ゴンズイ粘液中の致死因子の精製

誌名	日本水産學會誌
ISSN	00215392
著者	塩見, 一雄
巻/号	53巻7号
掲載ページ	p. 1275-1280
発行年月	1987年7月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

Nippon Suisan Gakkaishi 53(7), 1275–1280 (1987)

Purification of a Lethal Factor in the Skin Secretion from the Oriental Catfish *Plotosus lineatus*

Kazuo Shiomi,* Mitsuru Takamiya,* Hideaki Yamanaka,* and Takeaki Kikuchi*

(Accepted December 3, 1986)

One lethal factor in the skin secretion from the oriental catfish *Plotosus lineatus* was purified successively by DEAE-cellulose, CM-cellulose and Sephadex G-75 and its homogeneity was supported by analytical disc electrophoresis. The purified lethal factor had a LD_{50} of 0.71 mg/kg (intravenous injection into mice). In addition, it exhibited a strong edema-forming activity; the minimum edema dose inducing 130% edema ratio was as small as 0.89 μg. The molecular weight was estimated to be 11,000 by gel filtration on Sephadex G-75. Unexpectedly the molecular weight determination by SDS disc electrophoresis was unsuccessful because no band was detected under the various tried conditions. Although the purified lethal factor was a basic protein, it was richer in acidic amino acids than basic amino acids, indicating that the acidic amino acids exist in the molecule as an amide form.

Contact with the dorsal and pectoral stings of many catfishes inflicts painful wounds, which are often accompanied by edema, lymphadenopathy, numbness and so on.[1] These symptoms have been believed to be induced only by venom in the glands surrounding the dorsal and pectoral spines. Extracts from the stings are certainly toxic[2-4] but little is known about chemical properties of venoms. On the other hand, we recently found that the oriental catfish *Plotosus lineatus*, the most representative venomous catfish in Japan, contains at least one hemolysin, two lethal factors and two edema-forming factors in the skin secretion.[5] Similarly, Al-Hassan *et al.*[6,7] also reported that the Arabian Gulf catfish *Arius thallasinus* possesses a proteinaceous toxin in the skin secretion, together with a gland venom. It is possible that when injured by catfishes, not only the gland venom but also the skin secretion enters the wound. Therefore, these findings strongly suggest that the symptoms induced by at least *P. lineatus* and *A. thallasinus* are due to the combined effects of gland venom and skin toxin. In this respect, for a better understanding about the symptoms, it is important to disclose detailed properties of the skin toxin. This study was initiated to purify and characterize lethal factors in the skin secretion from *P. lineatus*.

Materials and Methods

Sample

Specimens of *P. lineatus* were collected along the coast of Katsuura, Chiba Prefecture, in May 1985 and immediately frozen. They were transported to our laboratory and kept at −20°C until use.

Purification Procedure of a Lethal Factor

From 132 specimens (7–22 cm in total length) 180 g of the skin secretion was obtained by scraping off with a spatula. The skin secretion contaminated with a large amount of sea water was first centrifuged at 15,000 rpm for 5 min. To the precipitate was added 60 m*l* of water, homogenized and then centrifuged similarly. The combined supernatant was used as crude toxin.

The crude toxin was dialyzed against 0.01 M Tris-HCl buffer (pH 7.4). Dialysis was performed with a Zeineh dialyzer (model L-D-1, Funakoshi) throughout this study. The dialysate was applied to a DEAE-cellulose column (3×50 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.4). After washing with the same buffer, the column was eluted by a linear gradient of 0–1.0 M NaCl in the buffer (1,500 m*l* in total volume). Fractions of 15 m*l* were collected at a flow rate of 45 m*l*/h. Lethal factors were detected in both the unadsorbed and adsorbed fractions. The unadsorbed fraction was layered onto a CM-

* Department of Food Science and Technology, Tokyo University of Fisheries, Konan-4, Minato, Tokyo 108, Japan (塩見一雄，高宮　満，山中英明，菊池武昭：東京水産大学食品生産化学科).

cellulose column (3×50 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) and elution was achieved in the same manner as adopted for the above DEAE-cellulose column. Lethal fractions were combined, dialyzed against water and lyophilized. The dried material was dissolved in 4 m*l* of 0.01 M phosphate buffer containing 0.15 M NaCl (pH 6.8) and chromatographed on a Sephadex G-75 column (2.5×90 cm), which was equilibrated and eluted with the same buffer. Fractions of 5 m*l* were collected at a flow rate of 30 m*l*/h. Active fractions were combined and used as the purified lethal factor after dialysis and lyophilization.

