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HALYSIN, AN ANTIPLATELET Arg-Gly-Asp-CONTAINING SNAKE VENOM PEPTIDE, AS FIBRINOGEN RECEPTOR ANTAGONIST

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Abstract—By means of Sephadex G-75 and CM-Sephadex C-50 column chromatography and reverse-phase HPLC, a low molecular weight ($M_r = 7500$), cysteine-rich peptide, halysin, was purified from *Agkistrodon halys* (mamushi) snake venom. Halysin is a potent platelet aggregation inhibitor that concentration-dependently inhibited human platelet aggregation stimulated by ADP, thrombin and collagen ($IC_{50} = 0.16$ to $0.36\ \mu M$) without affecting platelet secretion. It was active in inhibiting platelet aggregation of platelet-rich plasma and whole blood. Halysin had no effect on thromboxane B_2 formation of platelets or intracellular Ca^{2+} mobilization of Quin 2-AM loaded platelets stimulated by thrombin. It inhibited the fibrinogen-induced aggregation of elastase-treated platelets. Halysin concentration-dependently inhibited the ^{125}I-fibrinogen binding to ADP-stimulated platelets in a competitive manner ($IC_{50} = 0.16\ \mu M$). ^{125}I-Halysin bound to resting platelets ($K_d = 1.6 \times 10^{-7}$ M) and to ADP-stimulated platelets ($K_d = 3.4 \times 10^{-8}$ M) in a saturable manner. EDTA, the Arg-Gly-Asp (RGD)-containing snake venom peptides trigamin and rhodostomin, Arg-Gly-Asp-Ser (RGDS), and Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val blocked both ^{125}I-fibrinogen binding and ^{125}I-halysin binding to ADP-stimulated platelets. The monoclonal antibody, $7E_3$, raised against glycoprotein IIb–IIIa complex blocked both ^{125}I-fibrinogen and ^{125}I-halysin binding, whereas $10E_5$ had no significant effect on halysin binding to ADP-stimulated platelets, indicating that $7E_3$ and halysin bind to an epitope which is different from that of $10E_5$. RGDS concentration-dependently inhibited ^{125}I-halysin binding in a competitive manner. We determined the primary structure of halysin which is a single peptide chain of 71 amino acid residues. An RGD sequence appeared in the carboxy-terminal domain of halysin. Halysin showed about an 85% identical sequence with trigamin which is a specific antagonist of fibrinogen receptor associated with glycoprotein IIb–IIIa complex. In conclusion, halysin inhibited platelet aggregation by interfering with fibrinogen binding to the fibrinogen receptor of the activated platelets. The RGD sequence of halysin plays an important role in the expression of its biological activity.

The participation of platelets in the process of hemostasis is well established [1]. When a blood vessel is injured, platelets are activated, releasing biologically active substances, and aggregated. However, abnormal hyperaggregability of the platelets results in arterial thromboembolism [2, 3].

Upon being stimulated by agonists such as ADP [4, 5], epinephrine [6], thrombin [7] or prostaglandin endoperoxides [8, 9], platelets expose the latent fibrinogen receptors associated with glycoprotein IIb–IIIa complex, enabling the binding of fibrinogen to these receptors and subsequently leading to platelet aggregation [10, 11]. Thus far, there are two postulative binding domains of the fibrinogen molecule toward fibrinogen receptors, namely Arg-Gly-Asp (RGD) of the α chain and carboxy-terminal dodecapeptide of the γ chain. Attempts have been made to synthesize fibrinogen fragments, structurally mimicking these two peptides in order to exhibit greater antiplatelet activity. However, the synthetic peptides usually exhibit lower affinities toward fibrinogen receptor and thus are rather weak in inhibiting platelet aggregation [12–14]. On the other hand, the monoclonal antibodies raised against platelet glycoprotein IIb–IIIa complex have been shown to exhibit a potential antithrombotic activity in both *in vitro* and *in vivo* experiments [15–18]. However, the antigenicity of these monoclonal antibodies *in vivo*, especially in long-term use, is one drawback in terms of large molecular weight. Recently, we found that there are several antiplatelet components in hemorrhagic snake venoms [19]. Trigramin, an RGD-containing peptide from the venom of *Trimeresurus gramineus*, was demonstrated to inhibit platelet aggregation without affecting release reaction when platelets were stimulated by a variety of agonists, including ADP, epinephrine, thrombin, U46619, collagen and the ionophore A23187 [20–22]. Trigamin was shown to be a specific fibrinogen receptor antagonist with a very high binding affinity (K_d, 10^{-8} M), comparable to the monoclonal antibodies raised against glycoprotein IIb–IIIa complex. Owing to its relatively small molecular weight (7500 daltons) and its highly selective action, an investigation of the interaction between trigramin and glycoprotein IIb–IIIa complex would be very helpful not only in elucidating the interaction of fibrinogen and this complex, but also in designing antithrombotic peptides or related derivatives. Shortly after this finding, echistatin, a trigramin-like peptide, was reported [23]. Recently,

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several similar peptides have been isolated and characterized [24–27]. However, most of these reports deal with amino acid sequences and antiplatelet function. Few experiments were performed to see if the trigramin-like peptides had any effect on the intracellular events during platelet activation.

An earlier study of ours showed that a polypeptide purified from *Agkistrodon halys* venom, possessing phospholipase A activity, inhibits rabbit platelet aggregation stimulated by a variety of agonists with similar IC_{50} values [28]. In the present paper, we further purified this antiplatelet component, halysin, which is devoid of phospholipase A activity. We found that halysin is an RGD-containing single chain polypeptide of 71 amino acid residues, sharing an 85% identical sequence with that of trigramin. In addition, we investigated its mechanism of action in detail and characterized its binding properties toward human platelets in comparison with those of other trigramin-like polypeptides and monoclonal antibodies raised against platelet glycoprotein IIb–IIIa complex.

