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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

A novel allergen Tab y 1 with inhibitory activity of platelet aggregation from salivary glands of horseflies

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aprase; basophil; CD63; horsefly allergen; insect allergy.

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The IUIS Allergen Nomenclature Sub-Committee (<http://www.allergen.org>) has assigned to the purified allergen (aprase) the official number Tab y 1.0101, and the suggested allergenicity results are listed under Tab y 1.

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Abstract

Background: Horsefly sting causes allergic reactions in human body. However, our knowledge on horsefly allergens remains poor.

Objectives: To identify the novel horsefly allergens and characterize their properties.

Methods: A native allergen protein Tab y 1 (aprase) was purified from the salivary glands of the horsefly *Tabanus yao* Macquart by gel filtration and ion exchange chromatography. Its sequence was determined by Edman degradation and cDNA cloning. Its allergenicity was assessed by immunoblotting for specific IgE, basophil activation test, skin prick test (SPT), and competitive enzyme-linked immunosorbent assay (ELISA).

Results: Tab y 1 showed a single diffusion band of 70 kDa on SDS-PAGE. Seventy percent (7/10) of patients with horsefly allergy tested positive to Tab y 1 in SPT; sera from 81% (30/37) of patients reacted to Tab y 1 on western blots. Purified Tab y 1 reduced approximately 42% sera IgE reactivity to horsefly salivary gland extract on a competitive ELISA. Tab y 1 upregulated the expression of CD63 and CCR3 on passively sensitized basophils by up to approximately 4.9-fold. Tab y 1 also showed enzymatic activity to hydrolyze ATP and ADP, and potent antiplatelet aggregation and antithrombotic activities.

Conclusion: The current work identified a novel major allergen of horsefly, Tab y 1, with antiplatelet aggregation and antithrombotic activities, which implicates Tab y 1 in helping horseflies suck host blood, meanwhile causing allergy in their human hosts.

Horseflies are potentially economically important blood-feeding arthropods. They rely heavily on the pharmacological propriety of their saliva to successfully obtain blood meal from their hosts. Many active compounds with anticoagulation, antiplatelet, anti-inflammatory, and immunosuppressant activities have been found from the salivary glands (SGs) of hematophagous arthropods (1–5). Recently, a wide range of

physiologically active molecules acting on blood and immune systems has been found in horsefly saliva (6, 7).

After the bites, anaphylaxis induced by insect venom is mostly caused by Hymenoptera stings, as well as by various blood-feeding arthropods such as horseflies (8, 9). Many allergens have been identified from the venoms of insects including bees, wasps, and ants (10). However, relatively little

advance has been made in recognizing the allergens of blood-feeding arthropods. Many cases of horsefly allergy with severe allergic reactions have been recorded following horseflies biting human beings, but they are underestimated by poor diagnostic means and understanding. Considering coexistent anaphylaxis to Diptera and Hymenoptera, concomitant sensitization to Hymenoptera venoms in subjects allergic to horseflies should be common, for example in the wasp-horsefly syndrome (9, 11).

To understand further the interactions between horseflies and their hosts, we purified and characterized a novel allergen (Tab y 1) with antiplatelet activity from the SG extracts of *Tabanus yao* and investigated its allergenicity in this study.

Material and methods

Study population

This project was approved by the faculty committee on the use of human subjects in research in Kunming Institute of Zoology, Chinese Academy of Sciences, and the participants gave written informed consent before beginning the study. As reported in our previous work, sera were obtained from 37 patients with horsefly allergy and 12 controls who tested negative to horsefly bite tests (11).

Preparation of horsefly and salivary gland extract (SGE)

Preparation of SGE and collection of horseflies of *T. yao* were performed according to our previously reported method (6, 7).

Purification of apyrase from the SGE

The apyrase (Tab y 1) was purified from the SGE of *T. yao* as described previously (7). The purity was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

Sequence analysis

The amino acid sequences of the N-terminus and partial interior amino acid fragments recovered from the trypsin hydrolysis were determined by the automated Edman degradation on a pulsed liquid-phase sequencer (Applied Biosystems, Model 491, Foster City, CA, USA). A matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) AXIMA CFR (Kratos Analytical, Manchester, UK) in a positive ion and liner mode was employed to analyze the mass fingerprints, and α -cyano-4-hydroxycinnamic acid was used as the matrix (6, 7).