Estimation of Lethal and Edema-forming Activities

Lethal activity was examined by intravenous injection of sample solutions into the tail vein of ddY male mice weighing about 20 g. The injection volumes ranged from 0.1 to 0.5 m*l*. The LD_{50} of the purified lethal factor was determined using four doses and six mice for each dose by the method of Litchfield and Wilcoxon.[8] In order to semi-quantitatively evaluate the recovery in activity during the purification procedure, the minimum lethal dose was roughly estimated and defined as 1 mouse unit (MU). The edema-forming activity of the purified lethal factor was assayed according to the method of Yamakawa *et al.*[9] In brief, groups of 4 mice were injected with 25 μl of toxin solution in the right foot pad and with 25 μl of buffered saline (0.15 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4) in the left foot pad. After 2 h, the edema ratio, which was expressed as the percentage in weight of the envenomated foot relative to the saline injected one, was calculated.

Electrophoresis

Analytical polyacrylamide disc electrophoresis was performed on 7% gels by the methods of Reisfeld *et al.*[10] and Davis.[11] The solvents used were β-alanine-acetic acid buffer (pH 4.5) in the former method and Tris-glycine buffer (pH 8.3) in the latter. Proteins were stained with Amido Black 10B at 96°C for 10 min.

Sodium dodecyl sulfate (SDS) polyacrylamide disc electrophoresis was carried out by the method of Weber and Osborn[12] under the following various conditions: denaturing reagent, 1% SDS or 1% SDS+2% 2-mercaptoethanol; denaturing condition, 100°C for 10 min; gel concentration, 7.5, 10 or 12.5%; staining reagent, Coomassie Brilliant Blue R or Amido Black 10B; staining condition, 100°C for 10 min or room temperature for 24 h.

Molecular Weight Determination

The molecular weight of the purified lethal factor was determined by gel filtration on a Sephadex G-75 column (1×50 cm) with buffered saline. The flow rate was maintained at 0.25 m*l*/min by a constant pump and the eluate was continuously monitored for proteins at 280 nm by a UV detector. In order to calibrate the column, four standard proteins, bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen A (MW 25,000) and ribonuclease A (MW 13,700), were chromatographed in the same manner.

Amino Acid Analysis

The purified lethal factor (75 μg and 150 μg) was hydrolyzed with 5.7 N HCl in an evacuated tube at 110°C for 24 h and applied to a Hitachi 835 amino acid analyzer. The mean value of two determinations was calculated for the amino acid composition. Tryptophan was separately estimated according to the method of Goodwin and Morton.[13]

Results

Purification of a Lethal Factor

In Table 1 are summarized the protein amount, total lethal activity, specific activity and recovery in activity in each purification step.

The crude toxin contained a total lethal activity

Table 1. Summary of purification

Purification step	Protein amount (mg)	Total lethal activity (MU)	Sepcific activity (MU/mg)	Recovery in activity (%)
Crude toxin	5880	6250	1.1	100
DEAE-cellulose*	552	3000	5.4	48
CM-cellulose	60	1000	16.7	16
Sephadex G-75	4.6	240	52.2	4

* Estimated with the unadsorbed fraction.

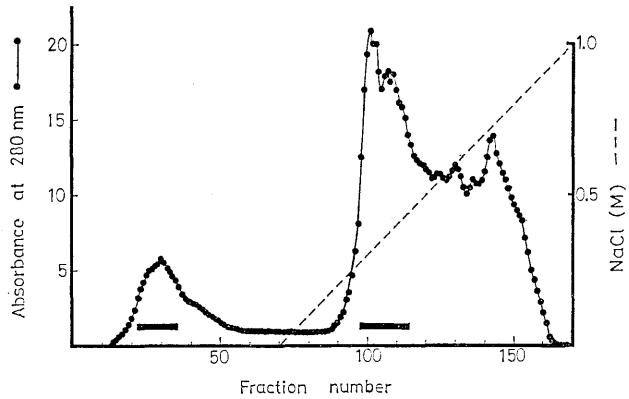

Fig. 1. DEAE-cellulose column chromatography of the crude toxin.

The crude toxin was applied to a DEAE-cellulose column (3×50 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.4). After washing with the same buffer, a linear gradient elution of 0–1.0 M NaCl in the buffer was started at fraction 70. Fractions of 15 m*l* were collected at a flow rate of 45 m*l*/h. Lethal fractions were indicated by the bars.