EXPERIMENTAL PROCEDURES

Materials. The lyophilized venom of *A. halys* was purchased from the Sigma Chemical Co. Apyrase, Quin 2-AM, luciferase–luciferin, human thrombin, bovine serum albumin, heparin, collagen (Type I, bovine achilles tendon), phosphatidylcholine, prostaglandin E_1 (PGE_1), ADP, ATP, elastase (porcine pancreas) and indomethacin also were purchased from Sigma. Sephadex G-10, -25, -50, and -75 and CM-Sephadex C-50 were from Pharmacia, Sweden. U-46619 (Biomol Research Lab. Inc.), Enzymobeads (BioRad), synthetic peptides, Arg-Gly-Asp-Ser (RGDS), Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (pentadecapeptide) (Peninsula Lab. Inc., California), $7E_3$ and $10E_5$ (monoclonal antibodies against glycoprotein IIb–IIIa complex, donated by Dr. Barry Coller, State University of New York, Stony Brook, NY), trifluoroacetic acid (TFA, Merck), acetonitrile (LC grade, Alps Chemical Co.), human fibrinogen (Kabi, Sweden), endoproteinase Lys-C (Boehringer-Mannheim), and V_8 protease (*Staphylococcus aureus*, Pierce Chemical Co.) were used. A reverse-phase C_{18} column (μBondapak, 3.9 mm × 30 cm) was purchased from Waters.

Trigramin was purified according to previously described methods [21]; rhodostomin, a trigramin-like peptide, was purified from *A. rhodostoma* ([29] and unpublished data).

Purification of halysin from the venom of A. halys. A crude preparation of halysin was obtained according to previously described methods [28], essentially consisting of Sephadex G-75 and CM-Sephadex C-50 column chromatography. This preparation which still exhibited phospholipase A activity was fractionated further by reverse-phase HPLC column chromatography (see Fig. 1). An appropriate amount of an aqueous solution of halysin (50–100 μL, containing 100–200 μg of protein) was injected onto a μBondapak C-18 column using a two-pump system for delivering solvents A and B. Solvent A consisted of re-distilled water containing 0.1% TFA (v/v), and solvent B was a mixture of 20% distilled water and 80% acetonitrile, containing 0.1% TFA. The eluent was monitored continuously at 208 nm and was collected manually. The flow rate was adjusted to 1 mL/min and the gradient was controlled as the present scheme shows in Fig. 1. The collected fractions were dried by a SpeedVac concentrator (Savant, model A160), rinsed with a small amount of distilled water, further lyophilized, and stored at −20°. Protein content was estimated by the method of Lowry *et al.* [30], using bovine serum albumin as standard.

Preparation of human platelets. Blood was obtained from healthy individuals who had not taken drugs for the previous 2 weeks. Blood collected in acid citrate dextrose (9:1, v/v) or in sodium citrate (3.8%, 9:1, v/v) was centrifuged at 100 *g* at room temperature for 10 min to obtain platelet-rich plasma (PRP). Human washed platelet suspension was prepared according to the methods of Mustard *et al.* [31] and Kornecki *et al.* [32] and suspended in Tyrode's solution (pH 7.35) containing 3.5 mg/mL bovine serum albumin (Sigma, Fraction V). Elastase-treated platelets were prepared according to the method of Kornecki *et al.* [33]. The concentration of elastase in the incubation mixture was 2 units/10^8 platelets, and the incubation was conducted in the presence of apyrase (0.5 unit/mL) at 37° for 50 min. After centrifugation at 170 *g* for 10 min, the pellets were washed once and recentrifuged and resuspended.

Platelet aggregation and ATP release reaction. Platelet aggregation and release reaction were performed at 37° by the turbidimetric method [34] using a Lumi-Aggregometer (Chrono-Log) in the case of platelet suspension and platelet-rich plasma. The platelet count was adjusted to 3.0–6.0 × 10^8 platelets/mL by a Coulter Counter (model ZM). Usually, platelet suspension or platelet-rich plasma (0.4 mL) was added to a silicon-coated cuvette and incubated at 37° with an appropriate amount of Tyrode's solution; then halysin or other inhibitory reagents were added 1 min prior to the addition of the aggregation agonist. The extent of aggregation was expressed in light transmission units. When measuring the ATP release reaction, 20 μL of a luciferase–luciferin mixture was added 1 min prior to the addition of agonist and the amount of ATP released was measured quantitatively by the peak of the fluorescence change compared to exogenously added ATP. Whole blood aggregation was measured by the impedance method using a whole blood aggregometer (Chrono-Log).

Clot retraction. Platelet-rich plasmas (0.6 mL) were added to clean, uncoated glass tubes in the absence or presence of halysin and incubated at 37° for 3 min; then 0.2 mL of human thrombin (final concentration, 4 units/mL) was added, mixed well, and incubated at 37° for 2 hr. Finally, the residual volume of serum was measured as an index of clot retraction.

$$\% \text{ Retraction} = \text{serum volume (test)}/\text{serum volume (control)} \times 100.$$

Intracellular Ca^{2+} mobilization of Quin2-AM

loaded platelets. Quin 2-AM loaded platelets were prepared essentially according to the method of Rink *et al.* [35]. Citrated whole blood was centrifuged at 100 *g* at room temperature for 10 min, and the platelet-rich plasma obtained was incubated with Quin 2-AM (30 μM) at room temperature for 1 hr. Then the preparation of platelet suspension was prepared as mentioned. The intracellular Ca^{2+} mobilization was measured by the fluorescence change monitored by a fluorescence spectrophotometer (Hitachi) (excitation, 339 nm; emission, 492 nm).

Radiolabeling of halysin and fibrinogen. Fibrinogen was further purified by ammonium sulfate precipitation, according to method of Lipinska *et al.* [36]. The purified fibrinogen and halysin were labeled with Na-^{125}I (Amersham) using Enzymobeads (BioRad), and the labeled fibrinogen and halysin were separated from free ^{125}I by Sephadex G-25 and G-10 columns, respectively. The specific activity of ^{125}I-fibrinogen was 20,000 cpm/μg protein and that of ^{125}I-halysin 80,000 cpm/μg protein. The clottability and precipitability of the ^{125}I-fibrinogen was about 90 and 97%, respectively. The ^{125}I-fibrinogen comigrated with unlabeled fibrinogen on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The labeled halysin was shown to be equally active in inhibiting platelet aggregation.

