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Purification, Characterization, and cDNA Sequence of Halysetin, a Disintegrin-like/Cysteine-Rich Protein from the Venom of *Agkistrodon halys* Pallas

Jie-Wu Liu,*,[1] Xiao-Yan Du,*,[1] Ping Liu,† Xin Chen,† Jian-Min Xu,† Xiang-Fu Wu,* and Yuan-Cong Zhou*,[2]

**Institute of Biochemistry, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China; and †Zhongshan Hospital, Shanghai Medical University, Shanghai, China*

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By means of DEAE–Sepharose CL-6B column chromatography, gel filtration on Sephadex G-75 and Superose 12 FPLC, halysetin, an antiplatelet protein, was purified from the venom of *Agkistrodon halys* Pallas with molecular mass of 29 kDa on SDS–PAGE and 23,168 Da by mass spectrometry. The p*I* was about 5.0. Halysetin was devoid of phospholipase A2, fibrino-(geno)lytic, esterase, hemorrhagenic activities. Halysetin dose-dependently inhibited the aggregation of human platelet, which was stimulated by collagen with IC_{50} of 420 nM, but not that stimulated by ADP. The N- and C-terminal sequences of halysetin were characterized. Its full-length cDNA was cloned by RT-PCR from the total RNA extracted from the snake venom gland. It encoded a protein of 212-amino-acid residues with disintegrin-like/cysteine-rich domains and was highly homologous with SVMPs (snake venom metalloprotease).

Key Words: **snake venom (*Agkistrodon halys* Pallas); platelet aggregation inhibitor; disintegrin-like; metalloprotease; cDNA sequence.**

Snake venoms, especially from Crotalidae and Viperidae destroy the vascular system in a complex way. They can decrease the level of fibrinogen (1, 2) or fibrin (3) in blood, induce blood platelet coagulation (4) or act as inhibitors of platelet aggregation (5–9). Recently, more and more reports demonstrate that disintegrins from snake venoms act specifically on the human integrins family, for example, $\alpha_{IIb}\beta_3$ (10) $\alpha_5\beta_1$ and $\alpha_V\beta_3$ (8) receptors on platelet surface. Integrins play a crucial role in signal transduction and cell-matrix interaction (11), therefore, disintegrins have been shown to be potentially useful tools for investigating cell-matrix and cell–cell interactions. Moreover, they may be useful for the development of drugs in view of their anti-adhesive, antimetastasis, and antiangiogenesis activities (12). Here report the purification of a disintegrin-like/cysteine-rich protein from *Agkistrodon halys* Pallas. It was named halysetin distinguished from halysin, a peptide containing RGD sequence (13) from the same kind of snake. Halysetin was characterized and its cDNA gene was cloned by means of RT-PCR. The cDNA sequence analysis was carried out.

MATERIALS AND METHODS

Purification of halysetin. Lyophilized crude venom from *Agkistrodon halys* Pallas was purchased from the Jing-de-zhen snake farm (Jiangxi Province, China). 1.0 g crude venom was dissolved in 0.05 M Tris–Cl buffer (pH 8.0) and applied to a DEAE–Sepharose CL-6B column (3.6 × 40 cm, Pharmacia Co.) equilibrated with the same buffer. The column was washed with the same buffer and eluted with a linear gradient from 0 to 0.8 M NaCl at a flow rate of 40 mL/h. The active fractions with antiplatelet activity were collected and further applied to a Sephadex G-75 column (1.6 × 120 cm, Pharmacia Co.), and eluted with the same buffer at a flow rate of 30 mL/h. The active fractions were loaded on Superose 12 FPLC (Pharmacia Co.) and eluted with the same buffer at a flow rate of 0.4 mL/min. The active fraction was collected, lyophilized, and stored at −20°C.

Electrophoresis. SDS–PAGE was performed on 10% polyacrylamide gel using the method of Laemmli (14). Isoelectric focusing (IEF) electrophoresis was performed with Model 111 Mini IEF Cell (Bio-Rad) according to the manufacturer's protocol.

Mass spectrometry. Mass spectral analysis was performed with a LCQ mass analyzer (Finnigan Co.) according to the manufacturer's protocol.

