PURIFICATION OF THREE ANTIHEMORRHAGIC FACTORS FROM THE SERUM OF *HERPESTES EDWARDSII*

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ABSTRACT

Three antihemorrhagic factors (AHF-1, AHF-2 and AHF-3) were purified from the serum of *Herpestes edwardsii,* a mangoose, by a combination of gelfiltration on a Sephadex G-200 column and a high performance liquid chromatography with a TSK gel DEAE-5PW column. Each of the purified antihemorrhagic factors showed a single band on a polyacrylamide gel disc electrophoresis. The three antihemorrhagic factors neutralized the toxicity of the hemorrhagic venoms of 6 species of *Trimeresurus* and *Agkistrondon blomhoffi.*

The factors inhibited also the hemorrhagic activity of HR 1 and HR 2, the hemorrhagic principles of *T. flavoviridis* Okinawa. AHF-1, AHF-2 and AHF-3 were stable at the temperature from 0°C to 60°C and at the pH between 2.0 and 11.0. The molecular weight of the three antihemorrhagic factors were estimated to be 65,000 and 69,000 by a gel filtration and a SDS-polyacrylamide gel electrophoresis, respectively. None of precipitin line was found for the purified antihemorrhagic factors with the venom of *T. flavoviridis* Okinawa or its hemorrhagic principles, HR 1 and HR 2.

INTRODUCTION

It has been frequently observed that certain animals are resistant to snake venoms and contain antitoxic factors for the venoms in their sera (Noguchi, 1909 ; Ovadia and Kochva, 1977 ; Philpot *et al.,* 1977 ; Perez *et al.,* 1978). Omori-Satoh *et al.* (1972) have purified and characterized antihemorrhagic factor in the serum of *Trimeresurus flavoviridis* against its own hemorrhagic principles, HR 1 and HR 2. The antihemorrhagic fators in the sera of hispid cotton rat *(Sigmondon hispidus)* and opossum *(Didelphis virginiana)* also have been purified and characterized (Pichyangkul and Perez, 1981; Menchaca and Perez, 1981). These antihemorrhagic factors are not immunoglobulins but natural immunity possessed by the animals. We have shown the presence of antihemorrhagic factors in the sera of *Dinodon semicarinatus,* a non-venomous snake, and *Herpestes edwardsii* a mangoose (Tomihara *et al.,* 1987). The sera of both animals neutralized the toxic activity of various hemorrhagic snake venoms, but did not show antilethal activity for the venom of *T. flavoviridis.*

In this paper we describe the purification of three antihemorrhagic factors from the serum of *H. edwardsii.* These factors are not distinguished in their molecular weight, but by a high performance liquid chromatography with a TSK gel DEAE-5PW. Some of their properties also are characterized.

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 (7.5 × 75mm) from Toyo Soda Manufacturing Co.,; Amberlite CG-50 from Sigma Chemical Co. Standard proteins for molecular weight

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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. The venoms of *T. flavoviridis* Okinawa, *T. flavoviridis* Amami, *T. elegans, T. okinavensis, T. tokarensis* and *T. mucrosquamatus* were pools of dried venoms taken from each snake. The venom of *Agkistrondon blomhoffi* was given by Dr. Y. Kawamura, the Japan Snake Institute. HR 1 and HR 2, the hemorrhagic principles 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 (Omori-Satoh *et al.*, 1967; Takahashi and Ohsaka, 1970). The blood of *H. edwardsii* was collected by heart puncture method, and the serum was separated by centrifugation at 3000 rpm for 15 min and stored at — 20°C. 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 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 *H. edwardsii* was incubated at room temperature for 1 hr. The 0.2-ml 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 factors were determined by a gel-filtration on a Sephadex G-200 as described by Andrews (1964). The column (3.3 × 70 cm) was developed with 5 mM Tris-HCl buffer (pH 8.5) at 4°C, and 4.5ml fractions were collected. The standard proteins 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 factors were determined by a SDS-polyacrylamide gel electrophoresis as described by Weber and Osborn (1969). The antihemorrhagic factors were denatured by incubating in 0.1% sodium dodecyl sulfate (SDS), and then subjected to a SDS-disc electrophoresis with a monomer (MW 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 factors.

