ISOLATION AND SOME PROPERTIES OF THE HEMOLYSINS FROM THE CRUDE VENOM EXTRACT OF SCORPION TELSON, *HETEROMETRUS GRAVIMANUS*

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The hemolytic and enzymic investigations of the crude venom from *Heterometrus gravimanus* scorpion have revealed the presence of both direct and indirect (phospholipase A) hemolysins.

The direct hemolysins were isolated from the lyophilized crude venom extract by Sephadex G-100 gel filtration, SP-Sephadex C-25 column chromatography, gel filtration of Sepharose 4B and Sephacryl S-300.

The two direct hemolysins having high molecular weights, P (A) (M.W.: nearly 200×10^4) and P (B) (M.W.: 22×10^4), were obtained with the activity recoveries of 8% and 39.4%, respectively. P (B) was further separated into three fractions by Sephadex G-200 gel filtration, namely F (A) (M.W.: 17×10^4), F (B) (M.W.: 4.5×10^4) and F (C) (M.W.: below 1×10^4), with the significant losses of direct hemolytic activities. However, F (B) had phospholipase A activity and this activity was partially inhibited by *p*-bromophenacyl bromide. F (B) and F (C) were soluble, even after the treatment of dialysis and lyophilization, in the isotonic saline and distilled water, and were homogeneous in polyacrylamide gels after going through disc and vertical plate electrophoreses. Furthermore, the schlieren pattern of F (B) also showed homogeneity of the preparation.

The protein parts of hemolysins were found to be acidic ones. Although ultraviolet spectra of them were somewhat varied in accordance with their protein contents in the samples, the spectra of excitation and emission were almost identical.

The direct hemolysins are presumably glycoproteins, since they are positive in the sugar reactions, and the difference between P (A) and P (B) is supposed to consist in the sugar part rather than that of proteins.

Keywords—*Heterometrus gravimanus* scorpion; direct hemolysins; indirect hemolysins; phospholipase A; human red blood cells; isolation of hemolysins from crude venom; amino acid analysis of isolated hemolysins; schlieren pattern of hemolysin; ultraviolet and fluorometric spectra of hemolysins

INTRODUCTION

The scorpion venoms are generally known to contain few or no hemolysin although there are some exceptions¹⁾ such as the *Heterometrus* of India.¹⁻⁴⁾

Hemolysins have been classified into two major groups following the mode of their action: the direct hemolysin is capable of causing

hemolysis in red blood cells when they are washed and the indirect one is an enzyme, phospholipase A₂ (EC 3.1.1.4), which produces an intermediary lysin.

We have been interested in the biologically active substances of venom obtained from *Heterometrus gravimanus* scorpion.⁵⁾ Biochemical investigations of the crude venom extract from

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this scorpion telson have revealed the presence of both direct and indirect hemolysins in the venom.

In this paper, we deal with the isolation and characterization of the hemolysins isolated from the crude venom extract of this species.

MATERIALS AND METHODS

Lyophilized Crude Venom — The lyophilized crude extract from the telson containing the venom gland and the venom of *Heterometrus grivimanus* scorpion was obtained as described previously,⁵⁾ and we started from that material in this investigation.

Hemolysis Assay — The citrated human blood (supplied by Hokkaido Blood Center of Japanese Red Cross Society) was centrifuged for 15 min at $1500 \times g$ and the cells were washed three times in isotonic saline (pH 7.4) or buffered isotonic saline (the total osmolarity of 300–310 mOSM/kg with an osmometer, Fiske Associates, Ms., U.S.A.). The washed cells were resuspended in the same media to yield suspension of 1% (w/v) cell concentration, and 2 ml of the cell suspension was added to an equal volume of the test solution. And then, the mixture was incubated and treated as described previously.⁵⁾ The degree of hemolysis was determined by estimating the absorbance of hemoglobin, which was released in the supernatant resulting from centrifugation; the absorbance was estimated to be 543 nm (Hitachi Spectrophotometer, model 200-20, Japan). Hemolysis at the 100% level was performed by diluting 0.2 ml of a 10% (v/v) washed red blood cell suspension with 3.8 ml of distilled water and measuring the absorbance at 543 nm.

