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Chemical Investigation of the Hornet (*Vespa xanthoptera* Cameron) Venom. The Structure of a New Bradykinin Analogue "Vespakinin-X"

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The biological active substances in the venom of hornet (*Vespa xanthoptera*) on the rat uterus, the rat arterial blood pressure, the guinea pig ileum and the rabbit erythrocytes were investigated. A new type of bradykinin analogous peptide, vespakinin-X, was identified as hornet-kinin. The amino acid sequence of vespakinin-X has been found to be Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ile-Val. In addition to the presence of vespakinin-X, histamine, acetyl choline-like substance and large amounts of serotonin were demonstrated in the venom.

Keywords——hornet venom; *Vespa xanthoptera*; bradykinin; vespakinin-X; serotonin

The hornet venom contracts isolated smooth muscle preparation, loweres arterial blood pressure, increases capillary permeability and causes severe pain. Bhoola, *et al.* have been demonstrated the presence of histamine, serotonin, acetylcholine and bradykinin-like substance, hornet-kinin, which was less active than bradykinin on guinea pig ileum contraction, in the venom of European hornet, *Vespa crabro*.[2] Edery, *et al.* have been observed in the venom of *Vespa orientalis* the presence of noradrenaline, adrenaline and dopamine in addition to such active amines and also the presence of honet-kinin,[3] which is readily differentiated from bradykinin by the contraction of the chymotrypsin sensitized smooth muscle preparations.[4] However, the chemical structures of these kinins have not been alluded in both reports.

We have investigated the active substances in the venom of *Vespa xanthoptera*(Japanese name: Kiiro-suzumebachi) by means of the rat uterus contraction, vassodilation of the rat arterial blood pressure and haemolytic action of the rabbit erythrocytes. This paper describes the chemical characterization of active principles and the identification of hornet-kinin of *Vespa xanthoptera*, as a new bradykinin analogue.

Experimental

Preparation of the Hornet Venom Extract——Venom sacs of 370 hornets were homogenized in 3 ml of 6% trichloroacetic acid. The homogenate was diluted by adding 17 ml of H_2O and centrifuged at 3000 rpm for 5 min. The supernatant was used as the crude extract.

Assay Methods——Oxytocic and pressor activities were determined by the methods reported previously.[5] Guinea pig ileum contractile activity was assayed by the Magnus method.[6] The haemolytic action was examined according to the method of Breithaupt and Habermann[7] with slight modification of using the rabbit erythrocytes.

1) Location: *1-2-3 Kasumi, Hiroshima.*
2) K.D. Bhoola, J. Calle, and M. Schachter, *J. Physiol.*, **159**, 167 (1961).
3) H. Edery, J. Ishay, I. Lass, and S. Gitter, *Toxicon*, **10**, 13 (1972).
4) H. Edery, *Nature*, **217**, 70 (1968).
5) T. Nakajima, T. Nakayama, and H. Sokabe, *Gen. Comp. Endocrinol.*, **17**, 458 (1971).
6) I. Trautschold, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös, Springer-Verlag, New York, 1970, p. 57.
7) H. Breithaupt and E. Habermann, *Naunyn-Schmiedebergs Arch. Pharmakol. exp. Pathol.*, **261**, 252 (1968).

Dansylation and Dansyl-Edman Procedure——Dansylation of the amines or the peptides was employed by the procedure previously reported.[8] Dansyl-Edman procedure was performed by the method of Gray.[9] N-Terminal amino acid after hydrolysis of the dansylated peptide with 6N HCl at 90° for 16 hr was identified by thin-layer chromatography of Silica gel H and polyamide layer using solvent systems of *n*-BuOH: AcOH: H_2O (4: 1: 5), iso-PrOH: MeOAc: 28% NH_4OH (9: 7: 4), $CHCl_3$: benzyl alcohol: AcOH (70: 30: 3) and benzene: dioxane: AcOH (90: 25: 4) for Silica gel H, and benzene: AcOH (9: 1) and 1.5% HCOOH for polyamide layer, respectively.