Step 1. *Sephadex G-200 column chromatography.* The serum obtained from 4 mongoose (1310 mg of proteins per 21 ml) was applied to a Sephadex G-200 column (3.3 × 70 cm) equili-

brated with 5mM Tris-HCl buffer (pH 8.5) supplemented with 0.15 M NaCl. The column was developed with the same buffer at 4° C (Fig. 1). Fractions with antihemorrhagic activity (indicated with a bar in Fig. 1) were pooled, and concentrated to 10 ml by an ultrafiltration with a Millipore membrane filter PSAC under the pressure of nitrogen gas.

Step 2. *First high performance liquid chromatography.* The concentrated fraction was dialyzed against 0.05 M sodium acetate buffer (pH 8.3) at 4°C over night. The dialysate was subjected to the HPLC with TSK gel DEAE-5PW column (7.5 × 75mm). The column was developed with 0.05 M sodium acetate buffer (pH 8.3, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B), and 0.5-ml fractions 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 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. The active fractions (indicated with a bar in Fig. 2) were pooled, concentrated by an ultrafiltration as described in step 1, and dialyzed against 0.05 M sodium acetate buffer (pH 8.3).

Step 3. *Second high performance liquid chromatography.* The dialysate was applied to a column (7.5 × 75 mm) of TSK gel DEAE-5PW, and the HPLC was carried out with the same buffer system as described in step 2 (Fig. 3). After washing the column with 30% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 30% B and 60% B for

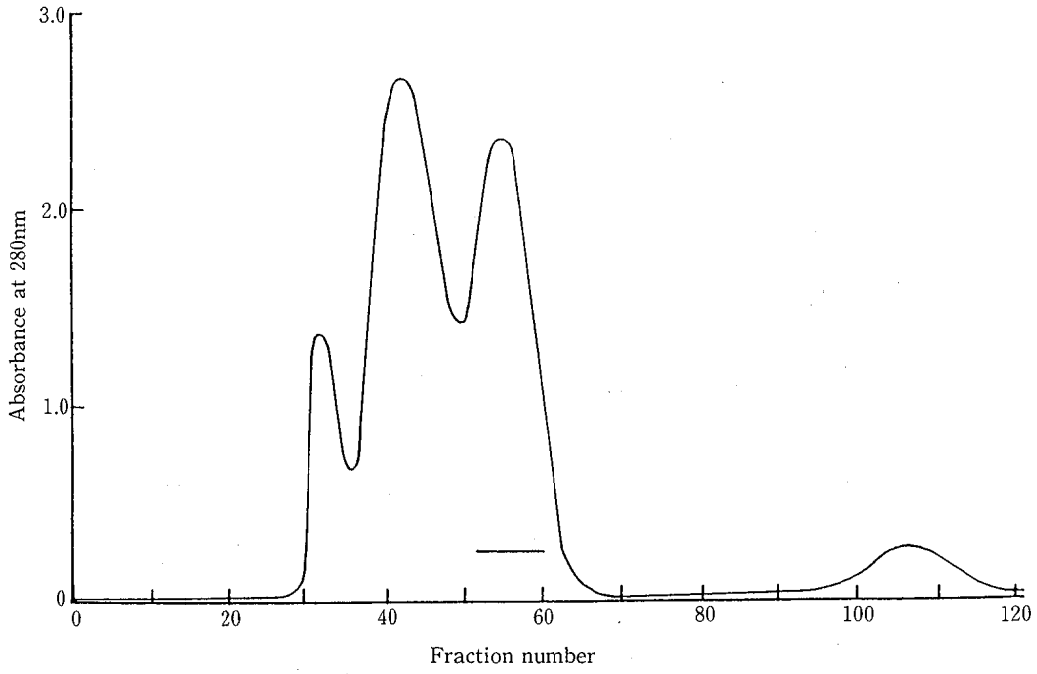

Fig. 1. SEPHADEX G-200 COLUMN CHROMATOGRAPHY OF THE SERUM OF *Herpests edwardsii.*
The serum (1310 mg of protein per 21 ml) of *H. edwardsii* was applied to a Sephadex G-200 column (3.3 × 70 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.5) supplemented with 0.15 M NaCl. The column was developed with the same buffer, and 4.2-ml fractions were collected at a flow rate of 40 ml/hr. The fractions containing antihemorrhagic activity (indicated with a bar) were pooled and concentrated as described in the text.

