. Y. Aramaki, T. Yashuhara, T. Nigashijima, A. Miwa, N. Kawai, and T. Nikajima, Biomed. Res., 8, No. 3, 167 (1987).
16. H. Determann, Gel Chromatography, Springer, New York (1968).
17. O. Lowry, H. S. Rosebrough, A. L. Farr, and R. S. Randall, J. Biol. Chem., 193, No. 1, 265 (1951).
18. R. Reisfeld, V. Lewis, and D. Williams, Nature (London), 195, 281 (1962).