cDNA cloning

cDNA library of horsefly salivary glands was constructed as reported previously (6, 7). A SMART™ PCR cDNA synthe-

sis kit (Clontech, Palo Alto, CA, USA) was used to synthesize the cDNA. A directional cDNA library was constructed using a plasmid cloning kit (SuperScript™ Plasmid System, GIBCO/BRL, Carlsbad, CA, USA) following the manufacturer's instructions, producing a library of about 2.3×10^5 independent colonies. PCR-based method for high stringency screening of DNA libraries was used to screen and isolate the clones. Two pairs of primers were used. The first pair was NTAPY-F (sense): 5'-aatgaaggcgtgggaggagtt-3' and CDS III-primer (antisense): 5'-attctagaggccgaggcggcatg-3'; and the second pair was 5'PCR-primer (sense): 5'-aagcagtgtgatcaacgcagagt-3' and NTAPY-R (antisense): 5'-ctctcccacgccttcattt-3'. The DNA polymerase was Advantage polymerase from Clontech. The PCR conditions were as follows: 2 min at 94°C, followed by 30 cycles of 10 s at 92°C, 30 s at 57°C (for the first pair of primers) or 44.5°C (for the second pair of primers), 10 s at 72°C. DNA sequencing was performed on a DNA sequencer, model ABI PRISM 377 (Applied Biosystems).

Apyrase assay

Unless otherwise indicated, samples (30 μ l) were incubated with reaction buffer (50 μ l) containing 100 mM Tris, 10 mM KCl, 450 mM sucrose, 20 mM glucose, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 7.5, and 20 μ l of 5 mM ATP, ADP, or AMP at 37°C for 20 min. The released inorganic phosphate (Pi) was quantified according to reported method (12).

Inhibition of platelet aggregation induced by ADP

Inhibition of platelet aggregation induced by ADP was determined according to the reported method (13).

Antithrombotic study by rat arteriovenous shunt thrombosis (RAST) model

RAST model was used to evaluate the antithrombotic activity as previously described (14).

Antithrombotic study by carrageenan-induced mouse tail thrombosis (MTT) model

To test antithrombotic function *in vivo*, a modified carrageenan-induced MTT model was used (15). All experimental protocols for the use of animals were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences.

Immunoblot analysis of IgE reactivity

Immunoblots for the detection of serum-specific IgE were performed using purified Tab y 1 as previously described (11).

Enzyme-linked immunosorbent assay (ELISA)

A 96-well plate was coated with purified Tab y 1 at 10 μ g/ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6) overnight

at 4°C, 100 µl per well. Human serum samples (1 : 20 dilution in PBS–Tween with 2% BSA) were then added to the plates and incubated at room temperature for 2 h. After IgE binding, plates were incubated with horseradish peroxidase–labeled goat anti-human IgE (1 : 2500 dilution), and the color was developed with tetramethylbenzidine peroxidase substrate. The plates were read on a microplate reader at absorbance of 405 nm. Two patients (10 and 11), whose sera recognized Tab y 1, were chosen for ELISA inhibition assay according to Ma et al. (11).

Skin prick testing (SPT)

For the skin prick testing, no patients were taking medications (antihistamines, steroids, and other drugs) for at least 2 weeks. They have no dermatographia or active skin disorders. The tests were carried out according to standard methods with allergens. The skin prick reaction was read at 15 min. The reaction wheal with diameter at least 3 mm larger than the negative control is considered positive.

Basophil activation test (BAT)

Expression of CD63 and CCR3 on basophil surface has been considered as the indicator of basophil activation (16, 17). We examined the influence of Tab y 1 on basophil activation. Briefly, peripheral blood mononucleated cells (PBMC) isolated from 20 ml blood donated by four allergic donors were

separated by Ficoll-Paque density gradient and treated with 10 ml LS (1.3 M NaCl, 0.005 M KCl, and 0.01 lactic acid, pH 3.9) for 2 min at 8°C. After neutralization with 12% Tris (pH 10.9), nonspecific IgEs on basophils were stripped off and cells from each donor were passively sensitized with sera from six different patients with horsefly allergy (1 in 10 dilution, 2 h at 37°C) and from six different healthy subjects as described by Pruzansky et al. (17), which means that the passive sensitization experiment was repeated six times and each time uses the serum from a different subject. The cells were then challenged with various concentrations of Tab y 1 for 15 min at 37°C. A goat anti-human IgE antibody (Serotec, Kidlington, UK) was used as a positive control. Anti-human CCR3-PE antibody (eBioscience Inc., San Diego, CA, USA) and anti-human CD63-FITC antibody (Invitrogen corporation, Camarillo, CA, USA) were added to cells and incubated at 37°C for 15 min. Flow cytometry analysis of surface markers was carried out at 488 nm on a FACSaria flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed by FACSDIVA software.

