PURIFICATION OF AN ANTIHEMORRHAGIC FACTOR FROM THE SERUM OF THE NON-VENOMOUS SNAKE *DINODON SEMICARINATUS*

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ABSTRACT

An antihemorrhagic factor was purified from the serum of *Dinodon semicarinatus*, a non-venomous snake (Akamata) by a series of high performance liquid chromatographies with a TSK gel DEAE-5PW column. The purified antihemorrhagic factor showed a single band on a polyacrylamide gel disc electrophoresis.The factor inhibited the hemorrhagic activity of HR 1 and HR 2, the hemorrhagic factors of *Trimeresurus flavoviridis* Okinawa.The purified antihemorrhagic factor was stable at the temperature from 0℃ to 60℃ and at the pH between 2.0 and 11.0. The molecular weight of the antihemorrhagic factor was estimated to be 59,000 and 52,000 by a gel filtration and a SDS-polyacrylamide gel electrophoresis, respectively, suggesting that the antihemorrhagic factor consists of a single subunit. No precipitin lines were found for the purified antihemorrhagic factor with the venom of *T. flavoviridis* Okinawa and its hemorrhagic factors, HR 1 and HR 2.

INTRODUCTION

It has been frequently observed that the serum of certain snakes, including venomous and non-venomous ones, neutralizes snake venom (Noguchi, 1909, Philpot and Smith, 1950). Omori-Satoh *et al* (1972) and Ovadia (1978) purified and characterized antihemorrhagic factor in the serum of the venomous snakes, *Trimeresurus flavoviridis* and *Vipera palaestinae*, respectively. The antihemorrhagic factor are not immunoglobulins but natural immunity possessed by the animals. We have shown the presence of antihemorrhagic factor in the serum of *Dinodon semicarinatus*, (Akamata) a non-venomous snake. (Tomihara *et al.*, 1987). The serum of *Dinodon semicarinatus* neutralized the toxic activity of various hemorrhagic snake venoms, but did not show antilethal activity for the venom of *T. flavoviridis*.
Antihemorrhagic factors in non-venomous snake, however, have not been isolated as a homogeneous preparation. In this paper we describe the purification and some of the properties of an antihemorrhagic factor from the serum of *Dinodon semicarinatus*.

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MATERIALS AND METHODS

Materials.

Sephadex G−100, G−200 and DEAE-Sephadex A−50 were obtained from Pharmacia

Fine chemicals; TSK gel DEAE−5PW column (7.5x75mm) from Toyo Soda Manufacturing Co.,; Amberlite CG−50 Sigma Chemical Co. Standard proteins for molecular weight determination by a gel filtration on a Sephadex G−200 and a SDS-polyacrylamide gel electrophoresis were purchased from Boehringer-Manheim and Oriental Yeast Co., Japan, respectively. HR 1 and HR 2, the hemorrhagic factors of *T. flavoviridis* Okinawa were partially purified by a column chromatography on a Sephadex G−100 followed by DEAE-Sephadex A−50 and Amberlite CG−50 column chromatographies (Omri-Satoh *et al.*, 1967; Takahashi and Ohsaka, 1970). The blood of *Dinodon semicarinatus* was collected by head decapitation and the serum was separated by centrifugation at 3000 rpm for 15 min and stored at−-20℃. All chemicals used were of analytical reagent grade.

High performance liquid chromatography

High performance liquid chromatography (HPLC) was carried out with a Schimadzu LC−4A liquid chromatography system consisting of UV detector SPD−2AS with a variable wavelength and a sample injector SIL−1A with a sample loop of 750 ul. All operations of HPLC were performed at room temperature unless otherwise stated.

Determination of antihemorrhagic activity

Antihemorrhagic activity was determined by the method developed by Kondo *et al.* (1960). One hundred minimum hemorrhagic dose (MHD) of the venom of *T. flavoviridis* okinawa was used as one test dose. One MHD is defined as the least quantity of the venom causing a hemorrhagic spot of 10 mm in diameter 24 hr after intracutaneous injection. The mixture (1.0 ml) of the venom and varing amount of serum of *Dinodon Semicarinatus* was incubated at room temperature for 1hr. The 0.2ml aliquot of the incubated mixture was injected into the depilated back skin of a rabbit. The rabbit was then killed 24 hr later and the skins were removed. The cross diameters of each hemorrhagic spot were measured. One unit of antihemorrhagic activity is defined as the least quantity of protein neutralizing the venom of one MHD.

