Hornetin: the lethal protein of the hornet (*Vespa flavitarsus*) venom

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By gel permeation on a Fractogel TSK HW 50 column followed by ion-exchange chromatography on carboxymethylcellulose CM 52, a lethal protein, designated hornetin, was purified from the venom of *Vespa flavitarsus*. Hornetin is a highly basic protein (p*I* 10.2) with a molecular mass of about 32 kDa. Its amino acid composition is characterized by a high content of lysine, aspartic and glutamic acid, and is devoid of tryptophan and cysteine. The lack of cysteine in the molecule is distinct from other known vespid venom proteins of comparable size. The i.v. LD_{50} of the toxin is 0.42 μg per g mouse. Assayed on the red blood cells of the mouse and guinea-pig as well as isolated nerve muscle preparations of the chick and mouse, hornetin showed direct hemolytic activity and presynaptic neurotoxicity at microgram level and displayed musculotropic effect at higher concentrations.

Lethal protein *Hemolysis* *Neurotoxicity* (Vespa flavitarsus) *Hornetin*

1. INTRODUCTION

Wasp and hornet venoms are known to be allergic [1–3]. However, deaths caused by vespid stings in human and animals do not all result from allergic reactions. When human or animals are attacked by a swarm of vespids, as they usually do, deaths may follow whether or not victims are allergic to the venom. While numerous neurotoxins and myotoxins (or cytotoxins) are well characterized in snake and bee venoms [4–6], few toxins from vespid venoms, except some protein allergens, have been purified and shown to be lethal to mammals [7,8]. However, in the oriental hornet (*Vespa orientalis*) venom, a lethal fraction containing a major component of M_r 43000 and several minor components had been eluted from an affinity column [9]. We report here the purification of a lethal protein (termed hornetin) with both neurotoxicity and direct hemolytic activity from the venom of *V. flavitarsus*, a hornet species commonly found in the mountain area of Taiwan.

Abbreviations: RBC, red blood cells; BSA, bovine serum albumin

2. MATERIALS AND METHODS

Venom sacs collected from 1100 hornet workers (*V. flavitarsus*) were extracted 3 times with 0.3% acetic acid (10 ml) and lyophilized to a dry weight of 2.4 g. Purification of the lethal protein was initially performed on a Fractogel TSK HW 50 (fine grade, Merck) column (2.5 × 115 cm) eluted with ammonium acetate buffer (0.05 M, pH 5.5). The most toxic fraction was further purified on a carboxymethylcellulose CM 52 (Whatman) column (1.5 × 20 cm) eluted with a non-linear gradient of ammonium acetate (0.05–1.0 M). Homogeneity of the protein was examined by SDS-polyacrylamide slab gel (14%) electrophoresis (SDS-PAGE) as described [10].

The isoelectric point of the toxin was determined by isoelectric focusing of the protein in polyacrylamide gel rods (15%) using ampholytes (LKB) for setting the pH range (7.5–10.5) [11].

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Amino acid composition were determined by the method of Spackman et al. [12] with an LKB-4150 amino acid analyzer. Hydrolysis was performed with 6 N HCl maintained at 110°C for 24 h. Tryptophan was determined after hydrolysis of the protein with 4 N methanesulfonic acid for 24 h [13]. N-terminal residue of the toxin was determined by the method of Chang et al. [14].

Direct hemolytic activity was assayed on the RBC of the mouse and guinea-pig as in [15]. Neurotoxicity and myotoxicity of hornetin were assayed on chick biventer cervicis nerve-muscle [16] and mouse phrenic nerve diaphragm preparations [17]. Both preparations were stimulated indirectly with supramaximal square pulses of 0.5 ms duration, at a frequency of 0.2 Hz for chick and 0.1 Hz for mouse nerve muscle preparations, respectively. The acetylcholine response of chick muscle was tested by adding acetylcholine to the resting muscle and washing with fresh Krebs solution after the evoked contraction had reached the maximum. The resting membrane potentials of the mouse diaphragm were recorded with a glass microelectrode attached to a volt-ohmmeter (W-P Instrument, model F-29) and displayed on a storage oscilloscope (Tetronix 5103-D13).

Lethality of the crude venom and the purified protein were estimated in mice (NIH strain) by i.v. injection. The LD_{50} value was calculated as described [18].