Statistics

Statistical significance between means was analyzed by one-way ANOVA or the Student's *t*-test using the SPSS 13.0 version. Data for direct ELISA are presented as scatter plot. Where Kruskal–Wallis analysis indicated significant differences between groups, for the preplanned comparisons of interest,

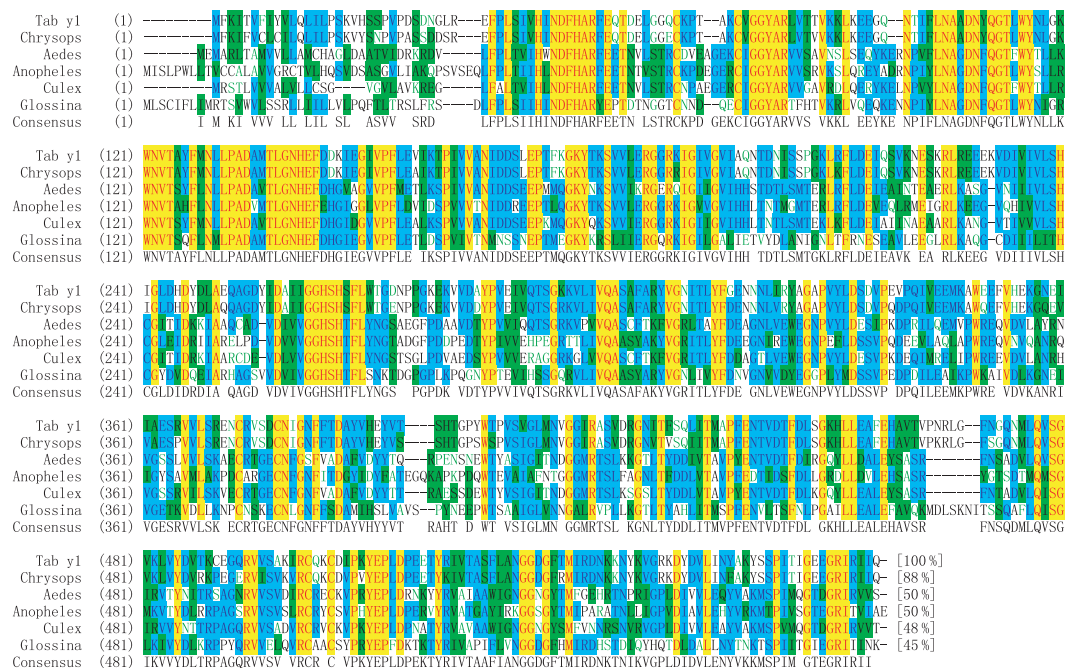


Figure 1 Alignment of Tab y 1 precursor with other apyrases of dipteran insect. Deduced amino acid sequence of Tab y 1 is aligned with venom allergen of other dipteran insects. Tab y 1 was cloned and sequenced in the present study. Chrysops is chrysopsin (34) from deerfly. Aedes is *Aedes aegypti* (23). Anopheles is *Anopheles*

gambiae (35). Culex is *Culex quinquefasciatus* (GenBank: XP_001858188). Glossina is *Glossina morsitans morsitans* (26). The identical residues are marked with Consensus. The percentage identity of each sequence is given in parentheses.

the paired Mann–Whitney *U*-test was employed. $P < 0.05$ was taken as statistically significant.

Results

Primary structure of Tab y 1

Following the isolation of substantial quantity of Tab y 1 (10 mg) from the SGE of the horsefly using the procedure reported previously (11), the structure and functions of Tab y 1 were investigated. After being digested by trypsin, the recovered peptide fragments were subjected to Edman degradation and MS/MS analysis. Amino acid sequences of six peptide fragments were determined. They are TPIVVANIDD-SLEPTFK, HLLEAFEHAVTVPK, IGVGVIAQNTDNISSPG, LKEEGQNTIFLNAADNYQGTLWYNLGK, YSSPITIGEEGR, and YVGNITLYFGENNNLIR. These sequences were found to share high similarity with those found in apyrases from blood-feeding insects. According to the determined amino acid sequences, two primers (NTAPY-F and NTAPY-R) were designed to screen cDNAs encoding Tab y 1 from the cDNA library. As illustrated in Fig. 1, the precursor encoding Tab y 1 is composed of 554 amino acid residues. The deduced sequence contains identical amino acid sequences to six peptide fragments as mentioned earlier. BLAST search reveals that Tab y 1 shares high similarity (45–88%) with other apyrases of blood-feed-

ing insects. It shows highest similarity (88%) with the apyrase from the deerfly, *Chrysops* sp.