Molecular weight determination

The molecular weight of the antihemorrhagic factor was determined by a gel filtration on a Sephadex G−200 as described by Andrews (1954). The column (3.3x70cm) was developed with 5mM Tris-HC1 buffer (pH 8.5) at 4℃, and 4.5ml fractions were collected. The standard protein employed were catalase (MW 232,000), aldolase (MW 158,000), bovine serum albumin (MW 68,000). ovalbumin (MW 44,000) and chymotrypsinogen A (MW 25,000). The molecular weight of the denatured antihemorrhagic factor was determined by a SDS-polyacrylamide gel electrophoresis as described by Weber and Osborn (1969). The antihemorrhagic factor was denatured by incubating in 0.1% sodium dodecyl sulfate (SDS), and then subjected to a SDS-disc electrophoresis with a monomer (M 12,400), a dimer (MW 24,000), a trimer (MW 37,200), a tetramer (MW 45,600) and a hexamer (MW 74,400) of horse heart muscle cytochrome C as a molecular marker.

Protein determination

Protein content was estimated by the measurement of the absorbance at 280 nm in a cuvette with 1.0 cm light path, assuming that the absorbance of 1.54 corresponds to 1.0 mg of protein per ml.

RESULTS AND DISCUSSION

Purification of the antihemorrhagic factor

Step 1. *First high performance liquid chromatography*

The serum obtained from 50 snakes (2700 mg of protein per 30 ml) was subjected to the HPLC with TSK gel DEAE-5PW column, (7.5x75mm). The column was developed with 0.1M sodium acetate buffer (pH8.3, solution A) and the same buffer supplemented with 0.5M NaCl (solution B), and 0.5ml fractions were collected at a flow rate of 1.0 ml per min(Fig. 1). After washing the column with the buffer of 5% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 5% B and 80% B for 15 min followed by elution and reequilibration of the column at 5% B for 5 min. Fractions with antihemorrhagic activity (indicated with an arrow in Fig.1) were pooled, and concentrated to 10 ml by an ultrafiltration with a Millipore membrance filter PSAC under the pressure of nitrogen gas.

Step 2. *Second high performance liquid chromatography*

The concentrated fraction was dialyzed against 0.1 M sodium acetate buffer (pH 8.3) at 4°C over night . The dialysate was applied

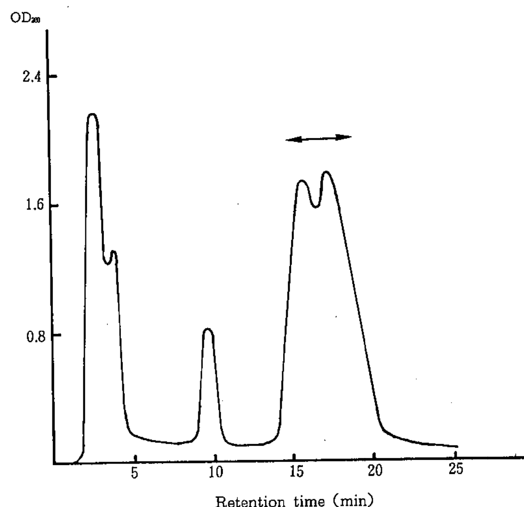

Fig.1. FIRST HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE－5PW.

The serum (2700mg of protein per 30ml) of *Dinodon semicarinatus* was applied to a TSK gel DEAE－5PW column (7.5 x 75 mm). The column was developed with 0.1M sodium acetate buffer (pH 8.3, solution A) and the same buffer supplemented with 0.5M NaCl (solution B) at room temperature. A 0.5ml fractions were collected at a flow rate of 1.0 ml／min. An antihemorrhagic factor was eluted under the condition described in the text. The fractions indicated with an arrow were pooled.

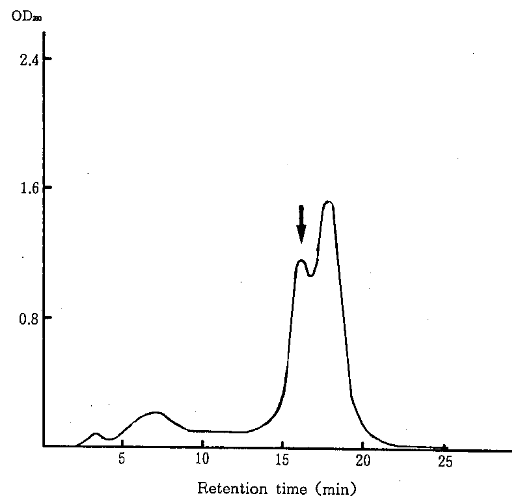

Fig.2. SECOND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE－5PW.