Binding of ^{125}I-fibrinogen and ^{125}I-halysin to platelets. Ligand-platelet binding studies were performed by a previously described method [21]. In brief, the incubation mixture was composed of 400 μL of platelet suspension (5×10^8 platelets/mL), 10 μL ^{125}I-fibrinogen or ^{125}I-halysin, 10 μL ADP (final concentration, 10 μM) and an appropriate amount of Tyrode's solution or inhibitor (total volume, 500 μL). ^{125}I-Halysin or ^{125}I-fibrinogen was added simultaneously with inhibitors (e.g. $7E_3$, $10E_5$, synthetic peptides, snake venom peptides, EDTA) to the platelet suspension at room temperature 3 min prior to the addition of ADP. Following the addition of ADP, the platelet suspension was shaken gently and incubated for another 10 min. Then 400 μL of platelet suspension was centrifuged through the sucrose solution (15%, w/v) at 14,000 rpm for 5 min. The radioactivities of the supernatant and the scissored tip containing pellets were counted by a gamma counter (LKB). The non-specific binding of ^{125}I-fibrinogen or ^{125}I-halysin was measured in the presence of EDTA (10 mM). The non-specific binding of ^{125}I-halysin measured in the presence of EDTA (10 mM) was relatively low (less than 5%); therefore, we did not subtract the non-specific binding of ^{125}I-halysin from its total binding. The number of halysin or fibrinogen binding sites per platelet and the dissociation constant (K_d) were calculated by the method of Scatchard [37].

Phospholipase A activity. This activity was estimated by the indirect hemolysis method following the method of Brown and Bowles [38] using phosphatidylcholine (Sigma) as the substrate.

SDS–polyacrylamide gel electrophoresis and autoradiography. Halysin was analyzed on a 15% polyacrylamide gel according to the system of Laemmli [39]. Following electrophoresis, gel was stained with silver after the mandatory fixation of protein with glutaraldehyde [40]. The purity of ^{125}I-halysin was assessed by a combination of SDS–polyacrylamide gel electrophoresis and autoradiography. After electrophoresis, the gels were stained and destained, dried, exposed to Kodak X-Omat R film (Eastman Kodak Co.) for about 20 hr, and developed in a Kodak Omat developer.

Automated NH_2-terminal sequencing. Sequencing was performed on a gas-phase sequencer (Applied Biosystems Inc., model 120A). The instruments are operated routinely by the Macromolecular Analysis and Synthesis Laboratory of the Temple University Health Science Center. Standard protocols of the manufacturer were followed with regard to both Edman degradation and separation of PTH-amino acids by HPLC. Cysteine was detected as *S*-(pyridylethyl) cysteine.

Pyridylethylation of halysin. The pyridylethylation of halysin was carried out by adding 1 μL of vinylpyridine to the reduced protein (50 μg in 99 μL of 6 M guanidine hydrochloride, 4 mM EDTA, 0.1 M Tris–HCl, pH 8.5, and 4.5 mM dithiothreitol). The reaction mixture was incubated for 2 hr at 22° in the dark under argon first for reduction and then a further 2 hr for pyridylethylation. Modified protein was isolated free of reagents by reverse-phase HPLC in 0.1% trifluoroacetic acid with acetonitrile as organic modifier.

Strategy of sequencing. *S*-Pyridylethyl halysin was incubated at 37° in the presence of the following proteases (final concentrations are given): V_8 protease (6%, w/v) in 0.03 M sodium phosphate, pH 7.8, 5 mM EDTA for 17 hr; endoproteinase Lys-C (3%, w/w) in 0.1 M ammonium bicarbonate, pH 9.0, for 20 hr. *S*-Pyridylethyl halysin (30 μg) in 20 μL of 88% formic acid was incubated with 5 μL of CNBr solution in acetonitrile (19 mg CNBr in 32 μL CH_3CN) at 37° under argon for 75 min and subsequently diluted with 140 μL H_2O, dried, and fractionated by reverse-phase HPLC. All these cleavaged fragments were fractionated by reverse-phase HPLC column and sequenced in order to establish the complete amino acid sequence from all these overlapping fragments.

RESULTS

Purification of halysin. By means of reverse-phase HPLC, the crude halysin was further fractionated into four fractions (Fig. 1). Two fractions with retention times of 25.5 and 27.0 min were found to be about equally active in inhibiting the collagen-induced aggregation of human platelets without any detectable phospholipase A activity, while the other two fractions with retention times of 45 and 47 min were found to possess phospholipase A activity with no inhibitory activity on platelet aggregation. The purified fraction I (retention time, 25 min) was named halysin, and was dried, lyophilized and stored at −20° for the following experiments.

N-terminal amino acid sequencing and construction of the complete amino acid sequence. The halysin preparation was found to possess the following N-terminal sequence, EAGEE, by the Edman

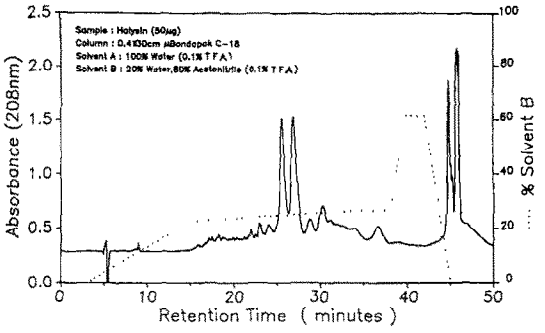

Fig. 1. Purification of halysin by reverse-phase HPLC. An aliquot (50 μg) of the active principle from a Sephadex G-50 column [24] was injected into a μBondapak C-18 column (0.4 × 30 cm, Waters) equilibrated in 0.1% trifluoroacetic acid. The chromatography was carried out with a two-solvent gradient as indicated at a flow rate of 1.0 mL/min, and eluent was monitored continuously at 208 nm with a Waters 490E Programmable Multiwavelength Detector. Two peaks with platelet aggregation inhibitory activity were eluted at retention times of 25.5 and 27.0 min and were free of any phospholipase A activity, whereas the fractions that eluted from 45–47 min possessed phospholipase A activity.