Inhibition of platelet aggregation induced by ADP

Effect of Tab y 1 on platelet aggregation induced by ADP is illustrated in Fig. 2A. At the concentration of 230 and 460 ng/ml, Tab y 1 inhibited 80 and 100% of platelet aggregation induced by ADP, respectively.

Antithrombotic activity

Using ozagrel as the positive control, the antithrombotic activity of Tab y 1 in the RAST model was evaluated (Table S1). Tab y 1 and ozagrel significantly inhibited thrombosis in a dose-dependent manner. At the dose of 0.6 mg/kg BW, Tab y 1 reduced 31.6% of protein weight in the thrombus, while the clinical antiplatelet agent ozagrel could exert similar antithrombotic activities at high doses (25 and 50 mg/kg BW). Tab y 1 also inhibited carrageenan-induced thrombosis in mouse tails in a dose-dependent manner (Fig. 2B). Up to approximately 70% of carrageenan-induced thrombosis in black tail was inhibited by 0.6 mg/kg BW of Tab y 1 at 48 h following injection.

Immunoreactivity to IgE

To determine the allergenicity of Tab y 1, we first examined the ability of Tab y 1 to bind IgE in the sera of the patients with horsefly allergy, by immunoblotting technique. The results showed that sera from 30 of 37 (81%) patients reacted to Tab y 1. IgE-binding activity of Tab y 1 in a representative group of 10 patients and two controls is illustrated in Fig. 3A. Most of IgE-binding bands were moderately positive with a molecular weight around 70 kDa (Tab y 1) for the patients, but completely negative in the control subjects. The specific immunoreactivity of IgE against purified Tab y 1 was confirmed by a direct ELISA (Fig. 3B). In comparison with the sera from the Tab y 1-negative patients and healthy control subjects, the IgE reactivity of Tab y 1 in the sera from the Tab y 1-positive patients increased by 4.6- and 5.4-folds, respectively.

Competitive ELISA

For ELISA inhibition assays, two patients (10 and 11), whose sera recognized Tab y 1, were chosen. Approximately up to 42% inhibition of IgE binding to the coated SGE (Fig. 4) was observed when the sera were preincubated with Tab y 1 for 1 h. In the same experiment, the horsefly SGE completely inhibited serum IgE binding to the coated SGE.

Skin prick testing

Seven of the 10 patients (70%) showed positive reactions to Tab y 1. The representatives of SPTs are illustrated in Fig. S1.

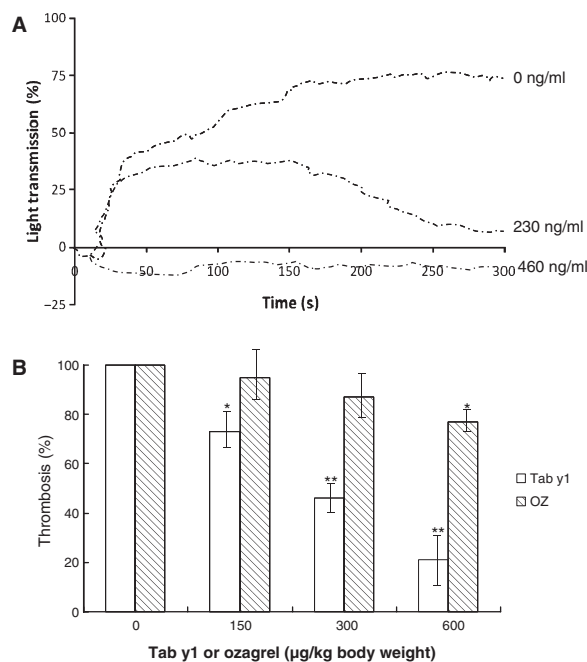


Figure 2 (A) Inhibition of ADP-induced platelet aggregation by Tab y 1. Human platelet-rich plasma (2×10^5 /ml) was incubated with Tab y 1 (230 and 460 ng/ml) for 1 min before the addition of ADP. The tracings represent a typical experiment. (B) Examination of anti-thrombotic activities of Tab y 1 *in vivo*. Tab y 1 and ozagrel inhibited carrageenan-induced thrombosis in mouse tails. * $P < 0.05$, ** $P < 0.01$ in comparison with the corresponding uninhibited control. OZ: ozagrel.

