Skin reactive substances from *Tabanus iyoensis* SHIRAKI

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INTRODUCTION

A great number of horse-flies, *Tabanus iyoensis* SHIRAKI, grows every summer in the mountainous area of 300 to 600 m altitude in Toyama Prefecture (Kamimura *et al.*, 1969, 1972). Stock-farming industries in this area are severely interfered because of the blood-sucking damage of the horse-flies to men and domestic animals.

The authors took notice of the strong skin reaction of the host after blood-sucking and aimed at clarifying active substances and elucidating the mechanism of the reaction (Kodama *et al.*, 1970 a, b). Crude extracts were prepared from female bodies, fractionated by column chromatography of various types of Sephadex and ion-exchange Sephadex, and the distribution of active substances was observed. Rabbits were immunized with fractions active after Sephadex G-25 gel filtration and the relationship between the skin reactivity and the immunological reactivity in gel diffusion was discussed.

MATERIALS AND METHODS

Body of horse-flies: Horse-flies were collected by a dry-ice light trap method in the middle of August, their peak period. Both live (feeding at 26°C in a chamber of 90 % relative humidity) and stored (−65°C until use) bodies were utilized. They were dissected into 3 parts, namely, head, thorax and abdomen.

Extraction: Ten to 40 ml of phosphate buffered saline solution, pH 7.0 with 0.01 M thioglycollate, was added to 300 to 1,200 bodies. After mashing, filtration through 2

layers of gauze, and centrifugation at 5,000 g for 30 min., the supernatants were stored at 4°C for 48 hrs. A significant amount of white precipitates formed during the storage was centrifuged off at the same speed. The supernatant thus obtained was used as crude extracts.

Determination of activity: 1) Skin reaction. Normal and sensitized (blood-sucked at the field 3 weeks before injection) rabbits and guinea pigs were injected i.c. with 0.1 ml (1 to 2 mg protein) of the crude extract. Toxicity to mice were observed after 0.2 ml (0.05 ml to new-borns) injection i.v. of the same extracts. One-hundredth ml of the crude or fractionated extract was injected i.c. to each volunteer (members of our institute) more or less seemed to have been sensitized during the horse-fly collecting work. 2) Activity *in vitro*. a) Hemolysis and hemagglutination. Two fold serial dilutions starting from the crude extracts were made with PBS, pH 7.0, and the hemolytic or hemagglutinating activity was determined using equal volume of 2 % human or sheep red blood cell suspensions. The maximum dilution where complete hemolysis or visible hemagglutination occurred was taken as an end point. b) Anti-coagulating activity. One tenth volume of the crude extracts was added to fresh human or sheep blood and the time required for coagulation was compared with the corresponding control at room temperature.

Gel filtration: Sephadex G-25, 50, 75, 100 and 200 were used. Two by 38 cm columns were employed for the former 3 types and 2.5 by 45 cm columns for the latter 2. Five to 15 ml volume of samples was applied at the speed of 15 to 30 ml per hr. in PBS, pH

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7.0. The coarser the bridge building of the Sephadex, the smaller the sample volume and the slower the elution speed. The void volume was experimentally determined before each run using 0.1% blue dextran solution. Every 5 ml effluent was collected, their absorbancy at 260 and 280 m μ was determined and the skin reactivity was qualitatively observed at each peak.

Rabbit antisera: The crude extracts from thorax and abdomen were fractionated by Sephadex G-25 gel filtration and the first peak having the skin reactivity was collected, concentrated to one-third of its original volume (C protein conc. 2 to 4 %) and used for immunization. Rabbits were injected i.v. 3 consecutive days for 8 weeks (1 ml each without adjuvant), and exsanguinated 5 days after the last injection.

Agar gel diffusion: Two ml of 1 % agar solution (Difco Special Agar, Noble, with 1/100 volume of 1 % merthiolate and several drops of saturated methyl orange solution per 100 ml agar) was applied to the ordinary microscope slides. The antigen and the

antibody wells, each 2 mm in diameter with the distance of 5 mm each other, made by a hexagonal template, were filled with the crude or fractionated extracts and rabbit antisera (0.025 ml each), and the reaction was carried out at 4°C for 1 to 3 days.

Ion-exchange Sephadex chromatography: Fifteen to 18 ml aliquots of the crude extracts were first fractionated with Sephadex G-25. The first peak was pooled, concentrated to one half of its original volume, dialyzed against Tris-HCl buffer solution, pH 7.8, and applied to a DEAE Sephadex A-50 column (1.4 by 20 cm) previously equilibrated with the same buffer solution. A stepwise elution was carried out with an increasing concentration of NaCl in the buffer. Every 5 ml effluent was collected and measurements similar to those in gel filtration were conducted.