The novel nucleotide sequence data have been submitted to the GenBank data bank and are available under Accession No. AF284093.

Abbreviations used: CAT-C, catrocollastatin-C, *C. atrox;* JRC, jararhagin-C, *Bothrops jararaca;* RVV-X-HC, *Vipera russelli;* HR1B; *Trimeresurus flavoviridis;* HR1A-32K, *T. flavoviridis;* HT-1-31K, *C. ruber ruber;* astrolysin A, *C. atrox.*

[1] These authors contribute equally to this work.

[2] To whom correspondence should be addressed at Institute of Biochemistry, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China. Fax: 86-21-64338357. E-mail: zhouyc@sunm.shcnc.ac.cn.

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Amino acid sequence of N- and C-terminal determination. Amino acid sequencing was performed on an auto-PE 491 protein sequencer.

Platelet aggregation assay. Human blood was obtained from healthy donors who had not taken any medications for the previous 10 days. A 3.8% sodium citrate solution was added into the blood to a final ratio of buffer to blood of 1:9. The mixture was centrifuged at 500*g* for 10 min. The PRP (platelet-rich plasma) was transferred into a clean tube. The concentration of platelets used in each assay was adjusted to 250,000 cells/μL in a final assay volume of 0.5 mL. The platelet aggregation assay was performed in an aggregameter (Chrono-Log, Co.) at 37°C with stirring (900 rpm). The chromatography fraction or halysetin was dissolved in PBS at pH 7.4 immediately before use. The antagonist solution was preincubated with PRP for 3 min prior to the stimulation of platelet aggregation by collagen (2 μg/mL) or ADP (2 μM). The extent of the inhibition of platelet aggregation was assessed by comparison with the maximal aggregation induced by the control dose of agonist (2 μM ADP or 2 μg/mL collagen). IC_{50} value was determined from dose-dependent curves. All experiments were performed in triplicate.

Phospholipase A2 activity. This was assayed by estimating the fatty acid released from PC (phosphocholine) according to the method of Novak (15).

Fibrinolytic activities. This was determined using the method of Astrup and Mullertz (16).

Fibrinogenolytic activities. The fibrinogenolytic activity was determined according to the method described by Ouyang and Huang (17).

Arginyl esterase activity. This was determined according to the method of Du (3).

Hemorrhagenic activity. Male Kunming mice (22–25 g) were injected subcutaneously near the center of the back with 100 μL halysetin solution of different concentration according to the method of Nikai (18). The hemorrhagic state was observed by opening mice abdomens after 24 h.

Purification of total RNA from venom gland and cDNA synthesis. The snake was sacrificed by decapitation. Venom glands were removed immediately, ground to powder, and quickly suspended in Trizol reagent (GIBCO BRL). The extraction of total RNA and the cDNA synthesis were performed according to the manufacturer's protocol (GIBCO BRL).

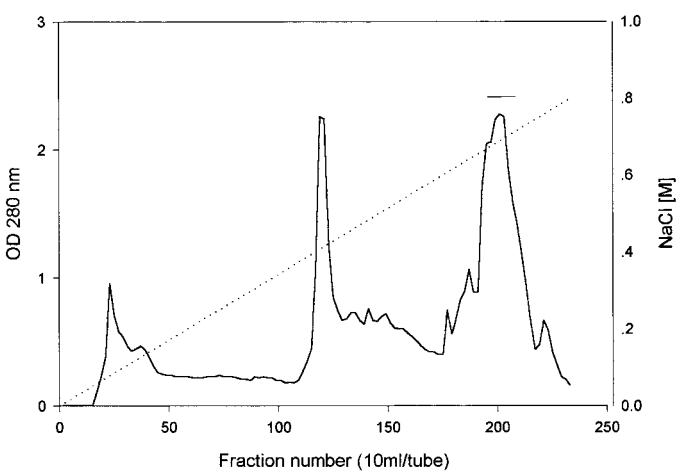

FIG. 1. Purification of halysetin. Lyophilized *Agkistrodon halys* Pallas venom (1.0 g) was loaded onto a DEAE–Sepharose CL-6B column. Elution was carried with 0.05 M Tris–Cl buffer, pH 8.0, at a flow rate of 40 mL/h. Dotted line represents salt concentration. Bars represents fractions collected which contain halysetin.