Enzymatic Degradation of Peptides——The enzyme solution was prepared as follows: TPCK-trypsin and γ-chymotrypsin from Worthington Biochemical Corp. were dissolved in 0.1N triethylamine bicarbonate buffer (pH 7.5) to make each 100 μl/ml solution. The peptide of *ca.* 10 nmoles was dissolved in 100 μl of the same buffer, adding 10 μl of the enzyme solution and incubated at 37° for 4 hr or 25° for overnight. After the incubation, the mixture was liophilized, or the solution was evaporated under the nitrogen stream. The residue was dissolved in 20 to 50 μl of MeOH and an aliquot was served for further chromatographical analysis.

All procedures including extraction, bioassay and purification were carried out with siliconized glass equipments.

Result and Discussion

Separation of the Active Principles

The crude extract was applied to a SE–Sephadex column and chromatographed by a linear gradient elution with water to 0.5 N ammonium formate (pH 6.5). The tightly adsorbed material on the column was finally eluted with 1.0 N ammonium formate(pH 9.5).

Pressor activity on the rat artery, contractile activity on the rat uterus and the guinea pig ileum, and haemolytic activity on the rabbit erythrocytes were assayed respectively by taking an aliquot of each fraction. The active principles in the crude extract were separated into four parts, H-I, H-II, H-III and H-IV as shown in Fig. 1.

Purification and Characterization of the Active Principle in H-I

Active principle in the H-I fraction was further purified with Sephadex G-10, SE–Sephadex and finally droplet counter current chromatography as shown in Chart 1. The purified principle did not show any remarkable absorption spectrum at ultraviolet(UV) and visible region, nor react with dansyl chloride to reveal yellow fluorescence. The principle did not lose its activity by treatment with trypsin nor chymotrypsin digestion. The principle, however, was inactivated when it was incubated with 0.01 N ammonia for 1 hr at room temperature. The ratio of contractile activity on the rat uterus to hypotensive activity of this principle was about a half and the activity was blocked in the presence of 10 μg/ml of atropine. These results indicate that the active principle in H-I was considered to be acetylcholine or closely related substance.

Purification and Characterization of the Active Principles in H-II and -III.

In the first step of separation by SE–Sephadex chromatography (Fig. 1), both fractions revealed hypotensive activity. The oxytocic action predominated over the guinea pig ileum contraction in the fraction 21 to 26, while the fraction 24 to 28 was *vice versa*. The fraction number on the column chromatogram showing hypotensive action(fraction 22 to 28) did not actually coincide to that of two kinds of smooth muscle contractile activity. This indicated that more than two kinds of active principles were overlapped in these regions. The fractions H-II and H-III were purified individually by gel permeation with Sephadex G-25 and the active principles were separated into three peaks(H-II-1, H-II-2 and H-III respectively) as shown in Fig. 2. Two active principles, in H-II-1 and -2, were eluted within a column volume of Sephadex G-25. The fraction, H-III, was eluted at the position of one

8) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and S. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).
9) W.R. Gray, "Method in Enzymology," Vol. XI, ed. by C.H.W. Hirs, Academic Press, New York, London, 1967, p. 469.

and a half volumes of the column. These active principles were purified further as shown in Chart 2.

The active principle in H-II-1 loweres the rat arterial blood pressure, contracts the rat uterus and the guinea pig ileum. The activity is lost by chymotrypsin, but not by trypsin treatment. These results indicate that the principle is closely related to bradykinin and considered to be hornet=kinin.

The active principle in H-II-2 was identified with histamine, by the following characteristics: the principle lowered the rat arterial blood pressure, contracted the guinea pig ileum, but on the rat uterus contraction, the activity was much less sensitive. The activity was not susceptible to trypsin nor chymotrypsin treatment, but was blocked in the presence of 200 μg of diphenhydramine hydrochloride. The dansylated principle showed the same