degradation method (Fig. 2). The reduced, ethylated-halysin was cleaved into eleven fragments by Lys-carboxylase. Furthermore, we obtained one fragment (fraction 9) from digests of V_8-cleaved halysin and two fragments (fractions 1 and 2) from digests of CNBr-treated halysin. By mapping the sequences of all of these fragments, we constructed the complete amino acid sequence shown in Fig. 2. Halysin is a cysteine-rich, RGD (position 51–53)-containing peptide, consisting of 71 amino acid residues. It shares about an 85% identical sequence with trigramin, including the exact numbering and positioning of half-cystine (see Fig. 9). In addition, it is a single chain peptide since its mobilities on SDS–polyacrylamide gel electrophoresis were the same in both the presence and the absence of 2% β-mercaptoethanol, with an apparent molecular weight of 7500 daltons (data not shown).

Effect on platelet aggregation. Halysin concentration-dependently inhibited the aggregation of human washed platelets stimulated by ADP (20 μM), thrombin (0.1 unit/mL) and collagen (10 μg/mL), with IC_{50} values of 0.16, 0.31 and 0.36 μM, respectively. On the other hand, halysin had no effect on the ATP release reaction of platelets stimulated by collagen and thrombin (0.1 to 1 unit/mL) (Fig. 3A). However, halysin markedly inhibited the ATP release reaction of platelets stimulated by a low concentration of thrombin (0.05 unit/mL) (Fig. 3B). Halysin appeared to have no inhibitory effect on the shape change of platelets induced by thrombin (compare the amplitude of the initial decrease of transmittance in the presence and absence of halysin, Fig. 3B). In a platelet-rich plasma preparation, halysin also blocked the aggregation of platelets challenged by ADP (5 μM), collagen (5 μg/mL) and the prostaglandin endoperoxide analogue, U46619 (4 μM), in a concentration-dependent manner with IC_{50} values of 0.28, 0.80 and 1.2 μM, respectively. Apparently, halysin exhibited a weaker activity in platelet-rich plasma than in washed platelets. Whole blood is a more physiological preparation than platelet-rich plasma and washed platelets. Thus, we also examined if halysin inhibited platelet aggregation of whole blood. As measured by the impedance method, halysin concentration-dependently inhibited

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          10        20        30        40        50        60        70
          :         :         :         :         :         :         :
EAGEECDCGSPGNPCCDAATCKLRQGAQCAEGLCCDQCRFMKKGTVCRIARGDDMDDYCNGISAGCPRNPF
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GEECDCGSPGNPCCDAATCK·····	LYS-C-2
LRQGAQCAEGLCCDQCRFMK·····	-5
EAGEECDC····	-3
GEECDC····	-11
KGTVCRIARGDDMDDYCNGISAGCPRNP···	-8
KGTVCRIARGDDM····	-6
GEECDCGS····	-4
GLCCDQCRFMKKGTVCRIARG····	V8-9
KKGTVCRIARGDDM····	CNBr-1
DDYCNGISAGCPRNPF	-2

Fig. 2. Amino acid sequence of halysin obtained from the venom of *A. halys* (mamushi). The amino acid residues of the fragments produced by CNBr, endoproteinase Lys-C and *S. aureus* protease (V_8) were determined by the Edman degradation method. From the mapping of the fragments, we determined the amino acid sequence of halysin. Dots correspond to the undetermined residues. The numbers 1–11 represent the fragments produced. The sequences of the peptides (some are not shown) were consistent with the structure presented.

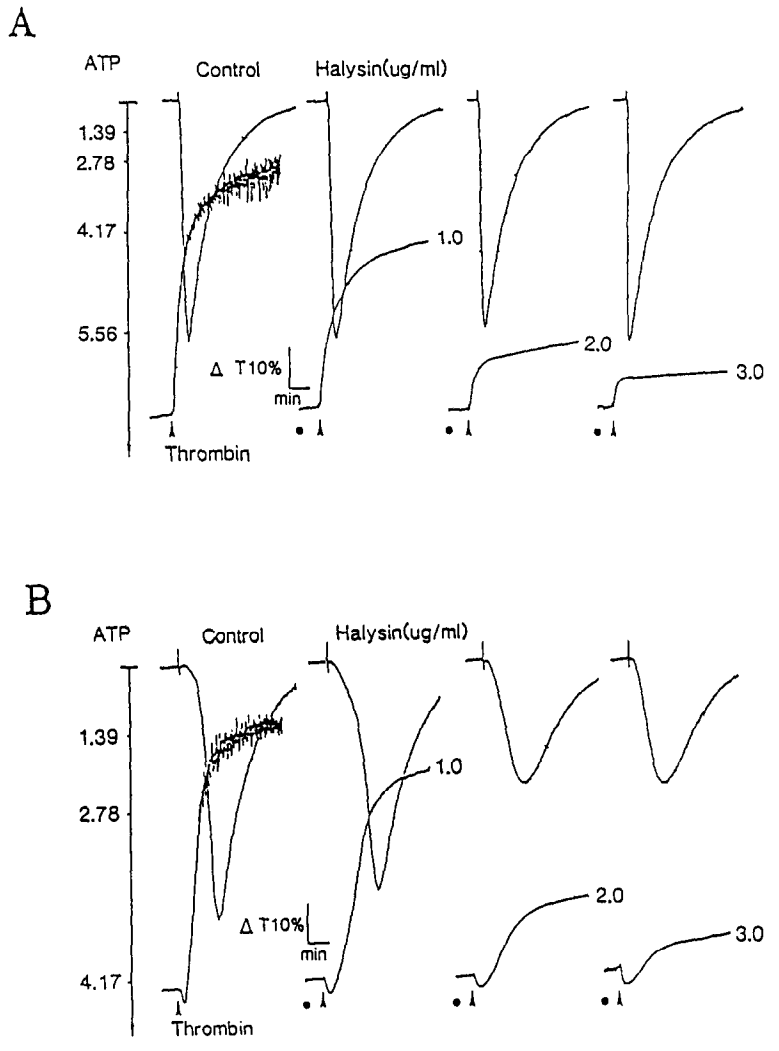


Fig. 3. Effect of halysin on thrombin (A: 1 unit/mL, B: 0.05 unit/mL)-induced aggregation (upward tracing, ΔT) and ATP release (downward tracing, nmol/10^9 platelets) in human platelet suspension. Halysin was added 1 min prior to the addition of thrombin.

platelet aggregation induced by ADP (10 μM), with an IC_{50} value of 0.032 μM. At a concentration of 0.067 μM, halysin completely inhibited platelet aggregation (Fig. 4).