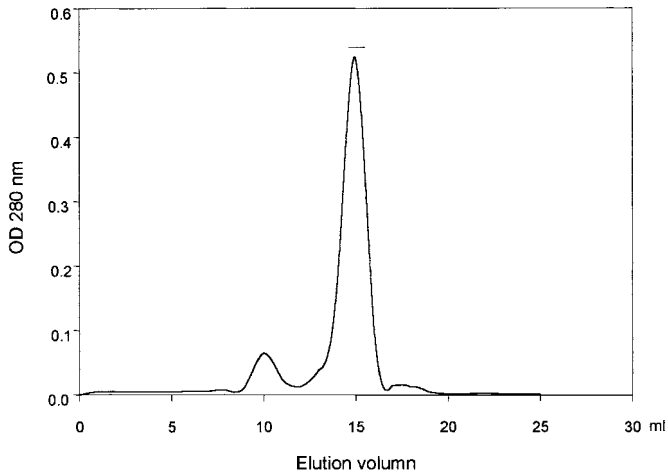

FIG. 2. FPLC Superose 12 chromatography. Active fractions after Sephadex G-75 column were further separated by FPLC Superose 12 column with 0.05 M Tris–Cl buffer, pH 8.0, at a flow rate of 0.4 mL/min. Bar represents fractions collected which contain halysetin.

Molecular cloning of halysetin cDNA. PCR was carried out to amplify cDNA of halysetin with total cDNA as a template and using *Taq* DNA polymerase (GIBCO BRL). Two primers were designed based on the sequence of the N- and C-terminal of halysetin. Primer 1 was 5′-CTGAATTCATGAT(TA)GTTTCACCTCC(ATCG)GT-3′, primer 2 was 5′-GTAAGCTTTTA(GA)TAGGCTGTAG(TC)(ATCG)AC(AG)TC-3′. PCR was performed for 30 circles with denaturation 1 min at 94°C, annealing 1 min at 52°C, then elongation 1 min at 72°C. After the treatment with a Klenow fragment, the PCR product was inserted into the *Eco*RV site of pBlueScript SK II vector and transformed into *Escherichia coli* strain TG1.

DNA sequencing and analysis. The nucleotide sequence was analyzed by the dideoxy chain-termination method using T3 and T7 universal primers. The DNA sequence and deduced amino acid sequence were compared with sequences in the GenBank database using BLASTN and BLASTP.

RESULTS

Purification of Halysetin

Following ion-exchange chromatography on DEAE–Sepharose CL-6B (Fig. 1) and gel filtration chromatography on Sephadex G-75 and Superose 12 FPLC columns (Fig. 2), a homogeneous halysetin preparation, as ascertained by SDS–PAGE, was obtained. From SDS–PAGE, its molecular weight was estimated to be around 29 kDa (Fig. 3). However, mass spectrometry analysis of the protein gave the molecular mass as 23,168. Isoelectric point was near 5.0 (Fig. 4).

N- and C-Terminal Sequence of Halysetin

The 10 N-terminal amino acid residues of halysetin were IVSPPVCGNE, while the 6 C-terminal amino acid residues were DVATAY.

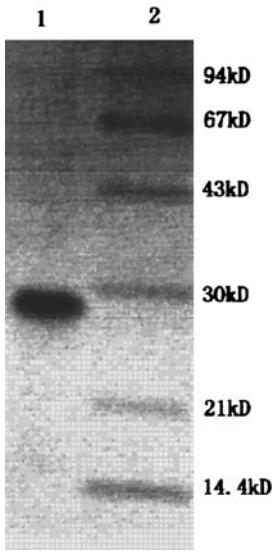

FIG. 3. SDS–PAGE of halysetin. Fraction following FPLC Superose 12 chromatography was performed on 10% polyacrylamide gel. The molecular weight was estimated to be 29 kDa.