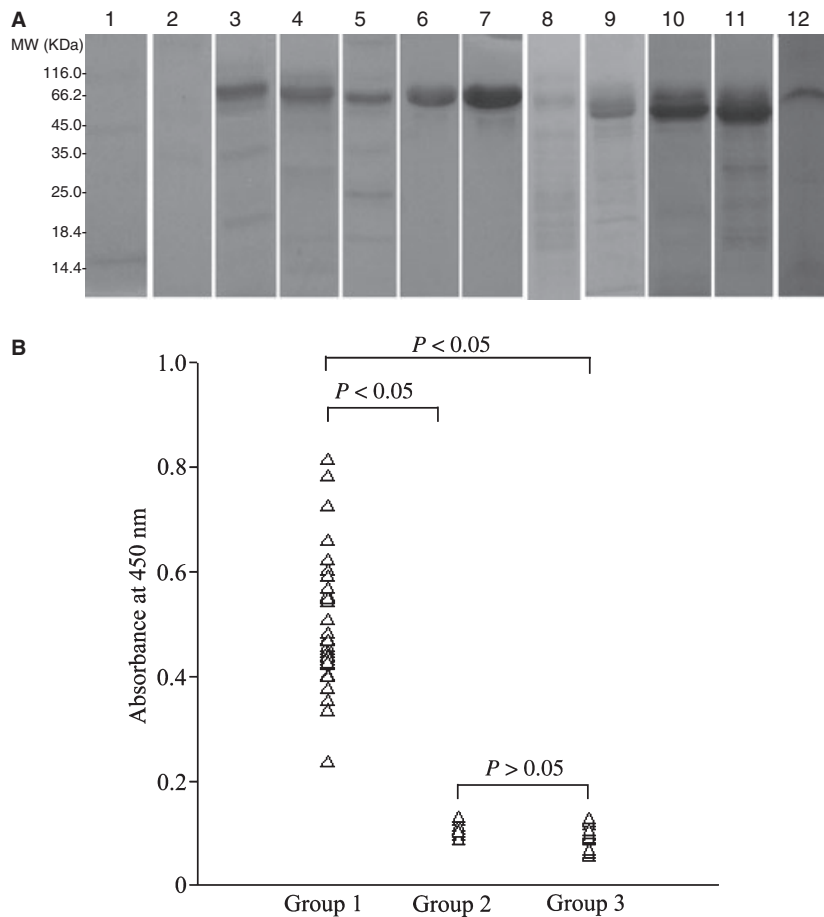


Figure 3 (A) Western blot analysis of IgE reactivity to Tab γ 1 in the sera from the patients with horsefly allergy. Lanes 1 and 2, the serum from healthy control; lanes 3–12, the serum from the patient. (B) Analysis of specific IgE reactivity of purified Tab γ 1 by

direct ELISA. The sera were collected from the patients with horsefly allergy and healthy control subjects. Group 1: the sera from Tab γ 1-positive patients; group 2: the sera from Tab γ 1-negative patients; group 3: the sera from healthy control subjects.

Tab γ 1-induced basophil activation

Throughout the experiment, 1.0 $\mu\text{g}/\text{ml}$ of Tab γ 1 was chosen as the optimal dose (Fig. S2). Tab γ 1 at 1.0 $\mu\text{g}/\text{ml}$ induced approximately up to 4.9- and 1.6-fold increase in CD63 and CCR3 double-positive cells following incubating Tab γ 1 with passively sensitized basophils from sera of the patients with horsefly allergy or from healthy subjects, respectively (Fig. 5).

Discussion

Although the fact that horsefly sting can induce allergic reactions has been widely accepted, little is known of major allergens from horseflies. Recently, we have purified and characterized two allergens (Ag5 protein and hyaluronidase) from the salivary glands of the horsefly (11). Since then, we have collected a large amount of horsefly of *T. yao*, which makes it possible to purify and characterize large quantity of native allergens. Here, a 70-kDa apyrase, Tab γ 1, from the

horsefly SGE was purified and characterized for the first time. It possesses platelet aggregation inhibitor and allergen activities.