The active fractions from lst HPLC were concentrated, dialyzed against 0.1M sodium acetate buffer (pH 8.3) and applied to a TSK gel DEAE－5PW column (7.5 x 75mm). The column developed with 0.1M sodium acetate buffer (pH 8.3,solution A) and the same buffer supplemented with 0.5M NaCl (solution B) at room temperature. A 0.5ml fractions were collected at a flow rate of 1.0ml／min. An antihemorrhagic factor was eluted under the condition described in the text. The fractions indicated with an arrow were pooled.

to a column (7.5x75mm) of TSK gel DEAE—5PW, and the HPLC was carried out with the same buffer system as described in step 1 (Fig. 2). The fractions (0.5 ml) were collected at a flow rate of 1.0 ml per min (Fig. 2). After washing the column with the buffer of 5% B for 5 min, the column was developed by a linear gradient between 5% B and 35% B for 15 min. The antihemorrhagic factor was eluted by continuing elution with 35% B for 15 min. The active fractions (indicated with an arrow in Fig. 2) were pooled, concentrated by an ultrafiltration as described in step 1, and dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) at 4°C over night.

Step 3. *Third high performance liquid chromatography*

The dialysate was applied to a column (7.5 x 75 mm) of TSK gel DEAE—5PW, and the HPLC was carried out with 0.01 M potassium phosphate buffer (pH 7.0, solution A) and the same buffer supplemented with 0.5 M NaCl (solution B). The antihemorrhagic factor was eluted by an elution at 10% B for 10 min. The fractions (0.5ml) were collected at a flow rate of 1.0ml per min. The fractions with the antihemorrhagic activity (indicated with an arrow in Fig. 3) were pooled and cocentrated by an ultrafiltration as described in step 1.

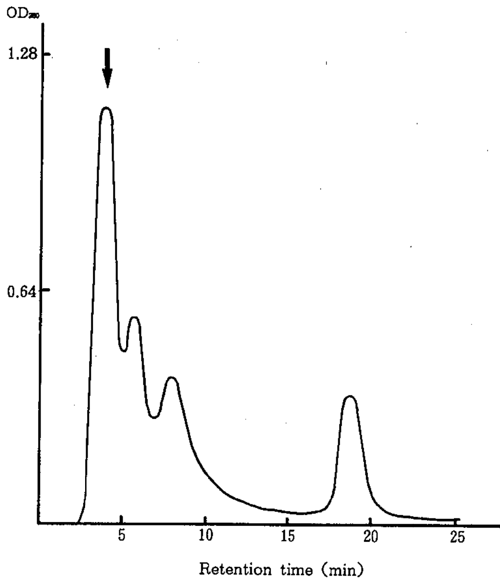

Fig.3. THIRD HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE—5PW.

The active fractions from 2nd HPLC were concentrated, dialyzed against 0.01 M potassium phosphate buffer (pH 7.0), and applied to a TSK gel DEAE—5PW column (7.5 x 75mm). The column was developed with 0.01 M potassium phosphate buffer (pH 7.0, solution A) and the same buffer supplemented with 0.5 M NaCl (solution B) at room temperature. A 0.5ml fractions were collected at flow rate of 1.0 ml/min. An antihemorrhagic factor was eluted under the condition described in the text. The fractions indicated with an arrow were pooled.

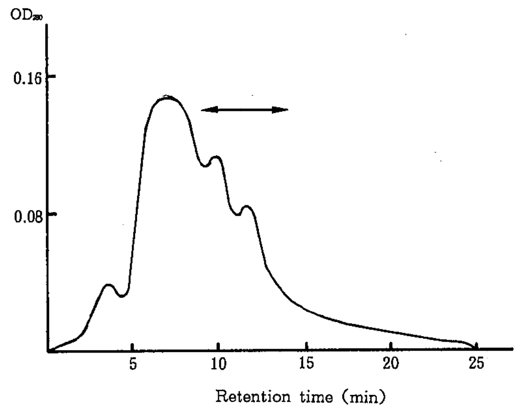

Fig.4. FOURTH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE—5PW.