Biochemical Character of Halysetin

Halysetin has less than a certain level of the following enzymatic activities, including fibrino-(geno)lytic, phospholipase A2 and arginyl esterase activities. No hemorrhagenic activity was observed even with the injecting 4 mg halysetin into mice. Halysetin was capable of inhibiting collagen-induced platelet aggregation (Fig. 5). The IC_{50} was determined to be 420 nM (Fig. 6). Halysetin did not significantly inhibit ADP-stimulated platelet aggregation (date not shown), indicating the selectivity of halysetin.

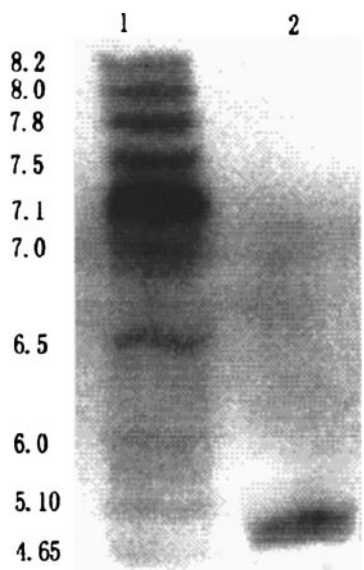

FIG. 4. Isoelectric focusing electrophoresis of halysetin. With Model 111 Mini IEF Cell (Bio-Rad), the isoelectric point was estimated to be near 5.0.

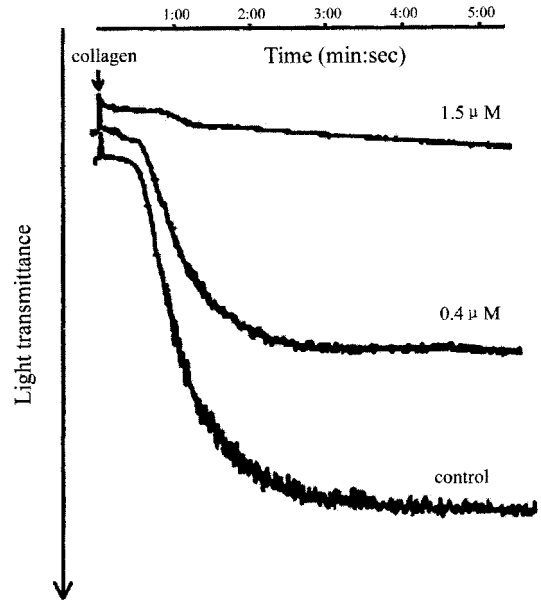

FIG. 5. Inhibition of collagen-induced platelet aggregation by halysetin. The platelet aggregation assay was performed in an aggregameter (Chrono-Log, Co.) at 37°C with stirring (900 rpm). Halysetin was dissolved in PBS at pH 7.4 immediately before use. The antagonist solution was preincubated with PRP for 3 min prior to the stimulation of platelet aggregation by collagen (2 μg/mL). Coagulation time was recorded 3 min after halysetin preincubated with the plasma. Concentration of halysetin was 0.4 and 1.5 μM, respectively.

Cloning and Sequence Determination of Halysetin

PCR amplification of total gland cDNAs achieved a fragment of about 650 bp. The fragment was cloned into the pBlueScript SK II vector and sequenced (Fig. 7). The mature halysetin covered an open reading frame of 636 nucleotides and encoded 212 amino acid residues.

DISCUSSION

Venom proteins of the Viperidae and Crotalidae families can cause hemorrhage. Hemorrhage is the result of the snake metalloproteases degrading the extracellular matrix surrounding capillaries to allow the escape of blood into the stroma (19). The presence of RGD-containing disintegrins exacerbated this effect by inhibiting platelet aggregation (8, 10, 20–22). SVMPs can be classified into four categories P-I to -IV based on the number of additional domains following the metalloprotease domain (23), although a new classification of low molecular weight SVMPs was reported recently by phylogenetic analysis (24). The P-I class has only a metalloprotease domain. The P-II class has both a metalloprotease domain and a disintegrin or disintegrin-