Effect on thromboxane B_2 formation. Since thromboxane A_2 formation via the activation of endogenous phospholipase A_2 is one of the important pathways leading to platelet activation and since thromboxane B_2 is a stable hydrolyzed product of thromboxane A_2, we examined if halysin inhibited thromboxane B_2 formation. However, halysin (1–3 μM) showed no inhibitory effect on thrombin (0.1 unit/mL)-induced thromboxane B_2 formation of platelets (control, 84 ± 11 vs treated, 91 ± 8 ng/10^9 platelets, N = 4).

Effect on intracellular free Ca^{2+} level. Thrombin (0.1 unit/mL) caused a rise in intracellular free Ca^{2+} of Quin 2-AM loaded platelets (114 ± 8 nM, N = 3). In the presence of halysin (3 μM), the rise in the Ca^{2+} level induced by thrombin remained unchanged (105 ± 11 nM, N = 3). Thus, halysin apparently did not affect the Ca^{2+} mobilization caused by thrombin.

Effect on fibrinogen-induced aggregation of elastase-treated platelets. Elastase treatment of platelets causes the exposure of fibrinogen receptors which are inaccessible to fibrinogen in intact platelets; therefore, the addition of fibrinogen to elastase-treated platelets causes aggregation of platelets. Hence, the elastase-treated platelet preparation is a suitable model for investigating whether halysin has an effect on the interaction of fibrinogen with fibrinogen receptors associated with glycoprotein IIb–IIIa complex on platelet membranes. Halysin concentration-dependently inhibited fibrinogen-induced aggregation of elastase-treated platelets with an IC_{50} of 0.033 μM. It completely inhibited aggregation at a concentration of 0.067 μM (data not shown), indicating that halysin blocked the interaction of fibrinogen and its receptor. When halysin was added at various time intervals (0, 1, 3

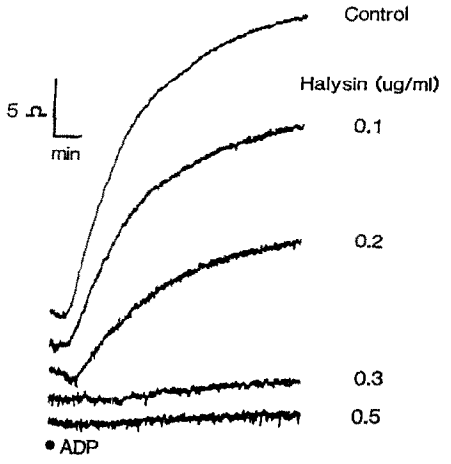


Fig. 4. Effect of halysin on ADP (10 μM)-induced platelet aggregation of human whole blood. Halysin was added 2 min prior to the addition of ADP. The aggregation was measured by the impedance method with a whole blood aggregometer (Chrono-Log).

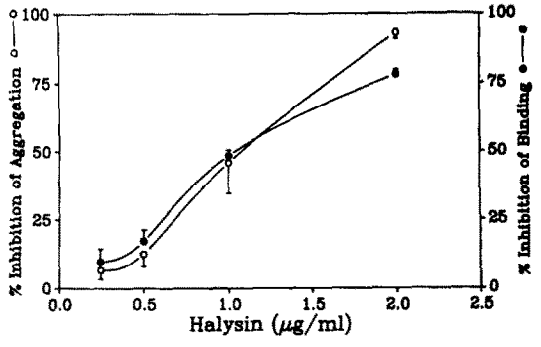


Fig. 5. Correlation between the concentration–inhibition curves of halysin on ADP (20 μM)-induced aggregation and ^{125}I-fibrinogen binding to human platelets. The final concentrations of fibrinogen in the binding and aggregation experiments were 100 and 200 μg/mL, respectively. The amount of fibrinogen binding in the control sample was 0.20 ± 0.015 μg/10^8 platelets. Values are means ± SEM, N = 5–8.

and 5 min) after fibrinogen, it disaggregated the aggregated platelets in 4, 13, 14 and 15 min, respectively. This result indicates that fibrinogen-induced aggregation is reversible in this preparation.

Effect of halysin on thrombin-induced clot retraction. Halysin inhibited the clot retraction of citrated platelet-rich plasma caused by thrombin (4 units/mL) in a concentration-dependent manner, with an IC_{50} of 0.70 μM.

Effect on ^{125}I-fibrinogen binding to ADP-stimulated platelets. Judging from the previous results, we inferred that halysin interferes with the interaction of fibrinogen and its receptors, and that it would inhibit ^{125}I-fibrinogen binding to ADP-stimulated platelets. Figure 5 shows that halysin concentration-dependently inhibited ^{125}I-fibrinogen binding to

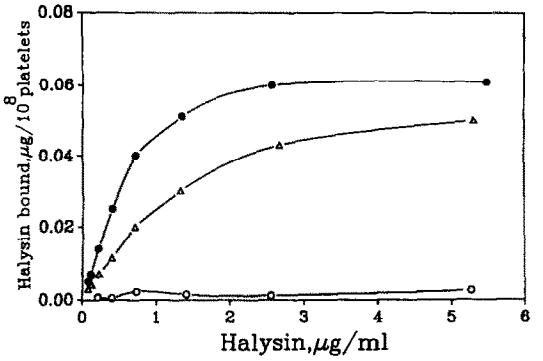


Fig. 6. Binding of ^{125}I-halysin to unstimulated (△) and ADP (20 μM)-stimulated platelets in the absence (●—●) or presence (○—○) of 5 mM EDTA.