One hemolytic unit (HU) is defined as the amount of venom protein required to produce 50% hemolysis of a 0.5% (w/v) suspension of washed red blood cells under the conditions specified, and the specific activity was expressed as the reciprocal of HU.

Measurement of Phospholipase A Activity — This enzyme activity was determined by measuring a decolorization of cresol red at 578 nm, which was due to the liberation of free fatty acid from the substrate lecithin at 25°C.⁶⁾ One unit is

defined as one μmol of acid formed per min per mg protein. For the inhibition experiment, the F (B) was preincubated with *p*-bromophenacyl bromide in 0.1 M sodium cacodylate-HCl buffer (pH 6.0) containing 0.1 M NaCl.

Determination of Protein Concentration — For a general purpose, protein content was determined spectrophotometrically, by measuring the absorbance at 280 nm, in a Hitachi spectrophotometer (model 200-20) with a cell of 1 cm light path, with the value assumed to be $E_{280\text{ nm}}^{1\%} = 10.0$. As to the purified preparation, it was estimated by the method of Lowry *et al.*⁹⁾ with crystalline bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis — By employing the electrophoresis apparatus developed by Raymond¹⁰⁾ (E.C Apparatus Corp., Pa., U.S.A.), vertical plate polyacrylamide gel electrophoresis was carried out according to the method described by Fujimoto *et al.*,¹¹⁾ in which a 7.5% (w/v) and 0.0825 M Tris-EDTA-borate buffer (pH 9.2) were used. Electrophoresis was performed for 60 min at 250 V and 50 mA. The gel was stained with Amido black 10B after the run and destained according to the standard procedure. Disc electrophoresis was carried out by the method of Orstein¹²⁾ and Davis¹³⁾ in 5.6% (w/v) polyacrylamide gel with the 0.05 M Tris-glycinate buffer (pH 8.3) employed for 90 min at 5 mA per glass tube (0.5×7.0 cm). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out by means of the glass columns (0.5×10 cm) and 3.3% (w/v) as described previously¹⁴⁾; the method is based on that of Weber and Osborn.¹⁵⁾

Measurement of UV and Fluorescence Spectra of Isolated Hemolysins — The pooled P (A), P (B), F (A), F (B), F (C) solutions were applied to a Shimadzu Multipurpose spectrophotometer, model MPS-5000. A Shimadzu spectrofluorometer (RF-503) was used to estimate the emission and excitation spectra in aqueous solution.

Amino Acid Analysis — Hydrolysis of isolated hemolysins, P (A), P (B), F (A), F (B) and F (C), was carried out in an evacuated test tube for 24 h

at 110°C with redistilled constant boiling 6 N HCl. Amino acid analysis was performed according to the modified method of Spackman *et al.*¹⁶⁾ in which a Nihon-Denshi amino acid analyzer, model JLC-6A, was employed.

Ultracentrifugal Analysis — Ultracentrifugation of the isolated hemolysin, F (B), was performed by means of a Spinco model E apparatus.

Chemicals — Acrylamide (Wako Pure Chemical Industries, Ltd., Japan), Amido black 10B (Merck, West Germany), Anthron (Wako), *p*-Bromophenacyl bromide (Wako), Coomassie brilliant blue R-250 (Merck), Cresol red (Kanto Chemical Co., Inc., Japan), Glycylglycine (free base, Sigma), Lecithin (Merck), β -Mercaptoethanol (Wako), α -Naphthol (Wako), Sodium dodecyl sulfate (Wako), Tween 20 (Wako), Sephadex G-100, SP-Sephadex C-25, Sepharose 4B, Sephacryl S-300, Blue dextran and Molecular weight markers for the gel filtration (H and L)

were purchased from AB Pharmacia, Uppsala, Sweden.

The other chemicals used were of analytical and reagent grades.

RESULTS

I. Enzymic and Hemolytic Activities of the Crude Venom

The lyophilized crude venom extract had phospholipase A activity of 3–5 U per mg, under the conditions specified concerning materials and methods. The effect of crude venom concentrations, non-treated and heated ones, in the hemolysis of washed human red blood cells is shown in Fig. 1. Both curves reveal that the degree of hemolysis depends upon the crude venom used. However, this effect in both cases shows saturation characteristics.