3. RESULTS AND DISCUSSION

The lyophilized venom of *V. flavitarsus* (500 mg) was first separated into 18 fractions by gel permeation on a Fractogel TSK HW 50 column (fig.1). Screening on mice, the lethal factor of the venom was found in fraction 7. Other fractions did not show any lethality in mice at doses 5-fold higher than the i.v. LD_{50} of crude venom (4.5 μg/g mouse). Fraction 7 (32.5 mg) was further purified by cation-exchange chromatography on a carboxymethylcellulose CM 52 column (fig.2). The last fraction eluted from this column showed an i.v. LD_{50} of 0.42 (0.31–0.56) μg/g mouse. The yield of the toxin was about 3% of the crude venom. The lethal protein, designated hornetin, was homogeneous on SDS-PAGE (fig.3a, lane E) and the isoelectrical focusing gel. The p*I* value thus obtained was 10.2. The M_r of the toxin estimated by gel filtration (Sephadex G-75) was 32000 ± 1000 (fig.3b). This value is in good accordance with the

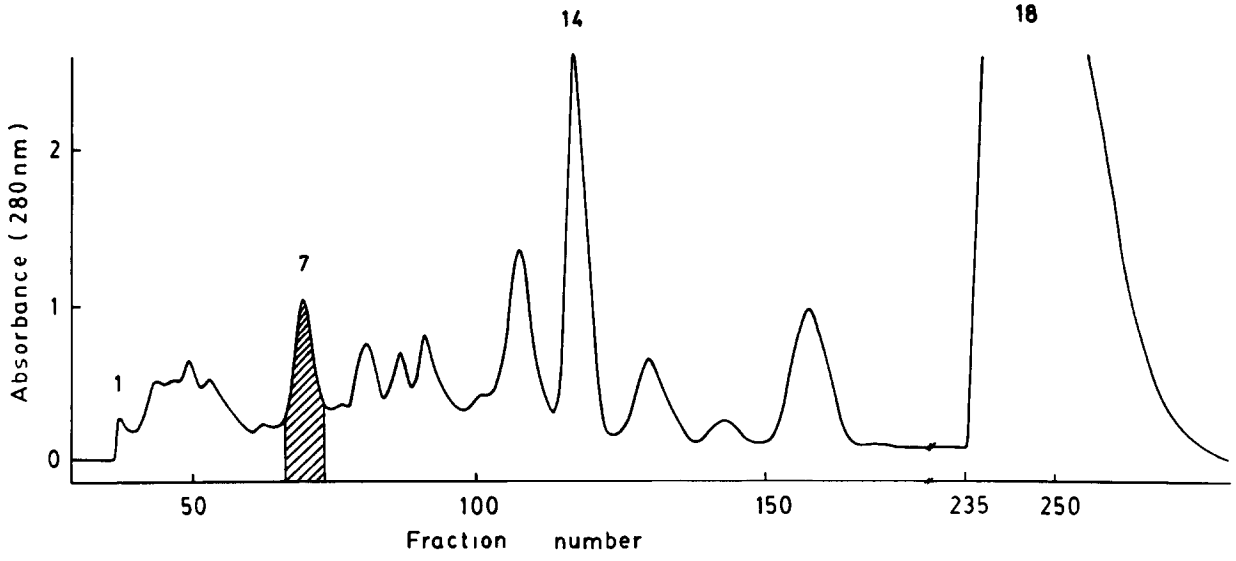

Fig.1. Gel permeation of the venom sac extract of *V. flavitarsus* on a Fractogel TSK HW 50 column. Venom sac extract (500 mg) was applied to the column and eluted with ammonium acetate buffer (0.05 M, pH 5.5). Fractions of 5 ml were collected at a flow rate of 45 ml/h. Fraction 7 (hatched) showed the highest lethality in mice.

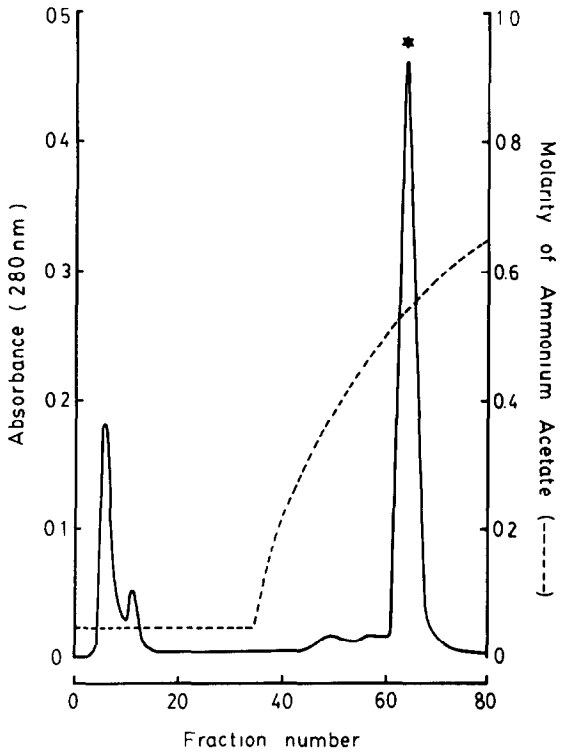

Fig.2. Ion-exchange chromatography of the toxic fraction (fraction 7) on a CM-cellulose CM 52 column. Elution was carried with one-step, non-linear gradient (0.05 M ammonium acetate, pH 5.5 vs 1.0 M ammonium acetate, pH 6.8). Fractions of 5 ml were collected at a flow rate of 45 ml/h. The last peak (*) was lyophilized for biochemical and pharmacological characterization.