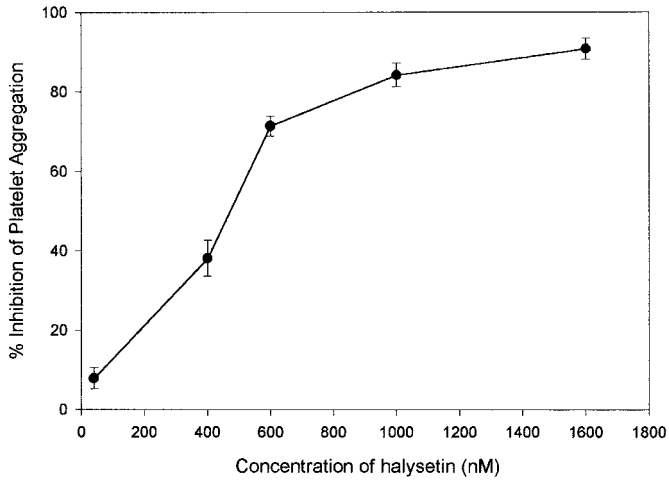

FIG. 6. Concentration dependence of collagen-induced platelet aggregation inhibition by halysetin. The extent of the inhibition of platelet aggregation was assessed by comparison with the maximal aggregation induced by the control dose of agonist (2 μg/mL collagen). The IC_{50} value was determined from the curve is 420 nM. The data are shown as mean ± SE. All experiments were performed in triplicate.

like domain. P-III proteins have a cysteine-rich domain carboxy to the disintegrin-like domain. The P-IV proteins have a fourth domain, a lectin structure. All of these subtypes are believed to be generated from a common precursor. That a mature protein with certain biological activity is also a precursor of another protein with different activities is very interesting (25). Comparison of the cDNA deduced protein sequence of halysetin with that of other SVMPs in GenBank indicates that halysetin is a new member of the disintegrin-like/cysteine-rich proteins. It has strong similarity with members of the SVMPs such as jararhagin (96%), catrocollastatin (96%), acutolysin e (92%), especially that with the disintegrin-like domain of a metalloprotease from *Gloydius halys* (98%). Moreover, the first amino acid residue of the N-terminal of halysetin is isoleucine which, as well as leucine, is the junction of the proteinase and the spacer region of the P-III class of venom metalloproteases (5). All of these findings support the hypothesis that the disintegrin-like/cysteins-rich proteins and SVMPs come from a same precursor. Although SVMP containing the complete sequence of halysetin has yet to be purified from *Agkistrodon halys* Pallas, we are confident of its presence.

The disintegrins peptides found in the venom of crotalid and viperid snakes are characterized by having a RGD motif in a flexible, hairpin loop structure between two β strands of the peptide backbone (7). They can bind to $\alpha_{IIb}\beta_3$ receptor on the surface of the platelet