II. Isolation of the Hemolysins

All steps were carried out at 0–4°C, except where otherwise illustrated. For the concentration of eluted active fractions by ultrafiltration, Diaflo membranes (type UM-2, Amicon Corp., Ms., U.S.A.) were used.

The lyophilized crude venom (1 g) was dis-

FIG. 1. Effect of the Crude Venom on Hemolysis of Washed Human Red Blood Cells

Assays were performed as described under materials and methods. ○ : non-treated crude venom, ● : heated (90°C, 3 min) crude venom.

FIG. 2. Isolation of the Hemolysins by Sephadex G-100 Gel Filtration

○ : protein (A_{280} /ml), ● : hemolysis.

TABLE I. *Isolation of Direct Hemolysins from the Crude Venom Extract of Scorpion Telson, Heterometrus Gravimanus*

Purification steps		Protein		Activity		
		A_{280}	Recovery (%)	Specific ^{a)} activity	Total activity	Recovery (%)
Crude venom		575 ^{b)}	100	1.7	977.5	100
1. Sephadex G-100 gel filtration		336	58	2.0	672.0	67.4
2. SP-Sephadex C-25 chromatography		248	43	2.2	545.6	58.9
3. Sepharose 4B gel filtration	P (A)	35	6	2.5	87.5	9.0
	P (B)	110	19	3.5	385.0	39.4
4. Sephacryl S-300 gel filtration	P (A)	30	5	2.6	78.0	8.0

a) Reciprocal of hemolytic unit (HU). b) 1000 mg.

solved in 5 ml of distilled water, and the solution was applied to a Sephadex G-100 column (2.6 × 100 cm) which had been equilibrated with the isotonic buffer (pH 5.8). The column was eluted by the same buffer at a flow rate of 10–12 ml per h and the fractions were collected. Hemolytic activity was eluted in the fractions of No. 10–30 and they were pooled (Fig. 2.)

The concentrated material was filtered through the following columns; SP-Sephadex C-25 (2.5 × 60 cm), Sepharose 4B (2.5 × 60 cm) and Sephacryl S-300 (1.6 × 32.5 cm). The columns were eluted by the same isotonic buffer (pH 5.8). Hemolytic activity coincides with the elution profiles of proteins on each occasion. The consecutive recoveries (from step 1 to step 4) of protein and hemolytic activity are given in tabular form (see Table I).

The pooled and concentrated P (B) solution (30 ml) obtained from Sepharose 4B gel filtration was applied to a Sephadex G-200 column (2.5 × 60 cm), and the column was eluted by isotonic buffer (pH 5.8). Somewhat contrary to our expectation, the active fraction was divided into three parts (Fig. 3). We pooled fractions of No. 13–17, 23–32 and 38–41 separately, tentatively classifying them into F (A), F (B) and F (C) in accordance with the respective elution rate.

Each pooled solution was dialysed against distilled water for 2 days and was lyophilized. Concerning the P (A) fraction and Sephadex G-200 column as well, similar elution profile was observed.

Protein recoveries of F (A), F (B) and F (C) were 3.8%, 10.3% and 3.4% of the crude venom, respectively. However, the reciprocal of F (A), F (B), F (C) activities (the specific activities) was 0.38, 0.32 and 0.31, respectively.

FIG. 3. *Sephadex G-200 Gel Filtration of the P (B) Fraction obtained from Sepharose 4B Gel Filtration* For details of the procedure, see the text. ○ : protein (A_{280} /ml), ● : hemolysis.

III. Criteria of Isolated Hemolysins

Isolated F (B) and F (C) preparations gave a single band respectively in both vertical plate

electrophoresis (Fig. 4-(a)) and disc-electrophoresis (Fig. 4-(b)), though the F (B), when it was made to go through the SDS polyacrylamide gel electrophoresis, showed several bands (Fig. 4-(c)).

Furthermore, the schlieren pattern of 0.3% (w/v) of isolated F (B) in distilled water indicates the homogeneity of this preparation (Fig. 5).

In both the vertical and disc-electrophoreses, the pooled P (A) and the F (A) showed less solubility in distilled water and even in isotonic saline.