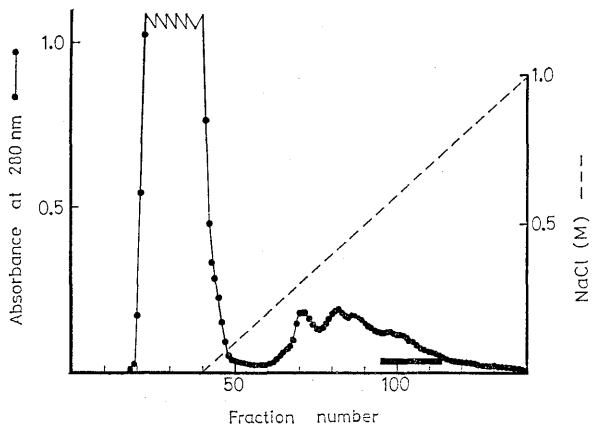

Fig. 2. CM-cellulose column chromatography of the lethal fraction unadsorbed by DEAE-cellulose.

The unadsorbed lethal fraction obtained by DEAE-cellulose column chromatography (Fig. 1) was put onto a CM-cellulose column (3×50 cm) equilibrated with 0.01 M Tris-HCl (pH 7.4). After washing with the same buffer, a linear gradient elution of 0–1.0 M NaCl in the buffer was started at fraction 40. Fractions of 15 m*l* were collected at a flow rate of 45 m*l*/h. Lethal fractions were indicated by the bar.

of 6,250 MU, which was equivalent to about 50 MU per fish. As reported previously,[5] at least two lethal factors were contained in the skin secretion; one was unadsorbed (3,000 MU in total lethal activity) and another adsorbed (1,200 MU) by DEAE-cellulose (Fig. 1). The lethal factor in the unadsorbed fraction was further purified on CM-cellulose. It was adsorbed by the column and eluted in fractions 97 to 113 (Fig. 2). A large amount of proteins had no interaction with the column and moreover colored proteinaceous materials were retained on it without being eluted by a linear gradient of 0–1.0 M NaCl. Although the recovery in activity was rather low, this step seemed to be very effective to remove impurities. In gel filtration on Sephadex G-75 the lethal factor appeared between fractions 72 and 86 in parallel to a relatively symmetrical protein peak (Fig. 3). Since it was preliminarily found that the second half of the peak was contaminated with a small amount of impurities, only the first half (fractions 72 to 80) was combined. Thus, 4.6 mg of the purified lethal factor was obtained with a low recovery in activity. About 50-fold purification was accomplished on the basis of specific activity. As shown in Fig. 4, the homogeneity

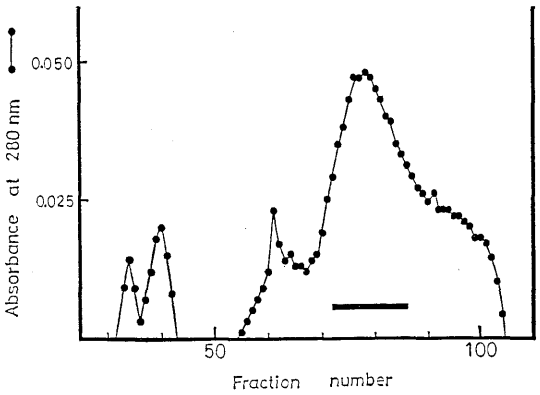

Fig. 3. Gel filtration on Sephadex G-75 of the lethal fraction obtained by CM-cellulose column chromatography.

The lethal fraction obtained by CM-cellulose column chromatography (Fig. 2) was subjected to gel filtration on a Sephadex G-75 column (2.5 × 90 cm). Elution was achieved by 0.15 M NaCl in 0.01 M phosphate buffer (pH 6.8). Fractions of 5 m*l* were collected at a flow rate of 30 m*l*/h. Lethal fractions were indicated by the bar.

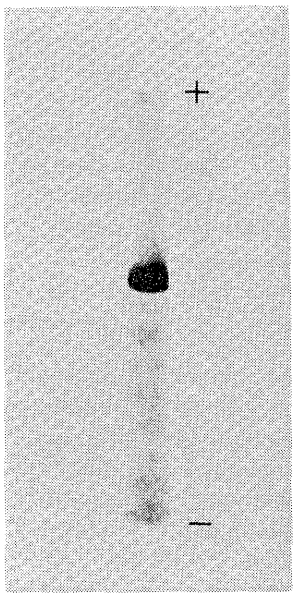

Fig. 4. Polyacrylamide disc electrophoresis of the purified lethal factor.