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  1 ATTGTTTCACCTCCCGTTTGCGGAAATGAACTTTTGGAGGTGGGAGAAGAATGTGACTGT   60
  1   I  V  S  P  P  V  C  G  N  E  L  L  E  V  G  E  E  C  D  C   20
 61 GGCACTCCTGAAAATTGTCAAAATGAGTGCTGCGATGCTGCAACGTGTAAACTGAAATCA  120
 21   G  T  P  E  N  C  Q  N  E  C  C  D  A  A  T  C  K  L  K  S   40
121 GGGTCACAGTGTGGGCATGGAGACTGTTGTGAGCAATGCAAATTTAGCAAATCAGGAACA  180
 41   G  S  Q  C  G  H  G  D  C  C  E  Q  C  K  F  S  K  S  G  T   60
181 GAATGCCGGGAATCAATGAGTGAATGTGACCCGGCTGAACACTGCACTGGCCAATCTTCT  240
 61   E  C  R  E  S  M [S  E  C  D] P  A  E  H  C  T  G  Q  S  S   80
241 GAGTGTCCTGCAGATGTCTTCCATAAGAATGGACAACCATGCCTAGATAACTACGGTTAC  300
 81   E  C  P  A  D  V  F  H  K  N  G  Q  P  C  L  D  N  Y  G  Y  100
301 TGCTACAATGGGAATTGCCCCATCATGTATCACCAATGTTATGCTCTCTGGGGGGCCGAT  360
101   C  Y  N  G  N  C  P  I  M  Y  H  Q  C  Y  A  L  W  G  A  D  120
361 GTTTATGAGGCTGAAGATTCATGTTTTGAGAGTAACACGAAAGGCAATTATTATGGCTAC  420
121   V  Y  E  A  E  D  S  C  F  E  S  N  T  K  G  N  Y  Y  G  Y  140
421 TGCAGAAAGGAAAATGGTATAAAGATTCCATGTGCACCAGAAGATGTAAAATGTGGAAGG  480
141   C  R  K  E  N  G  I  K  I  P  C  A  P  E  D  V  K  C  G  R  160
481 TTATACTGCAAAGATAATTCACCTGGACAAAATAATCCTTGCAAGATGTTCTATTCCAAC  540
161   L  Y  C  K  D  N  S  P  G  Q  N  N  P  C  K  M  F  Y  S  N  180
541 GAAGATGAACATAAGGGAATGGTTCTTCCTGGAACAAAATGTGGAGATGGAAAGGTCTGC  600
181   E  D  E  H  K  G  M  V  L  P  G  T  K  C  G  D  G  K  V  C  200
601 AGCAACGGGCATTGTGTTGATGTCGCTACAGCCTAT                          636
201   S  N  G  H  C  V  D  V  A  T  A  Y                          212
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FIG. 7. The cDNA and deduced amino acid sequence of halysetin. Fragment achieved by PCR amplification of total gland cDNAs was inserted into pBlueScript II vector. The nucleotide sequence was analyzed by the dideoxy chain-termination method. The SECD motif is indicated in a box.