FIG. 4. Schematic Presentation of (a) Polyacrylamide Vertical Plate Gel Electrophoresis, (b) Photograph of Disc-electrophoresis of the Isolated F (B) and F (C), and (c) SDS-Polyacrylamide Gel Electrophoresis

For the SDS-gel electrophoresis, the samples were preincubated at 100°C for 2 min with the mixture of 1% (w/v) SDS and 1% (v/v) β -mercaptoethanol in 0.01 M phosphate buffer (pH 7.0). The solubilized samples were electrophoresed as described in Materials and Methods.

FIG. 5. Schlieren Pattern of the Isolated Hemolysin

The isolated hemolysin F (B) (0.3% (w/v) in distilled water) was sedimented from left to right in a Spinco model E ultracentrifuge (56100 rpm, 20°C, bar angle 65°).

IV. Some Properties of Isolated Hemolysins

IV-1. Protein Contents and Qualitative Detection of Sugar — Protein contents were determined by the method of Lowry *et al.*⁹⁾ with crystalline bovine serum albumin as a standard. The weight percent (w/w) of proteins was 48.5%, 47.2%, 59.7%, 78.0% and 32.9% for P (A), P (B), F (A), F (B) and F (C), respectively. The detection of sugar by the methods of Morish, Phenol-H₂SO₄ and Anthron-H₂SO₄ showed positive reaction for all the hemolysins isolated. From those results, the hemolysins were found to be a kind of glycopro-

teins.

IV-2. Molecular Weight Estimation — The approximate molecular weights of P (B), F (A) and F (B) were determined by gel filtration in Sepharose 4B (2.5 × 60 cm) and Sephadex G-200 column (2.8 × 62.5 cm), according to Andrews.¹⁷⁾ The molecular weights of P (B), F (A) and F (B) were estimated to be 22×10^4 , 17×10^4 and 4.5×10^4 , respectively, on the basis of a linear relationship between the volumes of the protein markers and the logarithmus of their molecular weights (Fig. 6). The molecular weights of P (A) and F (C) seemed to be nearly 200×10^4 and below 1×10^4 , respectively.

IV-3. Amino Acid Composition — The results of amino acid analyses are shown in Tables II-(a) and II-(b). The amounts of hemolysins used were 105.0 μ g, 116.3 μ g, 108.25 μ g, 107.5 μ g and 112.8 μ g for P (A), P (B), F (A), F (B) and F (C), and those of the recoveries of amino acid (tryptophan was not determined) were 51.0%, 43.9%, 63.7%, 82.0% and 37.7%, respectively. Table II-(a) shows the mol % of amino acid, and the molar ratio of amino acids for the P (B) and F (B) were calculated based on the molecular weights of 22×10^4 and 4.5×10^4 which had been determined by the gel filtration (Table II-(b)), respectively.

IV-4. Ultraviolet and Fluorescence Spectra — The absorption spectra of the pooled P (A), P (B), F (A), F (B) and F (C) in the isotonic buffer (Fig. 7), and the excitation and emission spectra of hemolysins in doubly distilled water are shown (Fig. 8).

IV-5. Phospholipase A Activity — The isolated hemolysins, P (A), P (B), F (A) and F (C), had none of phospholipase A activity. However, the F (B) had this enzyme activity of 10–20 U per mg. The phospholipase A activity of F (B) decreased to 80% of the initial activity, when it (2.2×10^{-4} M) was treated with a 10-fold molar excess of *p*-bromophenacyl bromide at 30°C for 15 h, although the enzyme activity was constant while the F (B) was incubated under the same conditions in the absence of *p*-bromophenacyl bromide.

FIG. 6. Estimation of Molecular Weights of the P (B), the F (A) and the F (B) in the Columns of (I) Sepharose 4B and (II) Sephadex G-200

Authentic proteins of thyroglobulin (A: M.W., 67×10^4), ferritin (B: M.W., 44×10^4), catalase (C: M.W., 23.2×10^4), aldolase (D: M.W., 15.8×10^4), ovalbumin (E: M.W., 4.3×10^4), and ribonuclease (F: M.W., 13.7×10^3) were used.