ADP-stimulated platelets. The concentration–inhibition effect on both aggregation and fibrinogen binding correlated very well with each other (IC_{50} = 0.16 μM) (Fig. 5). The double-reciprocal plot of binding data in the absence and presence of halysin (1.0, 2.0 μg/mL) showed that halysin appears to block ^{125}I-fibrinogen binding to ADP-stimulated platelets in a competitive manner. Therefore, halysin may bind to the fibrinogen receptor or to a site very close to the fibrinogen receptor.

Binding properties of halysin to intact and ADP-stimulated platelets. As shown in Fig. 6, halysin bound to both unstimulated and ADP-stimulated platelets in a saturable manner. This binding is dependent on divalent cations since it was inhibited almost completely by EDTA (5 mM). The Scatchard plot of ^{125}I-halysin binding data showed that the binding sites of halysin in both unstimulated and ADP-stimulated platelets were about the same (44,758 ± 2200 and 45,981 ± 2279, N = 5, respectively). However, the binding affinity of halysin was increased markedly in the presence of ADP (5-fold). The K_d values of halysin in the presence and absence of ADP were estimated to be $3.4 \pm 0.4 \times 10^{-8}$ M and $1.6 \pm 0.8 \times 10^{-7}$ M, respectively.

Effect of RGDS on ^{125}I-halysin binding to ADP-stimulated platelets. Since there are many adhesive proteins, such as fibrinogen, von Willebrand factor, fibronectin and vitronectin, which are RGD-containing glycoproteins, the binding activities of all these proteins to their respective receptors are believed to be involved with the presence of the RGD sequence within their molecules [41]. The synthetic RGD-containing peptides inhibit fibrinogen binding to ADP-stimulated platelets. Because halysin is an RGD-containing peptide, we thought it would be interesting to examine if RGDS can inhibit halysin binding to platelets. As shown in Fig. 7, RGDS inhibited ^{125}I-halysin binding in a concentration-dependent manner. The double-reciprocal plot of ^{125}I-halysin binding data in the absence or presence of RGDS (25 and 100 μg/mL) showed that RGDS inhibited ^{125}I-halysin binding to ADP-stimulated platelets in a competitive manner (Fig. 8). A Scatchard plot of these data showed that the binding

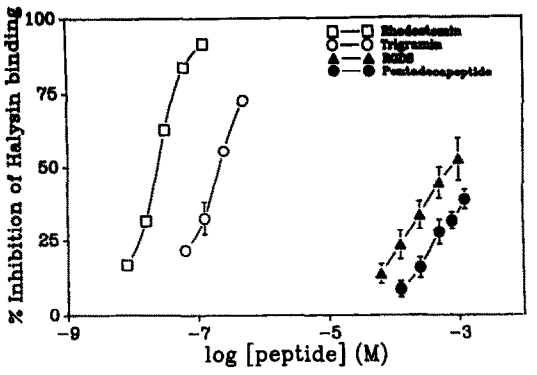


Fig. 7. Concentration–inhibition curves of rhodostomin, trigramin, Arg-Gly-Asp-Ser (RGDS) and pentadecapeptide on ^{125}I-halysin binding to ADP (20 μM)-stimulated platelets (3×10^8 platelets/mL). The peptides were added 3 min prior to the addition of ADP. In the control experiments, the amount of halysin bound to platelets was 27.5 ± 0.6 ng/10^8 platelets (N = 10). Rhodostomin and trigramin were purified from venoms of *A. rhodostoma* and *T. gramineus*, respectively. Pentadecapeptide is the synthetic peptide derived from the C terminus of the fibrinogen γ chain. Values are means ± SEM, N = 4.

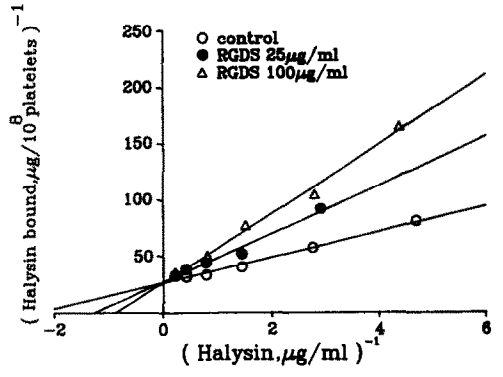


Fig. 8. Double-reciprocal plot of ^{125}I-halysin binding to ADP (20 μM)-stimulated platelets in the absence or presence of Arg-Gly-Asp-Ser (RGDS, 25 or 100 μg/mL). RGDS was added to the platelet suspension 3 min prior to the addition of ADP and ^{125}I-halysin.

sites of halysin remained unchanged; however, its binding affinity was decreased in the presence of RGDS.

Effects of synthetic peptides and trigramin-like peptides on ^{125}I-halysin binding. Rhodostomin and trigramin were very effective in displacing ^{125}I-halysin binding from ADP-stimulated platelets with IC_{50} values of 3.0×10^{-8} and 2.5×10^{-7} M, respectively (Fig. 7). On the other hand, RGDS and pentadecapeptide (carboxyl terminus of the fibrinogen γ chain) exerted rather weak activities compared to those of the snake venom peptides; their maximal inhibitory percentages in displacing halysin binding were 52.4 ± 7.4 and 38.7 ± 3.3, respectively, at concentrations of 9.3×10^{-4} and 1.1×10^{-3} M.

Effect of monoclonal antibodies against glycoprotein IIb–IIIa complex on ^{125}I-halysin binding to ADP-stimulated platelets. At a concentration of 20 μg/mL, $7E_3$ and $10E_5$ inhibited ^{125}I-fibrinogen binding to ADP-stimulated platelets by more than 90%. On the other hand, $7E_3$ inhibited ^{125}I-halysin binding by 67.9%, while $10E_5$ showed no significant inhibition (Table 1). In unstimulated platelets, $7E_3$ (20 μg/mL) exhibited 55.8% inhibition, while $10E_5$ showed only 10% inhibition of halysin binding.

