Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 167-171

PURIFICATION AND CHARACTERIZATION OF A PARALYTIC POLYPEPTIDE FROM LARVAE OF *MYRMELEON BORE*

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Received August 28, 1995

SUMMARY : A toxic substance was purified from larvae of the antlion, *Myrmeleon bore*, by DEAE Sephacel and Phenyl Superose column chromatography. The substance was a large polypeptide with a molecular weight of about 165-167 kDa. Its paralytic activity measured by injection against German cockroaches was about 130 times higher than that of tetrodotoxin on a molar basis.

Some *Myrmeleontidae* larvae make cone-shaped holes in the ground to trap insects. The larvae are called antlions because they often bite ants, which drop to the bottom of the holes. However, they frequently capture other insects, which are often much larger than themselves, especially in their last instar stage. From the behavior of the captured insects, we consider that antlions use venoms to paralyze their prey. In fact, Stäger has claimed that antlions have toxins (1), but since his report in 1925, toxic components have not been isolated. We therefore isolated a toxin and characterized it.

We selected the larvae of *Myrmeleon bore* as the toxin source, because over one thousand larvae were easily collected during May to October in Japan. Our preliminary experiments suggested that the toxic substances in the venom collected from the tip of mandibles of the larva could not be extracted with conventional organic solvents but precipitated with ammonium sulfate; that the toxicity was drastically decreased by heating in a boiling bath or by digestion with several proteases including trypsin and subtilopeptidase A attached to acrylic beads (Sigma, St. Louis, USA); that the toxic substances were not dialyzable through a membrane with a molecular weight cut-off of 100,000. In this paper, we purified the toxin from crude venom of larvae of *Myrmeleon bore* by monitoring the paralytic activity against German cockroaches. The purified toxin was a large polypeptide which had very potent paralytic activity against the cockroaches.

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0006-291X/95 $12.00

MATERIALS AND METHODS

Materials DEAE Sephacel, Phenyl Superose HR 5/5, Superdex 200 HR 10/30, and molecular weight markers for SDS-PAGE and gel filtration were obtained from Pharmacia Biotech (Uppsala, Sweden). The ultrafiltration membrane (cut-off molecular weight: 100,000) was from Millipore Japan (Tokyo, Japan). Tetrodotoxin (TTX) and Joro Spider Toxin-3 (JSTX-3) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Collection of toxic fluid The second and last instar larvae of *Myrmeleon bore* were collected in the north of Tottori Prefecture of Japan during mid-May, 1995. The tips of the mandibles of 700 larvae were cut by scissors and the venom was collected by glass capillaries from the cut ends. The crude venom (3.17 ml) was transferred to Eppendorf tubes cooled in an ice bath and diluted 2.46-fold (v/v) with ice-cold 25 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and 10% glycerol.

Column chromatography DEAE Sephacel and Phenyl Superose chromatography proceeded in an open column and in an FPLC System (Pharmacia Biotech), respectively. The buffer for the chromatography was 25 mM potassium phosphate buffer (pH 6.0 or 7.5) containing 10% glycerol. DTT (1 mM) was added to the buffer (pH 7.5) for the DEAE Sephacel column chromatography and the toxic component was eluted by increasing the phosphate concentration to 250 mM. The Phenyl Superose column chromatography was started with potassium phosphate buffer (pH 6.0) containing 1.7 M ammonium sulfate in addition to 10% glycerol and the toxic component was eluted by gradually decreasing the ammonium sulfate concentration. Elution of the toxic component from each column was monitored by UV absorption at 280 nm. The chromatography was performed at 4 °C.

Analytical methods Protein concentrations were determined by the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (3). SDS-PAGE was performed in 8% polyacrylamide slab gels using Tris/glycine buffer (pH 8.3) as described by Laemmli (4). Proteins were stained with Coomassie Brilliant Blue R-250. The molecular weight markers were myosin (212 kDa), α2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamate dehydrogenase (53 kDa). Gel filtration was performed using a Superdex 200 HR column with 25 mM potassium phosphate buffer (pH 6.0) containing 0.2 M KCl. The molecular weight standards were thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa).