Apart from strong platelet aggregation inhibition activity, Tab γ 1 showed potential antithrombotic activity as assessed by RAST and MTT models. ATP and ADP released from injured cells and activated platelets play key roles in the formation of primary hemostatic plug. They interact with purinergic P2 receptors to activate and aggregate platelets and strongly contribute to the amplification of the initial hemostatic response (18, 19). Salivary glands of blood-sucking insects have ATP/ADP-hydrolyzing enzymes (20). These enzymes are often called apyrases. Several apyrases have been identified from the saliva of a variety of blood-feeding arthropods such as bugs, ticks, mosquitoes, sand flies, reduviids, and tsetse flies (21–26). These blood-sucking arthropods can overcome ATP- and ADP-related host responses to successfully obtain blood meal using apyrases. We anticipate that the horsefly can also obtain its blood meal using antiplatelet aggregation and antithrombotic activities of Tab γ 1.

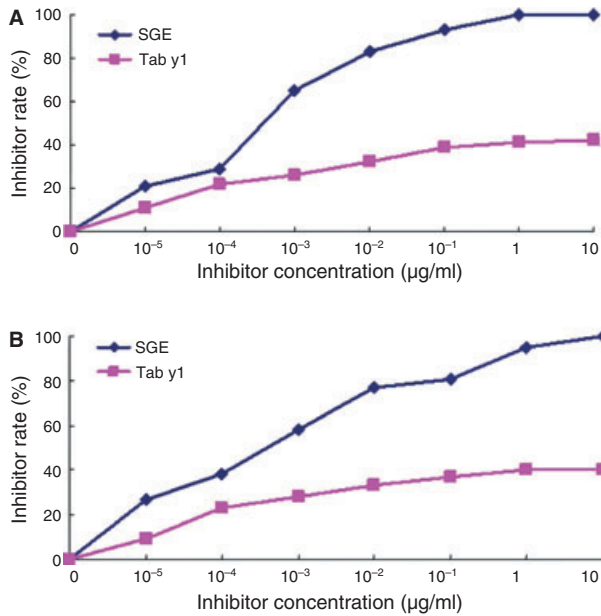


Figure 4 Examination of serum IgE reactivity to Tab y 1 by competitive ELISA. (A) and (B) represent the serum from the horsefly allergy patients 10 and 11, respectively. Inhibitor concentrations ranged from 0 to 10 μg/ml. SGE: salivary gland extracts of the horsefly *Tabanus yao*.

Two families of allergens including Ag5 proteins and hyaluronidases from insects have been extensively studied. Several reports have indicated that some insect venom Ag5 proteins, such as those from hornets, wasps, and fire ants, are allergens (11, 27, 28). Hyaluronidases are well-known venoms of Hymenoptera and represent clinically important allergens (29, 30). No native apyrase as an allergen has been reported before. The recombinant apyrase allergen is mosquito allergen Aed a 1 (31). Therefore, investigation into the allergenicity of Tab y 1 is primary in identifying native apyrase as an allergen. Using western blotting and SPT techniques, we proved that Tab y 1 is a major allergen of horsefly of *T. yao*, which reacted to sera from 81% of patients with horsefly allergy. However, Tab y 1 is not the only allergen in SGE, as it can only abolish up to 42% of binding capacity of serum IgE to the coated SGE, whereas SGE completely blocked the immunological reaction between the two in the competitive ELISA. The ability of Tab y 1 in activating horsefly-allergy-serum-sensitized basophils further indicates that this enzyme from horsefly possesses allergen activity.

Antagonistic host–parasite interactions lead to coevolution of host defenses and parasite virulence (32). Common means of avoiding or reducing parasitism include behavioral, mechanical, and physiological defenses (33). Horseflies develop many pharmacological or ‘functional’ compounds including apyrases in horsefly saliva to successfully obtain blood meal from their hosts. Apyrases interfere hemostasis system of hosts by inhibiting platelet aggregation. Hosts are