DISCUSSION

In this paper, we purified an antiplatelet peptide from the venom of *A. halys*. The purified peptide, halysin, is an RGD-containing peptide, consisting of 71 amino acid residues with a molecular weight of 7513 daltons. It is devoid of phospholipase A activity; therefore, the previously reported platelet aggregation inhibitor from the same snake venom contained a trace impurity of phospholipase A [28]. However, consistent with the previous report, its inhibitory activity on platelet aggregation was independent of phospholipase A activity. Halysin is a trigramin-like peptide since halysin shares about an 85% identical sequence with trigramin (see Fig. 9), a snake venom peptide acting as a fibrinogen receptor antagonist [21].

Halysin concentration-dependently inhibited the aggregation of platelets stimulated by a variety of agonists, such as thrombin, collagen, ADP and the prostaglandin endoperoxide analogue U46619, whereas it had no effect on ATP release reaction of platelets stimulated by collagen and thrombin (0.1 to 1 unit/mL). It also had no effect on the shape change of platelets stimulated by thrombin. Halysin apparently had no significant effect on the intracellular Ca^{2+} rise of Quin 2-AM loaded platelets stimulated by thrombin. Furthermore, halysin had no inhibitory effect on thromboxane A_2 formation of platelets stimulated by thrombin. All of these results indicate that halysin exerts no effect on the activation of endogenous phospholipase A nor does it interfere with intracellular events such as calcium mobilization and release reaction. This conclusion is consistent with the profile of other trigramin-like peptides such as rhodostomin, trigramin and arietin (*Bitis arietans*) (unpublished results) but contrasts with that of applaggin reported by Chao *et al.* [24]. Applaggin was shown to inhibit release reaction and thromboxane B_2 formation of the activated platelets. This discrepancy is not explained satisfactorily; however, applaggin may exist as a dimeric structure that is different from most of the trigramin-like peptides which occur as single chain peptides [20–27]. The reason why halysin partially inhibited the release reaction induced by a low concentration of thrombin (0.05 unit/mL) may be explained as follows. Usually the intercellular adhesion (or aggregation) induces release reaction, which triggers further aggregation. Therefore, its primary inhibitory effect on platelet aggregation would lead to the blockade of release reaction. It is consistent with the result reported by Coller *et al.* [42] that

Table 1. Effect of monoclonal antibody ($7E_3$ and $10E_5$) directed against glycoprotein IIb–IIIa complex on the binding of ^{125}I-fibrinogen and ^{125}I-halysin to ADP-stimulated and unstimulated platelets

	^{125}I-Fibrinogen binding (% Inhibition)		^{125}I-Halysin binding (% Inhibition)	
	$7E_3$	$10E_5$	$7E_3$	$10E_5$
ADP (20 μM)	90.4 ± 1.0	92.2 ± 3.0	67.9 ± 1.0	5.2 ± 2.2
Without ADP	ND*	ND	55.8 ± 1.8	10.0 ± 3.2

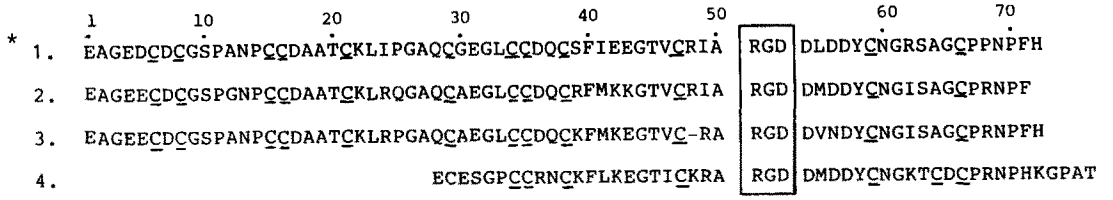
Monoclonal antibody ($7E_3$ or $10E_5$, 20 μg/mL) was added prior to the addition of ADP and binding ligand (fibrinogen or halysin). In the control experiments, the amount of fibrinogen bound was 0.120 ± 0.012 $\mu g/10^8$ platelets and the amounts of halysin bound in the presence and absence of ADP were 0.031 ± 0.001 and 0.020 ± 0.001 $\mu g/10^8$ platelets, respectively. Values are means ± SEM (N = 4).

* ND: not determined.

$10E_5$ blocked release reaction caused by a low concentration of thrombin (0.2 unit/mL) while it did not block release reaction caused by a high concentration of thrombin (1.0 unit/mL). Because the platelet release reaction is believed to be related to the hydrolysis of phosphoinositides [43], we thought that halysin did not affect the formation of inositol phosphates. Another trigramin-like peptide, triflavin, purified from the venom of *Trimeresurus flavoviridis*, was shown not to affect the formation of inositol monophosphate (manuscript in preparation). Regarding the relationship between glycoprotein IIb–IIIa complex and calcium mobilization, we found that halysin and trigramin did not affect the calcium mobilization of platelets stimulated by thrombin (this paper and unpublished data). Therefore, the binding sites of halysin and trigramin are not related to calcium mobilization. This result is consistent with the observation that the monoclonal antibodies $7E_3$, $10E_5$ and AP_2 did not affect the calcium flux in a glycoprotein IIb–IIIa incorporated liposome preparation [44]. However, another monoclonal antibody, M148, did inhibit calcium flux in the above preparation [44]. In addition, Brass [45, 46] reported that this complex is involved with calcium flux across the plasma membranes. It is likely that some specific site of this complex may be related to calcium flux, but the exact relationship between them is still unknown.