The purified lethal factor was run on a 7% gel using β-alanine-acetic acid buffer (pH 4.5).

of the purified preparation was evidenced by analytical disc electrophoresis performed according to the method of Reisfeld *et al.*[10]

Properties of the Purified Lethal Factor

The LD_{50} of the purified lethal factor was calculated to be 0.71 mg/kg (95% confidence limit 0.67–0.76 mg/kg). As previously reported for the crude toxin,[5] the mice injected with lethal doses showed characteristic signs, agitated behavior, increased heart rate, jumping and convulsion, and died of respiration arrest usually within 30 min. In addition, an apparent edema-

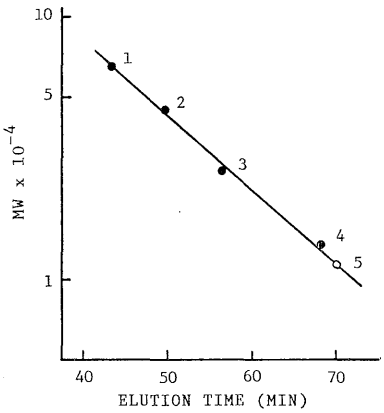

Fig. 5. Molecular weight determination on Sephadex G-75 of the purified lethal factor.

Column size, 1 × 50 cm; solvent, 0.15 M NaCl in 0.01 M Tris-HCl buffer (pH 7.4); flow rate, 0.25 m*l*/min. Preparations are: 1, bovine serum albumin; 2, ovalbumin; 3, chymotrypsinogen A; 4, ribonuclease A; and 5, purified lethal factor.

Table 2. Amino acid composition of the purified lethal factor

Amino Acid	Mole %*1
Asp	9.0
Thr	8.8
Ser	8.5
Glu	9.8
Pro	3.3
Gly	10.9
Val	7.0
Cys	0.4
Ala	6.9
Met	1.4
Ile	5.2
Leu	6.7
Tyr	2.4
Phe	4.2
Trp*2	1.6
Lys	8.6
His	1.9
Arg	3.8

*1 Mean value of two determinations.
*2 Determined optically.

forming activity was elicited by the lethal factor. The minimum edema dose, which had been defined as the dose inducing 130% edema ratio by Yamakawa *et al.*,[9] was as small as 0.89 μg.

In analytical disc electrophoresis the purified lethal factor gave a single band when analyzed by the method of Reisfeld *et al.*[10] (Fig. 4) while it afforded no band when analyzed by the method of Davis.[11] The molecular weight was determined to be 11,000 by gel filtration (Fig. 5). This value was comparable to that (12,000) obtained with the crude toxin.[5] Unexpectedly it was impossible to determine the molecular weight by SDS disc electrophoresis, in which no band was detected under all conditions tried. The amino acid composition of the purified lethal factor is shown in Table 2. Half cystine was poor in the molecule. The content of acidic amino acids (aspartic acid and glutamic acid) was higher than that of basic amino acids (lysine, histidine and arginine).

Discussion

One lethal factor was purified from the skin secretion of the oriental catfish *P. lineatus*, with a low recovery due to unstableness. In DEAE-cellulose column chromatography (Fig. 1) another lethal factor (1,200 MU in total activity) and a hemolysin were obtained in the adsorbed fraction. An attempt was made to purify another lethal factor using a CM-cellulose column equilibrated with 0.01 M phosphate buffer (pH 5.8). This lethal factor bound to the column and was eluted by a linear gradient of 0–1.0 M NaCl in the buffer. At this stage, however, its total activity was decreased significantly to below 10 MU and therefore its further purification was unsuccessful. The hemolysin was also very labile, which was an obstacle for further research. For the hemolysin and another lethal factor a skillful method to maintain their hemolytic or lethal activity should be devised.

The purified lethal factor seemed to be basic from its behavior in ion-exchange chromatography. This was confirmed by the results obtained in analytical disc electrophoresis, in which the lethal factor moved from anode to cathode on the gel (pH 4.3) with β-alanine-acetic acid buffer (pH 4.5)[10] while it did not enter the gel (pH 9.5) with Tris-glycine buffer (pH 8.3).[11] The isoelectric point, though not determined, was assumed to be over 9. In view of this fact, most of the acidic amino acids, which were richer in the molecule than the basic amino acids, appear to exist in the amide form. On the other hand, another lethal factor was a neutral to weakly acidic protein because it bound to both DEAE-cellulose at pH 7.4 and CM-cellulose at pH 5.8.