The active fractions from 3rd HPLC were concentrated, dialyzed against 5 mM Tris-glycine buffer (pH 8.5), and applied to a TSK gel DEAE—5PW column (7.5 x75 mm). The column was developed with 5 mM Tris-glycine buffer (pH 8.5, solution A) and same buffer supplemented with 0.25 M NaCl (solution B) at room temperature. A 0.5ml fractions were collected at a flow rate of 1.0ml/min. An antihemorrhagic factor was eluted under the condition described in the text. The fractions indicated with an arrow were pooled.

Step 4. *Fourth high performance liquid chromatography*

The concentrated fraction was dialyzed against 5mM Tris-glycine buffer (pH 8.5) at 4℃ over night, and applied to a column (7.5 x 75 mm) of TSK gel DEAE−5PW. The column was developed to with 5 mM Tris-glycine buffer (pH 8.5, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) (Fig. 4) After washing the column with 75% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 75% B and 80% B for 10 min followed by elution with 80% B for 5 min (Fig. 4). The fractions with antihemorrhagic activity (indicated with an arrow in Fig. 4)

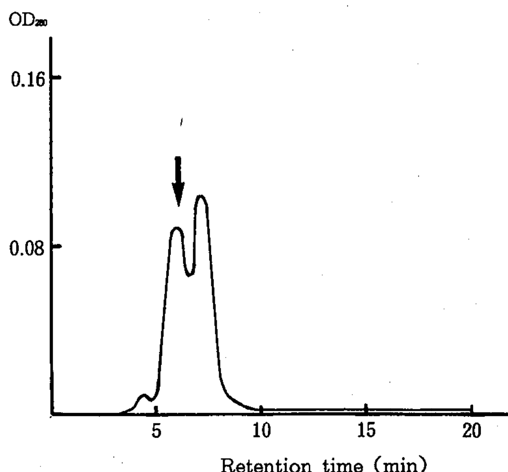

Fig.5. FIFTH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE−5PW.

The active fractions from 4th HPLC were concentrated, dialyzed against 5 mM Tris−glycine buffer (pH 8.5) and applied to a TSK gel DEAE−5PW column (7.5 x75 mm). The column was developed with 5mM Tris−glycine buffer (pH 8.5 solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) at room temperature. A 0.5ml fractions were collected at a flow rate of 1.0ml／min. An antihemorrhagic factor was eluted under the condition described in the text. The fractions indicated with an arrow were pooled.

were pooled and concentrated by an ultrafiltration as described in step 1.

Step 5. *Fifth high performance liquid chromatography*

The concentrated fraction was dialyzed against 5 mM Tris-glycine buffer (pH 8.5) at 4°C over night, and applied to a column (7.5 x75 mm) of TSK gel DEAE−5PW. The column chromatographies were performed with 5 mM Tris-glycine buffer (pH 8.5, solution A) and same buffer supplemented with 0.25 M NaCl (solution B) (Fig.5). After washing the column with 75% B for 5 min, the antihemonrrhagic factor was eluted by a linear gradient between 75% B and 80% B for 5 min. The column was reequilibrated at 75% B for 5 min. The fractions (0.5 ml) were collected at a flow rate of 1.0 ml per min

A summary of the purification is presented in Table 1. The antihemorrhagic factor

Fig.6. DISC ELECTROPHORESIS OF THE PURIFIED ANTIHEMORRHAGIC FACTOR OF DINODON SEMICARINATUS.

The electrophoresis was carried out under the condition of Davis (1964). The protein was migrated from cathode (upper) to anode (bottom).

Table 1 **PURIFICATION OF THE ANTIHEMORRHAGIC FACTOR FROM THE SERUM OF *DINODON SEMICARINATUS*.**

Step	Total protein* (mg)	Total activity† (units)	Specific activity† (units/mg)	Yield (%)
Crude serum	2,700	7,500±480	2.8±1.0	100.0
1st HPLC	570	5,100±350	8.9±2.1	68.0
2nd HPLC	70	2,500±290	35.7±4.2	33.3
3rd HPLC	25	1,250±250	50.0±7.8	16.7
4th HPLC	5	370±30	74.0±5.3	4.9
5th HPLC	2	170±20	85.0±6.8	2.3