Bioassay Male adult German cockroaches (*Blattella germanica*) were used as test insects. They were bred in a room controlled at 27± 2 °C and 70 ± 5% RH. The paralytic activity of test solutions was measured by injection into the ventral side of the abdomen between the third and fourth segments avoiding the central zone (2). Samples were injected in 25 mM phosphate buffer (pH 6.0) containing 1 mM DTT and 10% glycerol, except for the original toxic fluids, the pH of which was 7.5. TTX and JSTX-3 were injected as aqueous solutions. Injected insects were placed in a transparent cup and their symptoms were observed in the breeding room described above. Usually, three insects were used for each dose of toxin and at least 30 insects were used for each sample. The interval of the log doses was brought to 0.1 by varying the concentration and the injection volume (1.0 - 2.5 μl). When the dose was large, the injected insects were suddenly paralyzed and killed. With a moderate amount of the toxin, the paralytic symptom developed with time. Time to bring about the symptom was dose-dependent. We determined the MPD value, the minimum dose (ng protein/insect), required to paralyze all three insects within 10 min after the injection of each fraction. When the insects were unable to regain normal posture after turning them up, they were judged as paralyzed. The MPD values were given as an average from at least three runs, with the standard error within ± 0.2 in log units. Injection of the toxin-free solution (2.5 μl) did not induce the toxic symptoms.

RESULTS AND DISCUSSION

The crude toxin prepared as described above was centrifuged at 6,700 x *g* for 10 min at 4 °C and the supernatant was applied to a DEAE Sephacel column (ϕ = 1.2 cm, h = 7 cm), equilibrated with 25 mM potassium phosphate buffer (pH 7.5) containing 1 mM

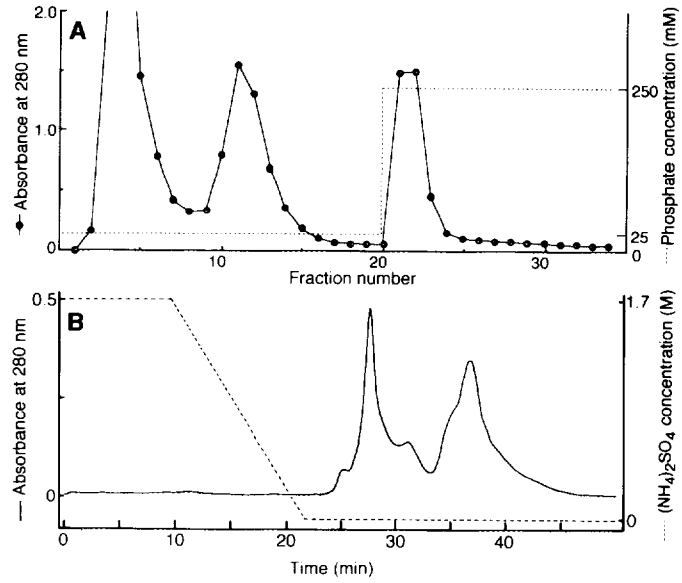

Fig. 1. Elution profiles of antlion toxin. (A) DEAE Sephacel column chromatogram. Each fraction contains 5.5 ml and the elution rate was 0.73 ml/min. Fractions No. 20 - 26 were pooled. No other fractions induced paralysis. (B) Phenyl Superose column chromatogram. The flow rate was 0.3 ml/min. The eluates with a retention time of 27.0 - 28.1 min were collected as the active fraction. No other fractions induced paralysis.