10 min followed by elution at 60% B for 4 min and then a linear gradient between 60% B and 70% B for 3 min. The column was reequilibrated at 30% B for 3 min. The fractions (0.5ml) were collected at a flow rate of 1.0 ml per min. The fractions with the antihemorrhagic activity (indicated with a bar in Fig. 3) were pooled and cocentrated by an ultrafiltration as described in step 1.

Step 4. *Third high performance liquid chromatography.* The concentrated fraction was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) at 4°C over night, and applied to a column (7.5 × 75 mm) of TSK gel DEAE-5PW. 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. After washing the column with 35% B for 5 min, the antihemorrhagic factor was eluted by a linear gradient between 35% B and 60% B for 15 min followed by elution and reequilibration of the column at 35% B for 5 min (Fig. 4). On this chromatography the antihemorrhagic activity was separated into three peaks. Peaks 1, 2, and 3 contain antihemorrhagic factors 1 (AHF-1), 2(AHF-2) and 3 (AHF-3), respectively. Each peak with antihemorrhagic activity (indicated with a bar in Fig. 4) was separately pooled and concentrated by an ultrafiltration as described in step 1.

Step 5. *Fourth high performance liquid chromatography.* The concentrated fractions were dialyzed against 5 mM Tris-glycine buffer (pH 8.5) at 4°C over night, and applied separately to a column (7.5 × 75 mm) of TSK gel DEAE-5PW. The column chromatographies were performed with 5 mM Tris-glycine buffer

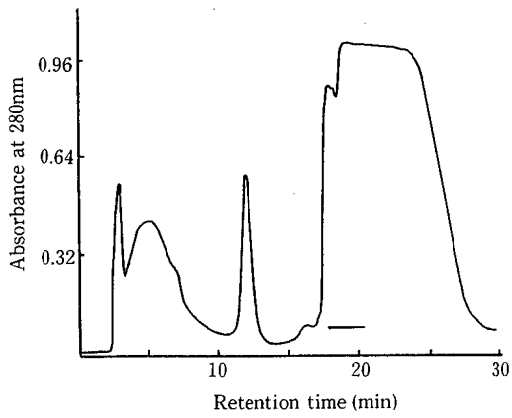

Fig.2. FIRST HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE-5PW.

The active fractions from Sephadex G-200 were concentrated, dialyzed against 0.05 M sodium acetate buffer (pH 8.3), and applied to a TSK gel DEAE-5PW column (7.5 × 75mm). The column was developed with 0.05 M sodium acetate buffer (pH 8.3, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) at room temperature. A 0.5-ml 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 a bar were pooled.

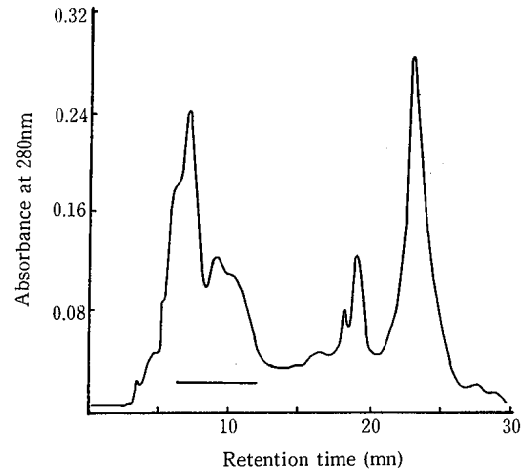

Fig. 3. SECOND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE-5PW.

The active frations from 1st HPLC were concentrated, dialyzed against 0.05 M sodium acetate buffer (pH 8.3), and applied to a TSK gel DEAE-5PW column (7.5 × 75mm). The column was developed with 0.05 M sodium acetate buffer (pH 8.3, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) at room temperature. A 0.5-ml 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 a bar were pooled.

(pH 8.5, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) (Fig. 5-A, 5-B, 5-C). After washing each column with 45% B for 5 min, the antihemorrhagic factors (indicated with a bar in Fig. 5-A, 5-B, 5-C) were eluted by a linear gradient between 45% B and 80% B for 10 min followed by elution at 80% B for 5 min. The column was reequilibrated at 45 % 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. AHF-1, AHF-2 and AHF-3 were purified to 66. 1-fold, 69.4-fold and 74.7-fold in a yield of 10.5%, 8.4% and 7.4%, respectively.