minimal M_r (32858) calculated from its amino acid composition (table 1) and with that estimated from SDS-PAGE (fig.3a). Amino acid analysis revealed that the toxin had a high content of Lys, Asp and Glu, and was devoid of Trp and Cys. The N-terminal residue of the toxin was solely Phe. The high basicity of the protein molecule suggested that most of its Asp and Glu residues must be in the amide form. The lack of Cys in the molecule is distinct from other vespid venom proteins of comparable size [7,8].

The mouse and guinea-pig injected with hornetin showed severe hemolysis immediately before death. The hemolytic potency of hornetin assayed on the washed RBC of the mouse was about 100-times higher than that of cobra (*Naja naja atra*) cardiotoxin (a direct lytic factor) (table

Table 1

Amino acid composition of hornet lethal protein (hornetin) purified from *Vespa flavitarsus* venom

Amino acid	Molar ratio[a]	Nearest integer
Try	0.0	0
Lys	27.6	28
His	10.9	11
Arg	7.4	7
Asp	28.7	29
Thr	13.8	14
Ser	7.7	8
Glu	20.9	21
Pro	13.4	13
Gly	21.7	22
Ala	17.6	18
Half-Cys	0.0	0
Val	19.3	19
Met	6.7	7
Ile	16.4	16
Leu	20.0	20
Tyr	10.7	11
Phe	10.2	10
Total		254
M_r		32858

[a] Average of three analyses on 6 N HCl hydrolysates except that tryptophan was estimated by methanesulfonic acid hydrolysis

Table 2

Comparison of the direct hemolytic activity of hornetin with that of cardiotoxin from cobra (*Naja naja atra*) venom

Toxin	Concentration (μg/ml)	Direct hemolysis (%)	
		Mouse RBC	Guinea-pig RBC
Hornetin	1.0	60.3 ± 5.0 (6)	11.4 ± 3.3 (4)
	3.0	83.2 ± 4.5 (6)	67.0 ± 14 (4)
	10.0	94.4 ± 2.2 (4)	88.3 ± 4.1 (4)
Cobra cardiotoxin	10.0	3.7 ± 0.5 (6)	7.9 ± 0.9 (3)
	30.0	3.9 ± 0.8 (6)	13.3 ± 1.5 (3)
	100.0	8.6 ± 1.2 (6)	60.7 ± 12.4 (6)

Washed RBC (2%) was incubated with the toxin in Tris-buffered saline (0.01 M, pH 7.4) at 37°C for 60 min. Cardiotoxin was isolated from *N. naja atra* venom [20]. Data shown are mean ± SE (number of experiments in parentheses)