Along with the finding that halysin had no effect on the intracellular events caused by thrombin, e.g. calcium mobilization and thromboxane B_2 formation, we also determined that halysin concentration-dependently inhibited fibrinogen-induced aggregation of elastase-treated platelets. All these results indicate that halysin directly interferes with fibrinogen binding to fibrinogen receptors. The fibrinogen receptors associated with glycoprotein IIb–IIIa complex are inaccessible to fibrinogen when platelets are intact; however, the treatment of platelets with elastase or chymotrypsin would expose the fibrinogen receptors available for fibrinogen binding [32, 33]. Halysin inhibited aggregation as well as fibrinogen binding to platelets in a concentration-dependent manner. Judging from the Scatchard plot of fibrinogen binding in the presence and absence of halysin, halysin inhibited the ^{125}I-fibrinogen binding to ADP-stimulated platelets in a competitive manner, indicating that halysin and fibrinogen may bind to a common epitope or to a site very near to each other. There are several common characteristics in terms of halysin and fibrinogen binding to ADP-stimulated platelets: (1) EDTA, monoclonal antibody $7E_3$ raised against glycoprotein IIb–IIIa complex, RGDS and synthetic pentadecapeptide (carboxyl-terminal peptide of the fibrinogen γ chain) all inhibited halysin and fibrinogen binding (Fig. 7 and Table 1; and [12, 13, 15, 47, 48]), (2) the binding of both halysin and fibrinogen was saturable (Fig. 6; [12, 14, 49]), and (3) RGDS inhibited both halysin and fibrinogen binding to ADP-stimulated platelets in a competitive manner (Fig. 8; [13]). However, there are also some differences between the binding properties of halysin and fibrinogen. First, halysin bound to both intact platelets and ADP-stimulated platelets, although it bound to stimulated platelets with a higher affinity. On the other hand, fibrinogen-like PAC-1 only binds to stimulated platelets [49]. Halysin behaved more like $7E_3$ and $10E_5$ in this respect [50]. Second, the binding of fibrinogen to ADP-stimulated platelets was inhibited by $7E_3$ and $10E_5$; however, the binding of halysin was blocked only by $7E_3$, and not by $10E_5$, indicating that halysin and $7E_3$ bind to a common epitope which is different from that of $10E_5$. It appears that one molecule of halysin binds to one molecule of glycoprotein IIb–IIIa complex because the binding sites of halysin to both intact and stimulated platelets are around 45,000 per platelet, consistent with the reported number of binding sites of fibrinogen. The binding affinity of halysin was increased markedly in the presence of ADP, indicating that ADP may induce a conformational change of glycoprotein IIb–IIIa complex or a microenvironmental change surrounding this complex. This is consistent with the finding that ADP increases the binding rate of $7E_3$, but not of $10E_5$ [15], further suggesting that $7E_3$ and $10E_5$ bind to different epitopes of this complex. Basing upon these results, we believe that $7E_3$ and halysin bind to a common or overlapping epitope which is different from $10E_5$.

The RGD sequence of the α chain and the dodecapeptide of the carboxyl terminus of the γ chain of the fibrinogen molecule play a very



*
1. Trigramin *(Trimeresurus gramineus or T. stejnegeri formosensis)*
2. Halysin *(Agkistrodon halys)*
3. Applaggin *(Agkistrodon piscivorus)*
4. Echistatin *(Echis carinatus)*

Fig. 9. Comparison of the amino acid sequences of snake venom peptides, including halysin, trigramin [22], applaggin [24] and echistatin [23]. Amino acid sequences use the standard single-letter code. Halysin shares about 85 and 89% identity of amino acid sequence with trigramin and applaggin, respectively. They are half-cystine rich, single chain polypeptides with an RGD sequence near the carboxyl terminus.

important role in mediating the binding of fibrinogen to glycoprotein IIb–IIIa complex of the activated platelets [13, 47–49]. Thus, both peptides inhibited fibrinogen and halysin binding to the activated platelets (Fig. 7). However, they were much weaker than the RGD-containing snake venom peptides, rhodostomin, halysin and trigramin. These trigramin-like peptides bound to a similar epitope of the glycoprotein IIb–IIIa complex. They were much more potent than RGDS or dodecapeptide in displacing halysin binding from ADP-stimulated platelets (at least a thousand-fold more potent). RGDS showed a more pronounced effect than pentadecapeptide in inhibiting the halysin binding to ADP-stimulated platelets (Fig. 7). However, RGDS was inactive in inhibiting halysin binding to the unstimulated platelets (unpublished observation). Obviously, RGDS showed a preferential binding to the activated glycoprotein IIb–IIIa complex than to the intact one, consistent with the report that ADP increases the extent of cross-linking between RGD peptides and glycoprotein IIIa [51]. However, other sequences of halysin may also be important in mediating its binding to the intact platelets, since RGDS only partially blocked halysin binding to ADP-stimulated platelets. Further investigation is required to elucidate this matter.

In addition to antagonizing fibrinogen binding to platelets, trigramin has been shown to inhibit von Willebrand factor binding to thrombin-stimulated platelets [22]. An examination of the amino acid sequences of halysin, applaggin and trigramin (Fig. 9), reveals the following similarities: (i) the three peptides exhibit exactly the same number and spacing of the half-cystine (12 half-cystine residues), (ii) there is a high degree of sequence homology, about 85% identity between trigramin and halysin and 88.7% between halysin and applaggin, and (iii) they are all RGD-containing peptides. The RGD sequence is very essential for their binding activity since RGDS inhibited halysin, applaggin, and trigramin binding to ADP-stimulated platelets (Fig. 7; [21, 24]). In addition, trigramin also has been shown to be very potent in inhibiting the adhesion of melanoma cells to fibronectin and fibrinogen-coated plates [52]; thus, halysin may also be active in this respect. Trigramin is active in prolonging mesenteric bleeding time when it is infused into the blood vessels of hamsters [53]. However, whether halysin is active as an antithrombotic agent needs further investigation. A comprehensive review dealing with the trigramin-like peptides (or disintegrins) from viper venoms was published recently [54].

In conclusion, halysin purified from *A. halys* venom was shown to be a trigramin-like peptide which belongs to the specific antagonist of the fibrinogen receptor of human platelets. The presence of an RGD sequence and their specific conformation maintained by the disulfide bonds may be essential for the expression of the biological activities of these tigramin-like peptides. Further studies on the structure and function relationship of trigramin-like peptides would be very valuable in the understanding of the interaction of the fibrinogen and glycoprotein IIb–IIIa complex on a molecular basis.

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