RESULTS

Various properties of the crude extracts from each part of the body were given in Table 1. Each, having a characteristic color,

Table 1 Various properties of crude extracts

	Head	Thorax	Abdomen
Weight (300)	1.2 g (5ml)	6.4 g (15ml)	3.5 g (10ml)
pH immediate	6.0	6.2	6.2
after 48 hrs at 4°C	5.8	5.4	5.0
Color	purple	pink	yellow
Activity <i>in vivo</i>			
Human 0.01 ml i.c.			
immediate*	+	##	##
delayed**	±	##	##
after 100°C, 10 min		±	±
acetone precipitates		##	##
Rabbit 0.1 ml i.c.	—	±	±
Guinea pig 0.1 ml i.c.	—	±	±
Mouse 0.2 ml i.p.	—	—	—
New-born mouse 0.05 ml i.p.	—	—	—
Activity <i>in vitro</i>			
Anticoagulation (human blood)	—	—	—
Hemagglutination (human, sheep)	—	—	—
Hemolysis (sheep)	—	+ (×4)	++ (×32)

* Localized white wheal, immediately after injection

** Diffused reddening and inflammation, delayed after injection

showed the pH value of 6.0 to 6.2 immediately after extraction. The pH of the thorax or the abdomen extracts further decreased after the storage at 4°C for 48 hrs. These two preparations exerted a strong human skin reaction, *i.e.* immediate swelling and delayed reddening and inflammation. The activity was destroyed after heating at 100°C for 10 min. and transferred to the precipitates by a cold acetone treatment. These extracts, however, were not quite skin reactive to rabbits and guinea pigs, nor toxic to mice. As to the activities *in vitro*, different degrees of hemolysis were observed with the abdomen and thorax extracts respectively, while anti-coagulating or hemag-

glutinating activity was not detectable. The head extracts exerting only a faint human skin reaction were discarded thereafter.

Sephadex fractionation patterns of the crude extracts were shown in Fig. 1. The human skin reactivity was observed always at the first peak in the fractionation by Sephadex G-25, 50 or 75. The active zone was diffused toward the right shoulder of the first peak by the G-100 fractionation and almost separated into 2 zones by the G-200, although the boundary was not clear. Neither a pink-colored substance in the thorax extracts having an absorption maximum at 410 m μ , nor a hemolytic substance observed in the abdomen extracts was related

Fig. 1 Fractionation of crude extracts by various types of Sephadex
 — 280 m μ , 260 m μ , - · - · - 410 m μ

Fig. 2 Schematic diagram of the distribution of skin reactivity in gel filtration
I, II and III correspond to Peak I, II and III in Fig. 1.

to the skin reactivity, since the former was transferred to the second peak by the G-50 fractionation and the latter in the second peak by the G-25 fractionation. A schematic diagram of the distribution of skin reactivity was shown in Fig. 2, where activities by larger molecule substances (L) and those by smaller ones (S) were, for convenience, distinguished.

The agar gel precipitation reaction between the crude extracts and their fractions after the gel filtration and rabbit antisera were schematically shown in Fig. 3. Two lines were formed between the abdomen or the thorax extracts and their corresponding antisera. Cross reactions were also observed, although there seemed to be quantitative differences. One of those lines, observed in the abdomen extracts and located toward the antigen side, was so faint that it could not be followed after the Sephadex fractionation. The other, being the main line and located toward the antibody side, was detected in Peak I after the G-50, in-between Peak I and II after the G-75, and at the left shoulder of Peak II after the G-200 fractionations respectively (Fig. 3 B, C and D).

In order to detect more sensitively the distribution of antigenicity in the agar gel precipitation after Sephadex fractionations, the original antisera were absorbed with various peaks after the Sephadex fractionation, and the absorbed antisera were subjected

to react with the corresponding crude extracts. Results with the abdomen extracts were shown in Fig. 3 E. The faint line observed between the crude extracts and the corresponding antiserum disappeared when the antiserum was absorbed with Peak I (tube No. 14 and 16) in the G-200 fractionation. On the contrary, the main line did so when the antiserum was absorbed with the left side of Peak II (tube No. 31).