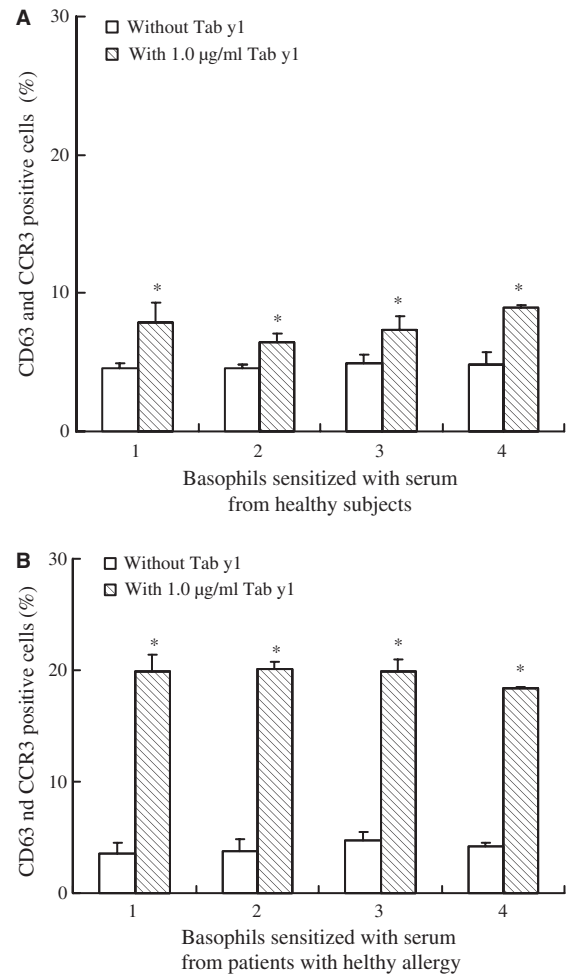


Figure 5 Induction of basophil activation by Tab y 1. Cells were obtained from four allergic individuals, each presented as a group of two columns. After nonspecific IgEs on basophils being stripped off, cells from each donor were passively sensitized with sera from six different healthy subjects (A) or from six different patients with horsefly allergy (B) and were then challenged with Tab y 1 at 1.0 μg/ml. The values shown are mean ± SE for the sera from six different subjects. * $P < 0.05$ in comparison with the corresponding carrier alone control.

not passive victims of horseflies but coevolve with them by developing efficient defenses that limit, reduce, or eliminate the damage caused by horseflies. To respond to the pressure of blood-sucking parasites, hosts develop multiple antiparasitic mechanisms including immune, hemostasis, inflammatory, and angiogenic responses. One effective defensive mechanism may be irritation to the parasite’s ‘functional’ compounds and induction of antibodies. In the current case, the antiplatelet Tab y 1 is found to be a major allergen to human being, which implies that horsefly-induced antibody production in host may not only neutralize or eliminate the antihemostasis effect of horsefly saliva, but also cause damages to the host body.

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Author contribution

R. L. and S. H. designed the project, supervised the experiments, and wrote the manuscript. Other authors performed the experiments and revised the manuscript.

Conflict of interest

The authors do not have any conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Figure S1. Skin prick test with Tab y 1.

Figure S2. Effect of Tab y 1 on expression of CD63 and CCR3 on basophils.

Table S1. Effect of Tab y 1 and ozagrel on thrombosis in rat arteriovenous shunt thrombosis model ($n = 9$).

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Figure legends for Supporting Information

Figure S1. Skin prick test with Tab y 1. Arrows indicate positive reactions induced by Tab y 1.

Figure S2. Effect of Tab y 1 on expression of CD63 and CCR3 on basophils. The values shown are mean $\pm$ SE for the sera from 4 different healthy subjects and from 4 different patients with horsefly allergy. Basophils were from one donor.

Table S1. Effect of Tab y 1 and ozagrel on thrombosis in rat arteriovenous shun thrombosis model (n = 9)

	Dosage (mg/kg body weight)	Protein weight in thrombus (g)	Inhibition rate (%)
Control		402±55	
	0.15	352±36 [*]	12.4
Tab y 1	0.3	305±61 ^{**}	24.1
	0.6	275±29 ^{**}	31.6
	12.5	337±52 [*]	16.2
Ozagrel	25	291±56 ^{**}	27.6
	50	266±35 ^{**}	33.8

Values shown are Mean ± SE for nine separate experiments. The animals were treated with various doses of Tab y 1, ozagrel or normal saline alone for 15 min before ADP being administrated. ^{*} $P < 0.05$, ^{**} $P < 0.01$ in comparison with the corresponding uninhibited control.

Figure S1



Figure S2