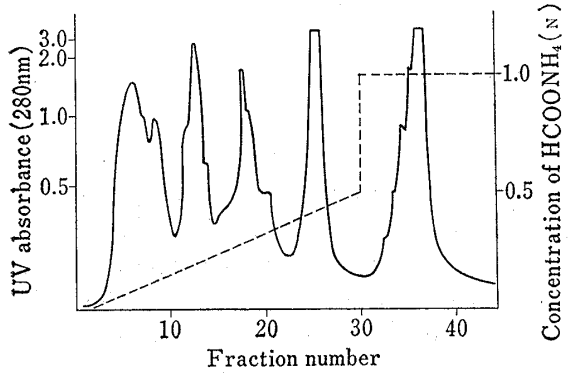

Fig. 1. SE-Sephadex Chromatogram of Crude Hornet Venom

The column (8×400 mm) was eluted with a linear gradient from H_2O to 0.5N $HCOONH_4$ (pH 6.5) (60—60 ml) and successively eluted with 1.0N $HCOONH_4$ (pH 9.5) (80 ml). 4 ml fractions were collected.
The biological activities were shown. by solid lines below the chromatogram.

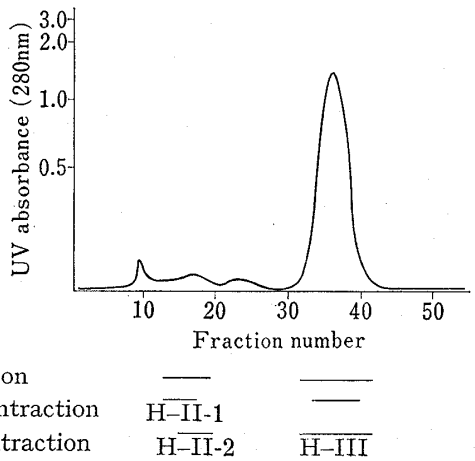

Fig. 2. Sephadex G-25 Chromatogram of H-II and III

The chromatogram showed the separation of H-III (fr. 26—29 of the first step of SE-Sephadex chromatogram of Fig. 1). The column (9×700 mm) was eluted with 0.1N $HCOONH_4$ (pH 6.5). 2 ml fractions were collected. 1 μl of each fraction was assayed for rat uterus contraction. 10 to 20 μl of each fraction were assayed for pressor activity and ileum contraction. The biological activities were shown by solid lines below the chromatogram.

active fraction (H–I)
↓ Sephadex G-25 chromatography
column size: 9×700 mm
elution: 0.1N $HCOONH_4$ (pH 6.5), 80 ml
fraction: 2 ml
active fraction (fr. 15—20)
↓ SE-Sephadex chromatography
column size: 8×400 mm, SE-Sephadex (NH_4^+)
elution: 0.1N $HCOONH_4$ (pH 7.0), 200 ml
fraction: 3 ml
active fraction (fr. 21—26)
↓ droplet counter current chromatography
tube number: 50 tubes
stationary phase: upper layer of *n*-BuOH:AcOH:H_2O (4:1:5)
moving phase: lower layer of the same solvent system
fraction: 2 g
active fraction (fr. 10—16)
acetyl choline-like substance (1110 ng equivalent to acetyl choline)