The antihemorrhagic activity was determined with HR 1 as a test toxin.
* : Protein was determined by absorbance at 280 nm in 1.0 cm light path cuvette, assuming that absorbance of 1.54 corresponds to 1.0 mg of protein per ml.
† : Antihemorrhagic activity was determined by the method of Kondo *et al.* (1960), and given as an average value of three experiments. One unit of activity is defined as the least quantity of protein neutralizing one MHD of the hemorrhagic activity of *Trimeresurus flavoviridis* Okinawa venom . One MHD is defined as the least quantity of venom causing a hemorrhagic spot of 10 mm in diameter, 24 hr after intracutaneous injection of the venom into the depilated back skin of rabbit. The specific activity is presented as units per mg of protein. Values represent means ± SD of activity.

was purified to 30.3 fold, in a yield of 2.3%.

Homogeneity of the purified antihemorrhagic factor of Dinodon semicarinatus

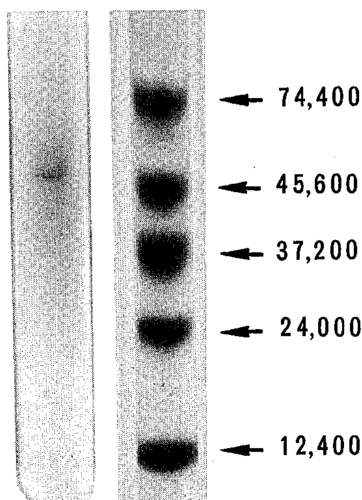

Fig.7. SDS-POLYACRYLAMIDE DISC ELECTROPHORESIS OF THE PURIFIED ANTIHEMORRHAGIC FACTOR OF DINODON SEMICARINATUS.
The SDS-Polyacrylamide gel disc electrophoresis was carried out under the condition of Weber and Osborn (1969).

Homogeneity of the purified antihemorrhagic factor was examined on a polyacrylamide gel disc electrophoresis under the condition of Davis (1964). The purified factor gave a single protein band stained (Fig. 6). Since Omori-Satoh *et al.*, (1972) have isolated the antihemorrhagic factor from the serum of *T. flavoviridis*, several authors have purified the antitoxic factors from the serum of snake (Ovadia *et al.*,1977; Ovadia, 1978) or mammals (Pichyangkul and Perez, 1981; Menchaca and Perez, 1981). The purified antihemorrhagic factor from *Dinodon semicarinatus*, may be the first evidence of the isolation from the serum of a non-venomous snake. The purified antihemorrhagic factor neutralized the toxicity of HR 1 and HR 2, the hemorrhagic factors of *T. flavoviridis* Okinawa.

Thermal and pH stability

The thermal stability of the purified antihemorrhagic factor of *Dinodon semicarinatus* was examined by heating at various temperatures for 15 min. The pH of the solution was kept at 7.0 in 0.01 M potassium phosphate buffer. The antihemorrhagic activities were stable at the temperature from

0°C to 60°C, though the activities were completely lost at the temperature above 70℃. The similar thermal stability was found for the antihemorrhagic factor of *T. flavoviridis* (Omori-Satoh *et al.*, 1972).

The pH of the purified antihemorrhagic factor was adjusted with 0.1 N HCL or 0.1 N NaOH, and the solution was kept at room temperature for 1 hr at various pH. The pH of the solution was then readjusted to 7.0. The antihemorrhagic factor was characterized by their stability in wide range of pH; the activities remained unchanged at the pH between 2.0 and 11.0. The similar pH stability was shown for the antihemorrhagic factor of *T. flavoviridis (Omori-Satoh et al.*, 1972), though the factors from other animals were more or less inactivated at the extreme pH (Ovadia, 1978; Menchaca and Perez, 1981; Pichyangkul and Perez, 1981).

Molecular weight

The molecular weight of the purified antihemorrhagic factor of *Dinodon semicarinatus* was determined to be 59,000 by gel filtration on a Sephadex G－200 as described by Andrews (1964). The molecular weight of the denatured proteins were estimated to be 52,000 for the Akamata factor by a SDS-polyacrylamide gel electrophoresis, suggesting that the antihemorrhagic factor consists of a single subunit.