DISCUSSION

It is illustrated that scorpion venoms generally contain few or no hemolysins except for some species: *Buthus martensis*, *Scorpio maurus* and *Heterometrus*.¹⁾ On the other hand, Rosin¹⁸⁾ reported the hemolysis by the scorpion venom of

Nebo hierichontiens. *Leirus quinquestratus* may cause the hemolysis, taking into consideration the report by Rabie *et al.*¹⁹⁾ of the inhibition of catalase in human erythrocytes caused by this scorpion venom. The intergeneric relationship of venoms from scorpions covering different

TABLE II-a. Amino Acid Analyses of the Isolated Direct Hemolysins

Amino acid	mol %				
	P (A)	P (B)	F (A)	F (B)	F (C)
Asp	11.2	11.2	11.2	11.9	10.5
Thr	5.8	6.7	6.1	5.9	5.5
Ser	5.4	6.0	5.6	5.6	5.1
Glu	11.1	10.0	11.6	11.7	10.3
Pro	4.9	4.8	5.6	5.9	4.9
Gly	7.4	9.0	7.2	7.4	9.7
Ala	7.1	9.2	7.0	7.1	11.2
Cys/2	2.1	3.8 ^{a)}	1.5	2.6	1.6
Val	6.0	6.0	6.3	5.8	6.8
Met	2.0	1.9	1.4	1.8	2.2
Ile	5.4	4.9	5.1	4.8	4.6
Tyr	3.5	2.9	5.2	3.5	2.6
Phe	4.5	3.7	4.5	4.3	3.4
His	3.7	1.6	3.3	2.7	2.1
Lys	6.7	6.8	6.0	7.1	7.1
Arg	5.0	3.6	4.8	4.3	4.7
Trp	N.D. ^{b)}	N.D. ^{b)}	N.D. ^{b)}	N.D. ^{b)}	N.D. ^{b)}

a) Corrected value. b) Not determined.

TABLE II-b. Amino Acid Compositions of the Isolated P (B) and F (B) (24 h Hydrolysate)

Amino acid	P (B) ^{a)}	F (B) ^{b)}	Amino acid	P (B) ^{a)}	F (B) ^{b)}
Asp	90	33	Ile	39	13
Thr	54	16	Leu	59	20
Ser	48	15	Tyr	23	10
Glu	80	32	Phe	30	12
Pro	39	16	His	13	7
Gly	72	20	Lys	54	19
Ala	74	19	Arg	29	12
Cys/2	29 ^{c)}	7	Trp	N.D. ^{d)}	N.D. ^{d)}
Val	48	16	Total	797 ^{e)}	272 ^{e)}
Met	16	5			

a) and b) Calculated based on the molecular weights of 22×10^4 and 4.5×10^4 , respectively. c) Corrected value. d) Not determined. e) Trp is not included.

geographical areas may exist, as described by Glenn *et al.*²⁰⁾

Recently, Malhotra *et al.*²¹⁾ reported the hemolysis and renal failure following the sting of the south Iranian scorpion (the species not illustrated), and Chadha and Leviav²²⁾ also observed the similar clinical case caused by the *Buthus sauloci* scorpion sting.

Heterometrus scaber, the south Indian scorpion, was known to have the hemolysin, and the presence of phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) in the venom was reported by Krup.²⁻⁴⁾ This enzyme is known to be the typical indirect hemolysin of bee and *Elapidae* snake venoms. We also found this enzyme in the lyophilized crude venom extract of *Heterometrus gravimanus* scorpion as illustrated in Results, and we will give the details concerning this enzyme elsewhere.

In this paper, we have treated especially the direct hemolysins in the venom of *Heterometrus*

gravimanus scorpion. The term of "direct hemolysin" is adopted in this study, since the isolation proceeded from assaying the active fractions for the hemolytic activity of washed human red blood cells.