Chart 1. Purification of the Active Principle in the Fraction H-I

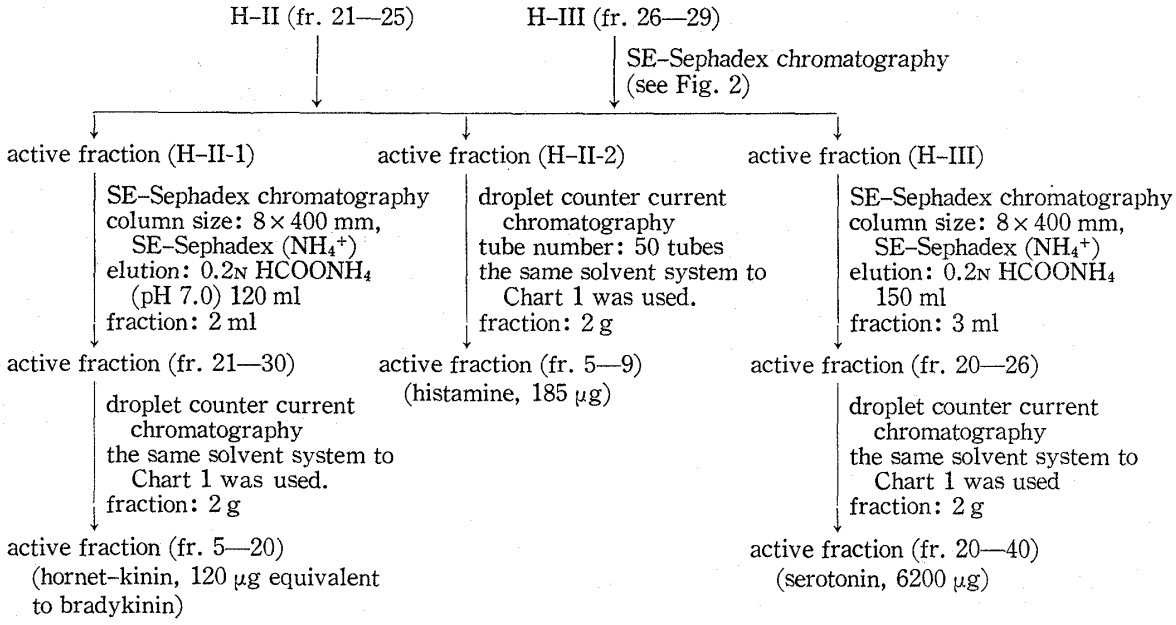

Chart 2. Purification of the Active Principles in the Fraction H-II and III

Rf values to those of dansylhistamine when chromatographed on a thin layer of Silica gel H with the solvent systems of benzene: dioxane: acetic acid and isopropanol: methyl acetate: 28% ammonia.

The active principle in H-III was not susceptible to chymotrypsin nor trypsin treatment. The principle showed a maximum absorption at 275 nm and a shoulder at 295 nm, which was a typical absorption spectrum of serotonin. In a thin-layer chromatography of Silica gel H, the dansylated derivative of the principle showed the overlapping to dansyl serotonin with the solvent system of benzene: dioxane: acetic acid. The active principle was identified with serotonin.

Sequence Analysis of Hornet-kinin

The peptide did not show any absorption spectra based upon tryptophyl nor tyrosyl residue, but week absorption at 252, 258 and 264 nm due to phenylalanyl residue. The peptide was hydrolysed with 6 N hydrochloric acid at 110° for 24 hr. The hydrolysate was assayed by an amino acid analyser (JEOL 5-AH). Amino acid composition of the peptide was as follows: Arg_2, Ser_1, Pro_3, Gly_1, Ala_1, Val_1, Ile_1, and Phe_2. This composition showed that the peptide contained each one mole of alanine, valine and isoleucine in addition to the amino acid composition of bradykinin. Dansyl-Edman procedure was carried out using 3.2 nmoles of the peptide and the N-terminal sequence was deduced to be Ala-Arg-Pro-. The remaining peptide core was hydrolysed with hydrochloric acid as an usual manner and amino acid composition of Ser_1, Pro_2, Gly_1, Val_1, Ile_1, Phe_2, and Arg_1 was obtained by amino acid analysis.

After the first cycle of Edman degradation of the intact peptide, the remaining peptide core, des Ala^1-peptide, was dansylated and the dansylated peptide was separated by thin layer chromatography of Silica gel H. The yellow fluorescent band was scratched and the silica gel powder was plugged in a small chromatotube. The dansyl derivative was eluted with a minimum amount of the mixture of acetone: water: acetic acid: pyridine(50: 50: 3: 1). The purified dansyl-des Ala^1-peptide was digested with trypsin and an aliquot of the digest was chromatographed on a thin-layer of Silica gel H. The dansyl-des Ala^1-peptide was changed by tryptic digestion to the another fluorescent peptide(DNS-T_1) showing the lower *Rf* value with the solvent system of *n*-butanol: acetic acid :water. After redansylation of the trypsin digest, the additional tryptic peptide(T_2) was observed as a fluorescent band using

the solvent system of chloroform: benzyl alcohol: acetic acid as shown in Fig. 3. DNS-T_1 was identical with dansyl bradykinin in the thin-layer chromatographical behaviors. DNS-T_1 was further digested with chymotrypsin and the digest was successively dansylated. Three dansylated chymotryptic fragments were also identified with dansyl-Arg-Pro-Pro-Gly-Phe, dansyl-Ser-Pro-Phe and dansyl arginine by co-chromatography with the authentic samples on a thin-layer of Silica gel H. These results indicated that this hornet-kinin contained the alanyl-bradykinyl sequence in the peptide, and an additional peptide, T_2, was located at C-terminal position. Sequence analysis for T_2, prepared by trypsin digestion of dansyl-des Ala[1]-peptide, was