Homogeneity of the purified antihemorrhagic factors of H. edwardsii.

Homogeneity of the purified antihemorrhagic factors, AHF-1, AHF-2 and AHF-3, was examined on a polyacrylamide gel disc electrophoresis under the condition of Davis (1964). Three purified factors gave a single protein band stained (Fig. 6). AHF-1 was migrated as a more acidic protein than the other two, although AHF-2 and AHF-3 could not be separated on the gel electrophoresis. 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) and mammals (Pichyangkul and Perez, 1981 ; Menchaca and Perez, 1981).

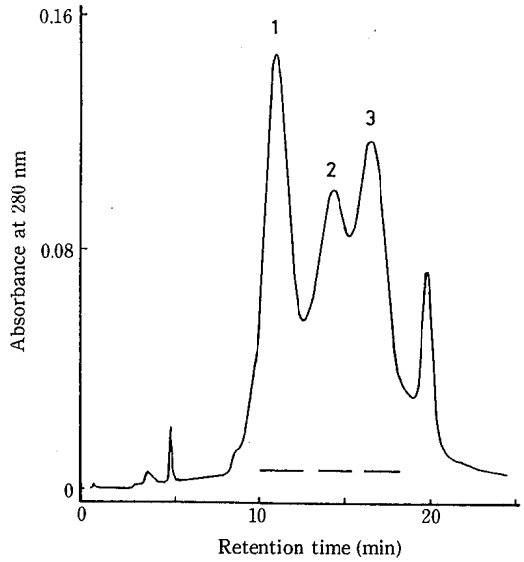

Fig. 4. THIRD HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE-5PW.

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 × 75 mm). 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 0.5-ml fractions were collected at a flow rate of 1.0 ml/min. Three antihemorrhagic factors were eluted under the condition described in the text. The fractions indicated with a bar were separately pooled.

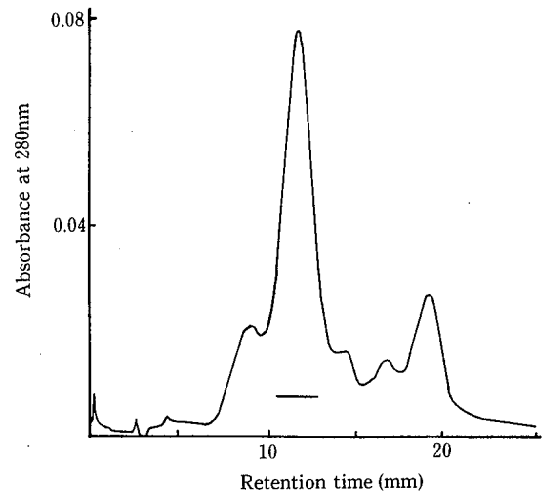

Fig. 5-A FOURTH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE-5PW.

The first 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 × 75 mm). The column was developed with 5 mM Tris-glycine buffer (pH 8.5, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) at room temperature. A 0.5-ml fractions were collected at a flow rate of 1.0 ml/min. An antihemorrhagic factor was eluted under the condition descried in the text. The fractions indicated with a bar were pooled.

These authors have found only single antihemorrhagic principle presented in the serum of each animal. The purified three antihemorrhagaic factors of *H. edwardsii,* therefore, may be the first evidence for the presence of different antitoxic factors for a snake venom in an animal serum. It, however, still remains to be clarified if the difference in the three antihemorrhagic factors is ascribed to a genetic variation in individual mangoose or a modification of protein structure in the course of purification of them, e.g. by a proteolytic hydrolysis, or separate factors.

Neutralization of various hemorrhagic snake venoms with the purified antihemorrhagic factors.

The capacity of the purified antihemorrhagic factors of *H. edwardsii* to neutralize various hemorrhagic snake venoms was studied. AHF-1, AHF-2 and AHF-3 completely inhibited the toxicity of the hemorrhagic venoms of *T. flavoviridis* Okinawa, *T. flavoviridis* Amami, *T. elegans, T. okinavensis, T. tokarensis, T. mucrosquamarus* and *A. blomhoffi* to the same extent. Three purified antihemorrhagic factors neutralized also the toxicity of HR 1 and HR 2, the hemorrhagic principles of *T. flavoviridis* Okinawa. This broad spectrum of the neutralization also has been observed for the antihemorrhagic factor from the other animals (Omori-Satoh *et al.,* 1978; Perez *et al.,* 1978).