Lazarovici *et al.*²³⁾ reported not only the presence of phospholipases but also of the direct lytic factors (DLFs) obtained from the venom of the scorpion, *Scorpio maurus palmatus*. Concerning the "direct lytic factors", mellitin²⁴⁾ and strongly basic polypeptides²⁵⁻²⁷⁾ (cardiotoxin, skeletal muscular depolarizing factor, cobramine A and B, toxin γ , direct lytic factor, peak 12B *etc.*) have been detected in venoms of bee and those of *Elapidae* snakes, respectively. As the basic polypeptides devoid of enzymic activity in snake venoms have been suggested possibly to be identical,²⁵⁻²⁹⁾ possessing the molecular weights of $6-7 \times 10^3$, so the "direct lytic factors" could be the same with the direct hemolysins. On the other hand, the isolated hemolysins in this experiment

FIG. 7. Schematic Illustration of Absorption Spectra of the Hemolysins, P (A), P (B), F (A), F (B) and F (C) in the Isotonic Buffer Solution

These studies were performed in the isotonic phosphate buffer (pH 5.8) at 25°C.

FIG. 8. Excitation and Emission Spectra of the Hemolysins

These studies were carried out in the doubly distilled water. Note the P (A), the P (B) and the F (A) were turbid solutions. The excitation spectrum with at 280 nm and the emission spectrum with excitation 350 nm are shown.

were acidic; more than 20% of the amino acid residues are acidic ones (Table II), differing from the direct lytic factors extracted from venoms of *Elapidae* snakes. Furthermore, the result obtained for the molecular weights of P (A) and P (B) showed that the direct hemolysins are the glycoproteins of very large sizes; P (A), which approximated nearly 200×10^4 , was eluted at the close fractions of void volumes on the gel filtrations of Sepharose 4B and Sephacryl S-300 and the molecular weight of P (B) obtained from the elution profile on the Sepharose 4B gel filtration approximated 22×10^4 , which was obtained by contrasting the elution profile with the molecular markers. P (B) was further separated into three fractions, F (A), F (B) and F (C), on Sephadex G-200 gel filtration (Fig. 3) and they had less hemolytic activities in washed red blood cells. This fact may suggest that the direct hemolysins isolated from the crude venom of the telson part required the high molecular weight for the direct hemolysis. And proteins in the aggregation form seem to act as indirect hemolysins (Table II-a, II-b and Fig. 4-(c)). During the purification process of direct hemolysins, the active high molecules were seemingly dissociated into the inactive forms. This may be one of the reasons why the specific activity did not increase so much, even though the purification was promoted progressively (Table I). On the other hand, to our surprise, the F (B) appears to have had phospholipase A activity. Furthermore, the F (B) was inhibited by the *p*-bromophenacyl bromide, one of the "active site" inhibitors of phospholipase A, although the degree of inhibition seemed to be lesser than that of phospholipases obtained from porcine pancreas⁷⁾ and snake.⁸⁾ Due to the immobility of the P (A), P (B) and F (A) in the course of the electrophoresis, and insolubility resulting from the treatment of dialysis and lyophilization, their homogeneity was impossible to check. However, the F (B) showed a single band in the vertical and disc-polyacrylamide gel electrophoresis (Fig. 4-(a) and -(b)), and further the schlieren pattern of the F (B) revealed the homogeneity of this preparation (Fig. 5). So, the F (B) was apparently a single

phospholipase A. On the other hand, it showed several bands when we run the SDS-polyacrylamide gel electrophoresis upon it (Fig. 4-(c)). Further experiments should be required to clarify whether these bands are subchains in the protein molecule or not.

The identification of the F (B) with the native phospholipase A in the crude venom remains to be seen.

Although the strongly basic polypeptides extracted from bee and snakes were observed to be active as the cationic detergents, the way in which the isolated direct hemolysins work remains to be clarified. Horie *et al.*³⁰⁾ described on the human red blood cells with hemolytic activity, and considered that the hemolysis would occur by the following process; from binding through membrane perturbation to hemolysis. Condrea *et al.*³¹⁾ isolated the direct lytic factor obtained from Cobra snake venoms, the factor which had a weak hemolytic activity not accompanied by that of any phospholipase, and they observed strong hemolysis and concomitant hydrolysis of erythrocyte phospholipids into lysophosphatides; the hemolysis and hydrolysis were conspicuous when they were combined with phospholipase A fractions obtained from venoms of the same species. So, it seems probable that the similar actions of the venom of *Heterometrus gravimanus* scorpion are to be observed on the red blood cells, though the isolated direct hemolysins were likely to be weakly hemolytic on the basis of protein contents.

On the other hand, the actions of the sugar parts of the direct hemolysins are not precluded.

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