Formation of precipitin line with the purified antihemorrhagic factor and the venom of T. flavoviridis

Formation of precipitin line with the purified antihemorrhagic factor and the venom of *T. flavoviridis* Okinawa was examined by the double diffusion technique on agar gel (Ouchterlony, 1949). No precipitin line was observed for the purified antihemorrhagic factor with the venom of *T. flavoviridis* Okinawa and its hemorrhagic factors, HR 1 and HR 2. This is coincident with the results obtained with the antihemorrhagic or antineurotoxic factors purified from the sera of various animals, suggesting that the antitoxic factors are not immunoglobulins (Omori-Satoh *et al.*, 1972; Ovadia *et al.*, 1977; Ovadia, 1978; Menchaca and Perez, 1981; Pichyangkul and perez, 1981).

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REFERENCES

Andrews, P. (1964) Estimation of molecular weight of proteins by sephadex gel filtration.*Biochem. J.* **95**, 222.

Davis, J. B. (1964) Disc electrophoresis-II. Method and application to human serum protein in gel electrophoresis. *Ann. N. Y.Acad. Sci.* **121**, 404.

Kondo, H., Kondo, S., Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of *Habu* snake venom. *Jap. Med. Sci. Biol.* **13**, 43.

Menchaca, J. M. and Perez, J. C. (1981) The purification and characterization of antihemorrhagic factor in opossum *(Didelphis virginiana)* serum. *Toxicon* **19**, 623.

Noguchi, H. (1909) Natural immunity of certain animals from snake venom. In *Snake Venom*, 268. Washington D.C., Carnegie Institute.

Omori-Satoh, T., Ohsaka, A., Kondo, S. and Kondo, H. (1967) A simple and rapid method for separating two hemorrhagic

principles in the venom of *Trimersurus flavoviridis*. *Toxicon* **5**, 17.

Omori-Satoh., T., Sadahiro,S., Ohsaka, A. and Marata, R. (1972) Purification and characterization of an antihemorrhagic factor in the serum of *Trimersurus flavoviridis, a crotalid. Biochim. Biophys. Acta*. **285**, 414.

Ouchterlony, O. (1949) Antigen-antibody reactions in gel. *Acta Path.Microbiol. Scand*. **26**, 507.

Ovadia, M. (1978) Purification and characterization of an antihemorrhagic factor from the serum of the snake *Vipera palaestinae*. *Toxicon*. **16**, 661.

Ovadia, M., Kochva, E. and Moav, B. (1977) The neutralization mechanism of *Vipera palaestinae* neurotoxin by a purified factor from homologous serum. *Biochim. Biophys. Acta* **491**, 370.

Perez, J., Haws, W., Garcia, V. and Jennings, B. (1987) Resistance of warm-blood animals to snake venoms. *Toxicon* **16**, 375.

Philpot, V. B., JR and Smith, R. G. (1950) Neutralization of pit viper venom by king snake serum. *Proc. Soc. exp. Biol*.

Pichyangkul, S. and Perez, J. C. (1981) Purification and characterization of a naturally occurring antihemorrhagic factor in the serum of the hispid cotton rat *(Sigmondon hispidus). Toxion* **19**, 205.

Takahashi, T. and Ohsaka, A. (1970) Purification and some properties of two hemorrhagic principles (HR 2a and HR 2b) in the venom of *Trimeresurus flavoviridis* , complete separation of the principles from proteolytic activity. *Biochim. Biophys. Acta*. **207**, 65.

Tomihara, Y., Yonaha,K., Nozaki, M., Yamakawa, M., Kawamura, Y., Kamura, T. and Toyama, S, (1987) Antihemorrhagic activity in the sera of *Dinodon semicarinatus* and *Herpestes edwardsii. The snake,* **19**, 19.

Weber, K and Osborn, M, (1969) The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.*, **244**, 4406.

アカマタ血清中の抗出血因子の精製

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アカマタ血清中の抗出血因子をTSK-gel DEAE-5pwを用いた高速液体クロマトグラフィーで約30倍に精製した。収率は2.3％であった。

精製標品はポリアクリルアミドディスク電気泳動的に均一であった。精製標品の分子量はゲル濾過法により59,000と求められた。またSDSディスク電気泳動により求められた分子量は52,000であることからアカマタ血清中の抗出血因子は単一のサブユニットから構成されていることがわかった。精製抗出血因子はハブ毒の出血因子であるHR1およびHR2の出血毒作用を阻害した。

精製抗出血因子は60℃まで安定であり、また、pH2～11で安定であった。ハブ毒との間に沈降反応が認められないことからこの因子はイムノグロブリンではなくその動物が本来保有している物質であることがわかった。

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