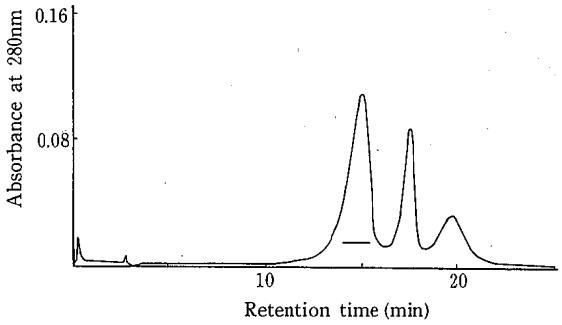

Fig. 5-B FOURTH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE-5PW.

The second active fractions from 3rd HPLC were concentrated, dialyzed against 5 mM Tris-glycine buffer (pH 8.5) and applid to a TSK gel DEAE-5PW column (7.5 × 75 mm). The column was developed with 5 mM Tris-glycine buffer (pH 8.5, solution A) and the same buffer supplemented with 0.25 M NaCl (solution B) at room temperature. A 0.5 ml 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 a bar were pooled.

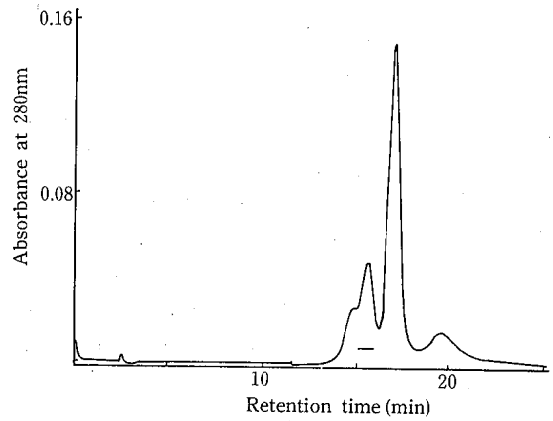

Fig. 5-C FOURTH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK GEL DEAE-5PW.

The third active fractions from 3rd HPLC were concentrated, dialyzed against 5 mM Tris-glycine buffer (ph 8.5) and applid to a TSK gel DEAE-5PW column (7.5 × 75 mm). The column was developed with 5mM Tris-glycine buffer (pH 8.5, solution A) and the same buffer suppplemented with 0.25 M 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 a bar were pooled.

Thermal and pH stability.

The thermal stability of the purified antihemorrhagic factors of *H. edwardsii* 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

TABLE 1. PURIFICATION OF THREE ANTIHEMORRHAGIC FACTORS FROM THE SERUM OF *Herpestes edwardsii*

Step	Total Protein* (mg)	Total activity† (units)	Specific activity† (units/mg)	Yield (%)
Crude serum	1310	19,000	14.4	100.0
Sephadex G-200	460	12,500	27.2	65.8
1st HPLC	70	9,500	135.7	50.0
2nd HPLC	25	8,100	324.0	42.6
3rd HRLC				
AHF-1	5.8	2,500	431.0	13.2
AHF-2	4.8	2,000	416.6	10.5
AHF-3	5.0	1,900	380.0	10.0
4th HPLC				
AHF-1	2.1	2,000	952.4	10.5
AHF-2	1.6	1,600	1000.0	8.4
AHF-3	1.3	1,400	1076.9	7.4

* Protein was determined by absorbance at 280 nm in 1.0 cm light path cuvette, assuming that the an absorbance of 1.54 corresponds to 1.0 mg of protein per ml.

† Antihemorrhagic activity was determined by the method developed by Kondo *et al.* (1960). One unit of activity is defined as the least quantity of protein neutralizing one MHD of the hemorrhagic activity of *Trimeresurus flavoviridis* Okinawa venem. 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 venom into the depilated back skin of rabbit. The specific activity is presented as units per mg of protein.