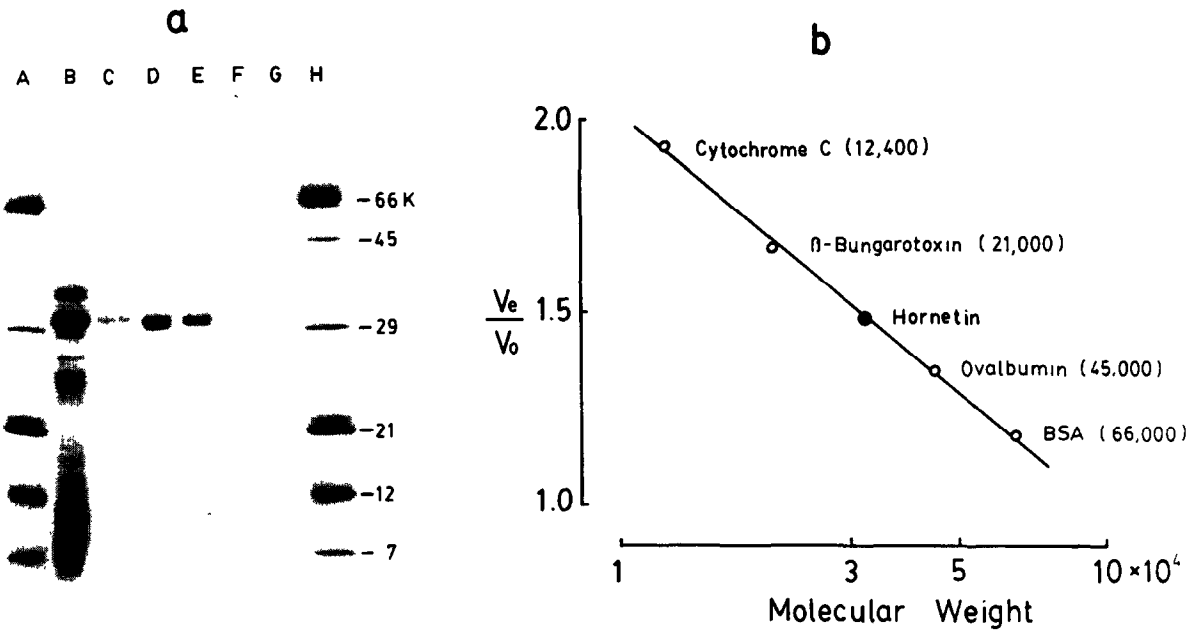

Fig.3. Homogeneity and M_r of hornetin estimated by SDS-PAGE (a) and gel filtration (b). (a) SDS-PAGE of crude venom and purified toxin. Lanes: A,H, standard protein markers (BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 21 kDa; cytochrome *c*, 12 kDa; cobra cardiotoxin, 7 kDa); B, crude venom (600 μg); C, venom protein (30 μg) obtained by passing the crude venom through a filter unit (Molcut II GC, Millipore) which retains proteins of >10 kDa; D, toxic protein (30 μg) from fraction 7 of Fractogel filtration; E, hornetin (20 μg) purified from CM-cellulose 52 column; F, blank; G, extract (250 μg) from other portion of the venom gland apparatus. (b) Estimation of the M_r of hornetin by gel filtration on Sephadex G-75 column (2.5 × 115 cm). V_e, elution volume; V_o, void volume.

Table 3

Inhibition of indirectly evoked contraction of the chick and mouse skeletal muscles by hornetin

Preparation	Hornetin concentration (μg/ml)	Time to block (min)	Contracture[a] (mg)	ACh response[b] (%)
Biventer cervicis nerve muscle of the chick	0.0	>600 (4)	0 (4)	105 ± 2 (4)
	1.0	273 ± 11 (5)	0 (5)	124 ± 6 (5)
	3.0	154 ± 18 (5)	14 ± 9 (5)	122 ± 10 (5)
	10.0	85 ± 3 (4)	53 ± 16 (4)	86 ± 4 (4)
Phrenic nerve diaphragm of the mouse	0.0	>600 (4)	0 (4)	–
	3.0	154 ± 13 (5)	300 ± 30 (5)	–
	6.0	119 ± 8 (3)	350 ± 20 (3)	–
	10.0	77 ± 7 (3)	610 ± 10 (3)	–

[a] Maximal increase in resting tension of the muscle after application of hornetin

[b] Acetylcholine (ACh) response tested after complete blockade (or at 600 min in control muscles) was expressed as % of that before the application of hornetin (or saline in control muscle)

Data shown are means ± SE (number of experiments given in parentheses)

2). Unlike cobra cardiotoxin which is known to be potently hemolytic on guinea-pig RBC but not on mouse RBC [19], hornetin showed higher hemolytic activity toward mouse RBC.

In the chick biventer cervicis nerve muscle preparation, hornetin, at lower concentrations (1–3 μg/ml), inhibited the indirectly evoked contractions of the muscle without depressing the postsynaptic acetylcholine response of the muscle, indicating a presynaptic site of blockade (table 3). At a higher concentration (10 μg/ml) the toxin caused contracture and depression of the acetylcholine response of the muscle, suggesting a musculotropic action of the toxin. The phrenic nerve diaphragm of the mouse showed similar neuromuscular blockade by the toxin. However, contracture of diaphragm muscle was more severe at the same toxin concentrations (3–10 μg/ml). Concomitantly with muscle contracture, the resting membrane potential of the diaphragm muscle was depolarized by hornetin (10 μg/ml) from 84 ± 1.6 mV (SE) to less than 20 mV in 2 h.

Mice treated with hornetin showed respiratory paralysis and circulation failure similar both in symptoms and in time course to those caused by crude venom. In contrast, the lethal factor of *V. orientalis* venom, which had a subunit molecular mass of 43 kDa, exhibited some CNS specific activities in the treated animal [9]. The differences in molecular size and biological activity between the two lethal factors indicate that vespid venom from different species may vary in their constituents.

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