The ion-exchange Sephadex chromatography of the active fraction (Peak I) after Sephadex G-25 gel filtration was shown in Fig. 4. The activity *in vivo* of each fraction was examined in the volunteers after each fraction was dialyzed against the initial buffer solution used in equilibrating the column and appropriately diluted (OD at 280 $m\mu$ = 0.2–0.3). The skin reactivity was widely distributed in 5 fractions, *i.e.* from the first unadsorbed fraction to the fifth fraction eluted with 0.5 M NaCl. Similar results were obtained with both the thorax and the abdomen extracts.

DISCUSSION

The mechanisms by which a strong human skin reaction is elicited after the blood-sucking by horse-flies are unknown. The authors attempted to extract active substances from the adult female of *Tabanus iyoensis* and pointed out that these substances

Fig. 3 Agar gel precipitation between Crude extracts and their fractions and corresponding rabbit Antisera

Note: A. Crude extracts vs. Antisera

Peripheral well: abdomen (A) and thorax (T) extracts.

Central well: antisera against abdomen (a) and thorax (t).

B. Fractions in G-50 vs. Antisera

Peripheral well: left, abdomen Peak I (tube No. 12), Peak II (18), Peak III (29) and original (0); right, thorax Peak I (13), Peak II (17), Peak III (31) and original (0).

Central well: as above.

C. Fractions in G-75 vs. Antisera

Peripheral well: left, abdomen Peak I (11), between Peak I and II (16), Peak II (22), Peak III (31) and original (0); right, thorax Peak I (11), between Peak I and II (16), Peak II (21), Peak III (31) and original (0). Central well: as above.

D. Fractions in G-200 vs. Antisera

Peripheral well: left, abdomen Peak I (15), left side of Peak I' (20), right side of Peak I' (24), Peak II (32) and original (0); right, thorax Peak I (15), left side of Peak I' (20), right side of Peak I' (20), Peak II (32) and original (0). Central well: as above.

E. Antisera absorbed with various G-200 fractions of abdomen vs. Crude abdomen extracts

Peripheral well: antisera absorbed with Peak I (14 and 16), with left side of Peak I' (20), with right side of Peak I' (25), with left side of Peak II (31), with right side of Peak II (39) and unabsorbed control (0). Central well: crude abdomen extracts.

Fig. 4 Fractionation of peak I in Sephadex G-25 gel filtration by DEAE Sephadex A-50
 — 280 $m\mu$, - - - 260 $m\mu$

could be obtained both in the thorax and in the abdomen, not only from the living bodies but also from the frozen ones (Kodama *et al.*, 1970a). Anatomically, salivary glands of this species extend over the thorax and the abdomen.

The fact that the activity *in vivo* (human skin reaction) was destroyed by heating and transferred to the precipitates by a cold acetone treatment, and that it was found in the first peak by the Sephadex G-25 gel filtration where the absorbancy at 280 $m\mu$ was slightly higher than that at 260 $m\mu$ suggest that the main role exerting the activity *in vivo* may be played by large molecule proteins. The results of various gel filtrations indicate that the active substances are divided into 2 categories in connection with the molecular weight, one having a molecular weight of more than 200,000, and the other in the range from 50,000 to 100,000, although a clear-cut border line could not be drawn between the two.

Purification by ion-exchange chromatography revealed that the activity was detected in several fractions both in the thorax and

in the abdomen extracts. The distribution of the activity during such purification process may lead to a conclusion that several substances, rather than a single one, are related to the human skin reaction.

Mckiel and Clunie (1960) found 4 water-soluble, non-dialyzable components in extracts of whole male and female *Aedes aegypti* which caused skin reactions in men. Sekigawa and Omori (1970) reported that crude extracts prepared from larvae of 3 horse-fly species exerted skin reactions to men as well as hemolytic and proteolytic activities. They also disclosed that 7 to 9 bands were distinguishable in the crude extracts by the disc electrophoresis, although whether or not all those bands were related to the skin reactions was unknown.

It may be attributed to the unsatisfactory sensitization that the authors could not observe distinct skin reactions in rabbits and guinea pigs. However, even those rabbits during the course of immunization with the crude extracts did not exhibit distinct skin reactions.

To characterize immunologically above

described skin reactive substances, the relationship between the skin reactivity and the antigenicity in the gel diffusion was examined using rabbit antisera. The antigens responsible for the main line observed between the crude abdomen extracts and the corresponding antiserum, seemed to be unrelated to the skin reaction, since they were separated from the skin reactive fraction after the Sephadex G-75 gel filtration. Experiments with the absorbed antisera also supported the difference in the distribution between the skin reactivity and the antigenicity after various gel filtrations. The other antigens responsible for the faint line observed in the crude extracts, which could be followed only in the experiments with the absorbed antisera, behaved similarly to the larger molecule skin reactive substances in various gel filtrations. The relationship between the two, therefore could not be denied.