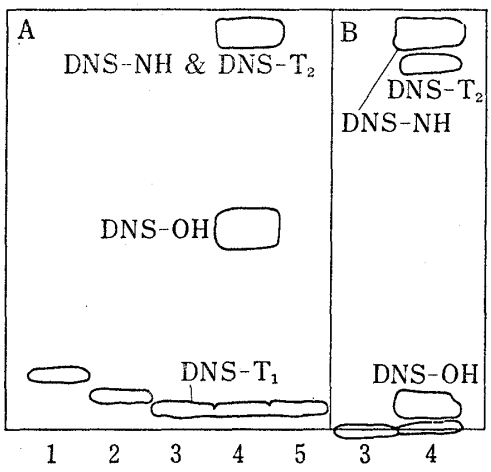

Fig. 3. Thin-Layer Chromatogram of the Dansylated Intact Peptide, des Ala[1]-peptide, and Its Tryptic Digest

solvent system
A: *n*-BuOH: AcOH: H_2O (4: 1: 5)
B: $CHCl_3$: benzyl alcohol: AcOH (70: 30: 3)
1. intact peptide, 2. des Ala[1]-peptide, 3. after trypsin digestion of 2, 4. redansylation of 3, 5. bradykinin
DNS-NH: dansylamide
DNS-OH: dansyl sulfonic acid

TABLE I. The Contents of Vasoactive Materials in One Venom Sac.

	Polistes rothneyi[a] (ng)	Vespa xanthoptera (ng)
Acetyl choline	—	3
Histamine	—	500
Serotonin	1200	16800
Kinins	830	560

a) M. Watanabe, T. Yasuhara and T. Nakajima, "Animal, Plant, and Microbial Toxines," Vol. II, ed. by Y. Sawai, Plenum Publishing Co., New York, London, 1976, p. 105.

performed by Dansyl-Edman procedure and Il-Val was resulted in this peptide. DNS-T_2 also coincided with authentic dansyl-Ile-Val by co-chromatography.

From these results, the structure of this hornet-kinin was deduced as follows:

Ala–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–Ile–Val
(Vespakinin-X)

The peptide is given a name "Vespakinin-X" based upon species of *Vespa xanthoptera*.

We have identified four vasoactive principles, histamine, serotonin, vespakinin-X and a trace of amount of acetyl choline-like substance in the venom of *Vespa xanthoptera*. The amounts of these materials in one venom sac were summerized in Table I. Comparing with the similar size of wasp (*Polistes rothneyi*) as shownin the Table, serotonin was contained more than ten times higher in the amount in this hornet. This may cause a severe pain by the hornet sting.

The other pharmacological activities and toxic properties of different species of the hornet venom have also been reported. The venom of oriental hornet (*Vespa orientalis*) effects on muscle transverse tubular system and muscle mitochondria of guinea pig[10]; shows haemolytic properties[3]; and changes the electrical activity of the cat brain.[11] The venom of *Vespa mandarinia* blocks neuromuscular junction of walking leg of lobster.[12] It is not yet clear whether these effects may result by an action of the single component or the multiples. The venom of *Vespa xanthoptera* also contained the unidentified haemolytic factor

10) J. Ishay, Y. Lass, and U. Sandbank, *Toxicon*, **13**, 57 (1975).
11) J. Ishay, Y. Lass, D. Ben-Shachar, S. Gitter, and U. Sandbank, *Toxicon*, **12**, 159 (1974).
12) N. Kawai and S. Hori, *Toxicon*, **13**, 103 (1975).

and some proteases. We could not characterize of these materials in this experiment and more hornets will be necessary for their elucidation.

Vespakinin-X is a new type of bradykinin analogue which differs from the other analogous peptides ever isolated in the hymenopteran venoms and elongates amino acid residue/s both in the N- and C-termini of the bradykinin molecule. Although the contractile activity of vespakinin-X on the rat uterus is less active than that of bradykinin, further pharmacological studies may reveal the other properties of this peptide.