DTT and 10% glycerol. After washing the column with the same buffer, the toxin was eluted with 250 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and 10% glycerol (Fig. 1A). Fractions No. 20-26 were pooled and concentrated by ultrafiltration under nitrogen gas. The solution on the membrane was exchanged by 25 mM potassium phosphate buffer (pH 6.0) containing 10% glycerol to give 5.9 ml. Solid ammonium sulfate (2.30 g) was added and completely dissolved. After placing the solution at 4 °C for 30 min, the precipitates were collected by centrifugation at 15,000 x *g* and suspended in 25 mM potassium phosphate buffer (pH 6.0) containing 1.7 M ammonium sulfate and 10% glycerol, then the suspension was centrifuged at 15,000 x *g*. The supernatant was discarded and the precipitates were dissolved in 100 µl of 25 mM potassium phosphate buffer (pH 6.0) containing 10% glycerol.

Immediately after adding 40 µl of 25 mM potassium phosphate buffer (pH 6.0) saturated with ammonium sulfate and containing 10 % glycerol, the toxic component (140 µl) was separated by Phenyl Superose chromatography equilibrated with 25 mM potassium phosphate buffer (pH 6.0) containing 1.7 M ammonium sulfate and 10%

Table 1. Purification of antlion toxin

Purification step	Total protein (mg)	MPD (ng protein/insect)	PAI (insects/mg protein)[1]
Crude venom	65.8	330	3030
DEAE Sephacel	14.7	120	8333
Phenyl Superose	0.069	40	25000

[1] PAI (Paralytic Activity Index) was calculated as 1/MPD x 10^6.

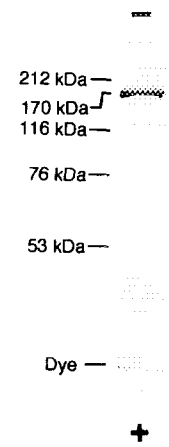

Fig. 2. SDS-PAGE of the purified antlion toxin. The conditions were as described in MATERIALS AND METHODS. The lane was loaded with 1.5 μg of protein of the purified toxin. The direction is from the top (cathode) to the bottom (anode).

glycerol. The toxin was eluted after reducing the concentration of ammonium sulfate from 1.7 M to zero (Fig. 1B). The eluates having a retention time of 27.0 - 28.1 min were collected as the active fraction.

The crude venom was purified 8.25-fold by the described above (Table 1). The purified sample migrated as a single band at 167 kDa on the SDS-PAGE (Fig. 2). The molecular weight of the toxin was estimated at 165 kDa by means of Superdex-200 HR chromatography, suggesting that the toxin is a single polypeptide. The amino acid sequence of the *N*-terminus of the toxin was elucidated to be NH_2-Ser-Tyr-Glu-Asn-Asp-Ala. In the present assay system, the toxin isolated from the venom of the antlion was about 3670-fold more active than Joro-Spider Toxin-3 (JSTX-3) and about 130-fold more than tetrodotoxin (TTX) on a molar basis (Table 2). We believe that the toxin purified here is one of the most important means by which the larva of *Myrmeleon bore* paralyzes insects.

In conclusion, we highly purified an antlion toxin from the larva of *Myrmeleon bore*. The "ALMB-Toxin" is a single polypeptide of about 165-167 kDa and it has a potent paralytic activity against the German cockroach. Other species of antlion may also secrete powerful proteinous toxins.

Table 2. Paralytic Activity against German cockroaches

Toxin	Molecular weight	Toxicity [1]	
		ng/insect	mol/insect
Antlion toxin	165,000[2]	40	2.4×10^{-13}
JSTX-3	565	500	8.8×10^{-10}
TTX	319	10	3.1×10^{-11}

[1] Expression similar to MPD.
[2] Estimated by gel filtration.

ACKNOWLEDGMENTS: We are grateful to Akiyoshi Shimada, Hiroaki Miyagawa, Takafumi Yura, Naoyuki Hosoya, Takenori Shimizu, Yoshinori Nishimura, Ken Hanai, Nobuo Koike, Akiko Suga and Naofumi Yoshida of Kinki University for collecting the antlions and supporting the purification of the toxin. We express our thanks to Professor Toshiaki Matsura of Kyoto University of Education for identifying the antlion as the larva of *Myrmeleon bore*. We also thank Dr. Michihiko Kobayashi of Kyoto University for advice regarding purification and analysis of the toxin.

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