In regard to the activities *in vitro*, the anticoagulating and the erythrocyte agglutinating activities which were first reported by Metcalf (1945) in *Anopheles quadrimaculatus* could not be detected in the crude extracts. However, a fairly strong hemolytic activity was observed in the abdomen extracts. This hemolytic activity was not related to the skin reaction, since it was located in the second peak after the Sephadex G-25 gel filtration where no skin reactivity was observed. Sekigawa and Omori (1970) also described a hemolytic activity in the extracts from larvae of *Tabanus chrysurus* and claimed that the hemolytic activity as well as the skin reactivity was detected in the second peak after the Sephadex G-25 gel filtration. This difference in the distribution of skin reactivity after the gel filtration may be attributable to the difference in age or in species, although strict comparison could not be made because of the difference in the detailed experimental conditions.

Benjamini *et al.* (1960) using the oral secretion of fleas, and Hudson *et al.* (1958) using mosquito extracts, described that the immediate and delayed reactions to the insect bites were allergic in nature. This may be applied also to the horse-flies. The fact that the crude extracts themselves were not very toxic to mice nor to the new-born ones, and

that both the immediate and the delayed reactions were observed in appropriate volunteers supports the concept.

SUMMARY

This paper describes the characterization of the skin reactive substances from the horse-fly, *Tabanus iyoensis*.

1. Strongly skin reactive substances were extracted from the thorax and the abdomen of adult female bodies. Activities both from the thorax and the abdomen distributed similarly in the further fractionation process, *i.e.* the gel filtration by various types of Sephadex and the chromatography by ion-exchange Sephadex.

2. The active substances seemed to be non-dialyzable proteins which, in respect to the molecular weight, were subdivided into 2 categories, one having the M. W. of more than 200,000 and the other the M. W. of 50,000 to 100,000. This, with the results of the ion-exchange chromatography, suggests that several substances rather than a single one, which are not very toxic themselves, are related to the human skin reaction.

3. The agar gel diffusion between the abdomen extracts and the corresponding rabbit antiserum (an active fraction after the G-25 gel filtration was used for immunization) revealed at least 2 lines, of which the main line located toward the antibody side seemed to be unrelated to the skin reaction. In the experiments with the absorbed antiserum, however, the other line disappeared when the skin reactivity (activity L) was present in the fraction used for the absorption, indicating that there may be a close connection between them.

4. Neither a hemolytic substance observed in the abdomen extracts nor a pink-colored substance in the thorax extracts having an absorption maximum at 410 m μ was related to the skin reactivity.

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摘 要

イヨシロオビアブ雌成虫から抽出した
皮膚反応活性物質について

本論文はイヨシロオビアブ雌成虫体から抽出した皮膚反応活性を示す物質について述べたものである。その概要は次のとおりである。

1. 虫体の胸部および腹部から、著しいヒト皮膚反応を起す物質を抽出したが、両活性物質は、各型のセファデックスによる分画およびイオン交換セファデックスによる精製過程において、同様の態度を示した。
2. これら活性物質は通常 $280\text{ m}\mu$ における吸収が $260\text{ m}\mu$ のそれよりやや高く、加熱あるいはアセトン処理などに対する態度から、蛋白質性のものと思われるが、各型のセファデックスによる分画では、分子の大きさという点で2大別される（分子量20万以上のものと5~10万の範囲のもの）。またイオン交換セファデックスによる精製では、5つの分画にわたって活性が分布していた。したがって、活性には虫体成分のある単一な物質というより、いくつかの成分が関与しているものと考えられる。
3. 粗抽出液はマウスおよび哺乳マウスに対して毒性を示さず、皮膚反応活性は、それ自身の毒性にもとづくものではなく、他の昆虫の刺咬の場合のように、アレルギー性のものと思われる。
4. 腹部粗抽出液と家兎免疫血清（腹部粗抽出液をG-25で分画した最初のピークを免疫原として使用）との間にはゲル内沈降反応において少くとも2本の沈降線が認められ、そのうちの主たる抗体側の線は皮膚反応と関係がないと思われたが、抗原側に見られる薄い線と皮膚反応活性の関連性については否定できなかった。
5. 粗抽出液中の溶血作用を示す物質および着色物質は、ヒト皮膚反応活性と無関係であった。