The molecular weight could be estimated by gel filtration but not by SDS disc electrophoresis. Since the purified lethal factor afforded a clear protein band in analytical disc electrophoresis, it was very difficult to account for why no band was detected in SDS disc electrophoresis. It may be possible that the lethal factor is composed of several small peptides which can dissociate in the presence of SDS and exude out of the gel during staining. The question will be answered more definitely only after the elucidation of the primary structure.

Some marine fishes possess toxic principles in the skin secretion, which are referred to as crinotoxins. The following several crinotoxins have been purified and well characterized: pahutoxin (choline ester) in boxfishes,[14] grammistins (peptides) in soapfishes,[15] grammistin-like substances in coral-gobies[16] and clingfishes[17] and pardaxin (protein) in the flatfish.[18] The skin toxin of *P. lineatus* may be classified into the category of crinotoxins. In fact the purified lethal factor is similar to pardaxin as to chemical properties. However, differing from the known crinotoxins, the skin toxin of catfishes does not appear to function as a defensive substance to predators by itself. According to the observation by Al-Hassan *et al.*,[7] the Arabian Gulf catfish *A. thallasinus* secretes epidermal mucus containing several toxic components in an apparently defensive type response but these components do not appear to function as toxic, repellant or fright substances. This may be true for *P. lineatus*.

Acknowledgements

We are indebted to Mr. J. Sato for collecting catfish samples and to ProfessorS. Konosu and Dr. A. Shinagawa, Faculty of Agriculture, The University of Tokyo, for amino acid analysis.

References

1) B. W. Halstead: Poisonous and Venomous Marine Animals of the World, Vol. 3, U. S. Government Printing Office, Washington, D. C., 1970, pp. 149–191.
2) T. Toyoshima: *J. Japan Protoz. Soc.*, **6**, 45–270 (1918).
3) W. S. Birkhead: *Copeia*, **1972**, 790–807 (1972).

4) G. J. Calton and J. W. Burnett: *Toxicon*, **13**, 399–403 (1975).
5) K. Shiomi, M. Takamiya, H. Yamanaka, T. Kikuchi, and K. Konno: *Toxicon*, **24**, 1015–1018 (1986).
6) J. M. Al-Hassan, M. Ali, M. Thomson, T. Fatima, and C. J. Gubler: *Toxicon*, **23**, 532–534 (1985).
7) J. M. Al-Hassan, M. Thomson, K. R. Criddle, B. Summers, and R. S. Criddle: *Mar. Biol.*, **88**, 117–123 (1985).
8) J. T. Litchfield and F. Wilcoxon: *J. Pharmac. Exp. Ther.*, **96**, 99–113 (1949).
9) M. Yamakawa, M. Nozaki, and Z. Hokama: in "Animal, Plant, and Microbial Toxins" (ed. by A. Ohsaka, K. Hayashi, and Y. Sawai), Vol. 1, Plenum Press, New York and London, 1976, pp. 97–109.
10) R. A. Reisfeld, V. J. Lewis, and D. E. Williams: *Nature*, **195**, 281–283 (1962).
11) B. J. Davis: *Ann. N. Y. Acad. Sci.*, **121**, 404–427 (1964).
12) K. Weber and M. Osborn: *J. Biol. Chem.*, **244**, 4406–4412 (1969).
13) T. W. Goodwin and R. A. Morton: *Biochem. J.*, **40**, 628–632 (1946).
14) D. B. Boylan and P. J. Scheuer: *Science*, **155**, 52–56 (1967).
15) Y. Hashimoto and Y. Oshima: *Toxicon*, **10**, 279–284 (1972).
16) K. Shiomi and Y. Hashimoto: in "Animal, Plant, and Microbial Toxins" (ed. by A. Ohsaka, K. Hayashi, and Y. Sawai), Vol. 1, Plenum Press, New York and London, 1976, pp. 303–310.
17) K. Hori, N. Fusetani, K. Hashimoto, K. Aida, and J. E. Randall: *Toxicon*, **17**, 418–424 (1979).
18) N. Primor, J. Parness, and E. Zlotokin: in "Toxins: Animal, Plant and Microbial" (ed. by P. Rosenberg), Pergamon Press, Oxford, 1978, pp. 539–547.