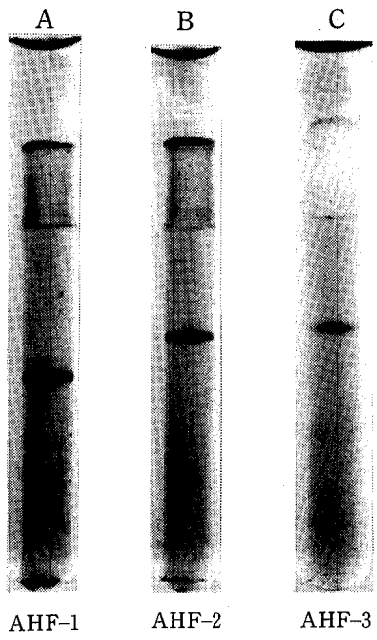

Fig. 6. DISC ELECTROPHORESIS OF THE PURIFIED ANTIHEMORRHAGIC FACTORS OF *Herpestes edwardsii*.

The electrohporesis was carried out under the condition of Davis (1964). A : antihemorrhagic factor 1 (AHF-1). B : antihemorrhagic factor 2 (AHF-2). C : antihemorrhagic factor 3 (AHF-3).

above 70°C. The similar thermal stability was found for the anihemorrhagic factor of *T. flavoviridis* (Omori-Satoh *et al.*, 1972).

The pH of the purified antihemorrhagic factors 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 factors were 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). No difference was found for the thermal and pH stabilities between AHF-1, AHF-2, and AHF-3.

Molecular weight.

The molecular weight of the purified antihemorrhagic factors of *H. edwardsii* were

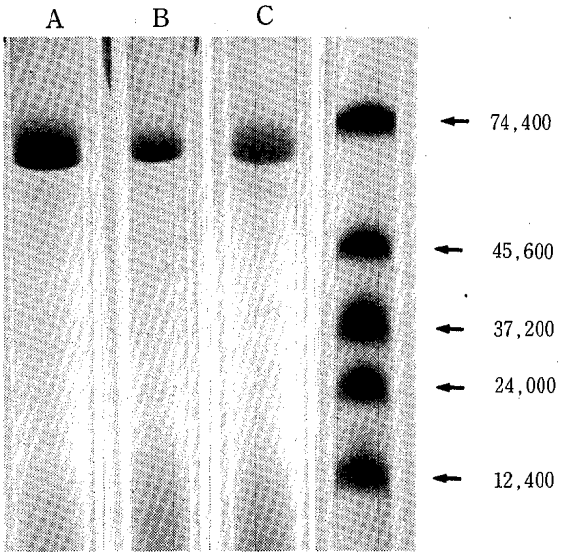

Fig. 7. SDS-POLYACRYLAMIDE GEL DISC ELECTROPHORESIS OF THE PURIFIED ANTIHEMORRHAGIC FACTORS OF *Herpestes edwardsii.*

The SDS-Polyacrylamide gel disc eletrophoresis was carried out under the condition of Weber and Osborn (1969). A: antihemorrgagic factor 1 (AHF-1). B: antihemorrhagic factor 2 (AHF-2). C: antihemorrhagic factor 3 (AHF-3).

determined by a gel filtration on a Sephadex G-200. The same molecular weight, 65,000, was calculated for AHF-1, AHF-2 and AHF-3 by a gel filtration. The molecular weight of the denatured proteins were determined to be 69,000 for AHF-1, AHF-2 and AHF-3 by a SDS-polyacrylamide gel electrophoresis, suggesting that these antihemorrhagic factors consist of a single subunit.

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

Formation of precipitin line with the purified antihemorrhagic factors and the venom of *T. flavoviridis* Okinawa were examined by the double diffusion technique on agar gel (Ouchterlony, 1949). None of precipitin line was observed for AHF-1, AHF-2 and AHF-3. Precipitin line was not formed also with HR 1 and HR 2 of the *Trimeresurus*. 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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マングース血清中の3種の抗出血因子の精製

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要　旨

マングース血清中の3種の抗出血因子をSephadex G-200およびTSK-gel DEAE-5PWを用いた高速液体クロマトグラフィーで約70倍に精製した。収率は約7％～10％であった。

各種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。各標品の分子量はゲル濾過法により65,000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69,000であることからマングース血清中の3種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。各精製抗出血因子はハブ毒の出血因子であるＨＲＩおよびＨＲ2の出血毒作用を阻害した。

精製抗出血因子は60°Cまで安定であり、また、pH 2～11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それら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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