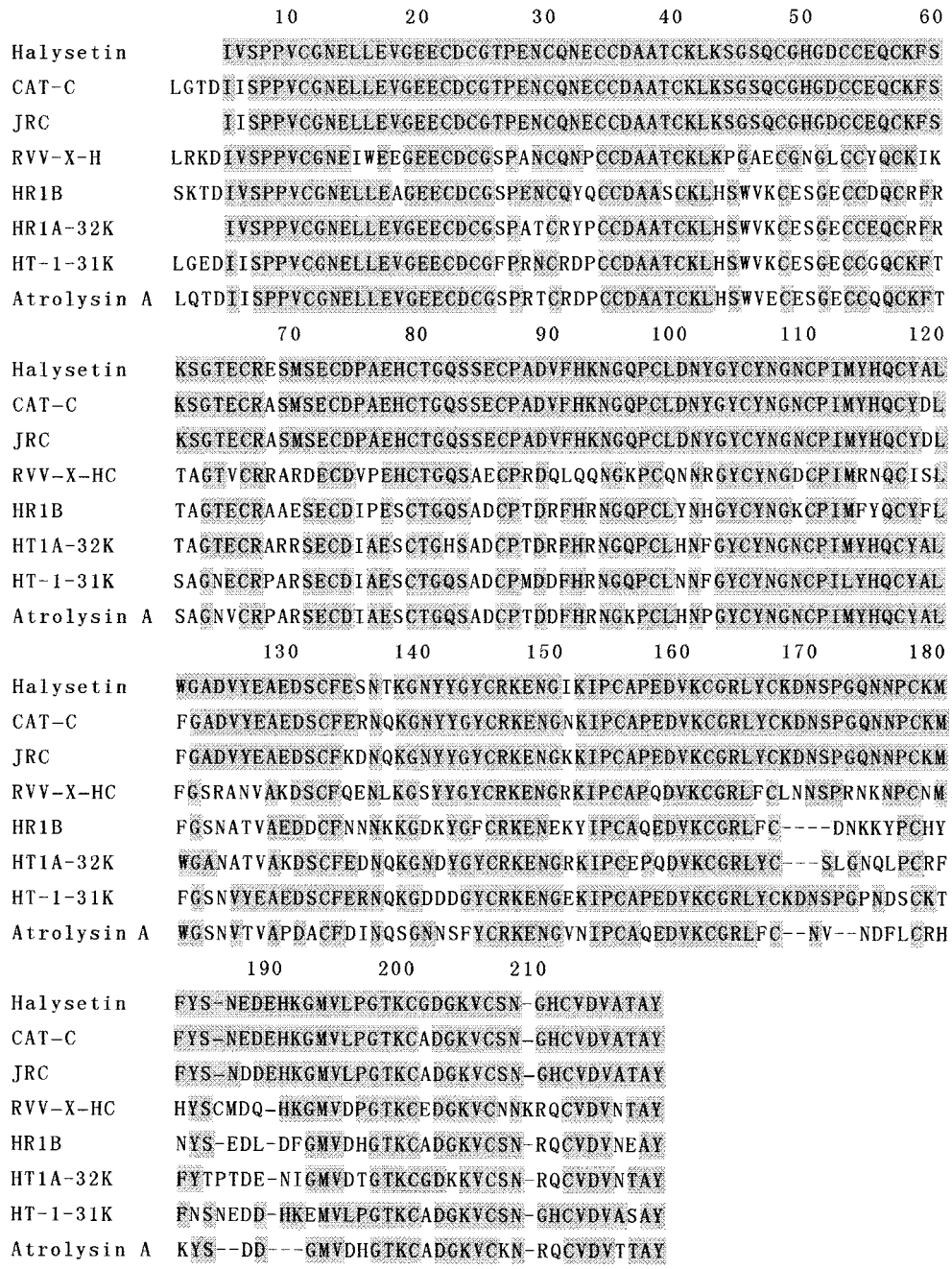

FIG. 8. Comparison of halysetin sequence with the sequences of the spacer/disintegrin-like/cysteine-rich domains of other class P-III venom metalloproteases. Residues identical with halysetin are shaded. It is noted that catrocollastatin-C has four more amino acid residues at the N-terminal than halysetin and jararhagin-C. Glu^{64} around the region of SECD in halysetin is replaced by Ala in catrollastatin-C and jararhagin-C, and Ala^{115} in cysteine-rich domain is replaced by Asp in these two proteins.

through this motif and inhibit the aggregation of platelets (26). The disintegrin-like/cysteine-rich proteins also have such activities but with the RGD sequence replaced by SECD (5, 26, 30), and maybe act on one certain receptor on the surface of platelet. For example, Catrocollastatin-C only inhibits collagen-induced platelet aggregation but not the ADP-stimulated ones (5). Some disintegrins genes containing RGD sequence obtained from cDNA of *Agkistrodon halys* (29) have been reported. As yet, no proteins having only the two disintegrin-like/cysteine-rich domains were purified from *Agkitrodon halys*. To our knowledge, halysetin is the first reported protein having only the two disintegrin-like/cysteine-rich domains from *Agkistrodon halys*. Similar to Catrocollastatin-C, halysetin was found to inhibit only collagen-induced platelet inhibi-

tion but not the ADP-stimulated ones. This suggests that the protein may be acting in the activation pathway prior to the activation site of ADP (5). It may act by binding with collagen, thereby blocking platelet adhesion to collagen with the result of inhibiting adhesion-initiated platelet aggregation (28). However, another intriguing possibility is that these proteins may also be binding to the platelet $\alpha_2\beta_1$ collagen receptor to block its interaction with collagen, hence preventing platelet stimulation. This possibility was supported in the case of MG-63 cells (5). The comparison of halysetin sequence with that of the spacer/disintegrin-like/cysteine-rich domains of other P-III venom metalloproteases (Fig. 8) showed that halysetin was highly homologous with catrocollastatin-C and jararhagin-C (30). However, their activities were a little difference: The IC_{50} value of halysetin inhibiting collagen-induced platelet aggregation was 420 nM compared with 66 nM of catrocollastatin-C (5). Jararhagin-C can inhibit ADP-stimulated platelet aggregation while halysetin cannot. The reason is not clear now. It maybe has some relationship with the differences of primary structures. It is noted that catrocollastatin-C has four more amino acid residues at the N-terminal than halysetin and jararhagin-C. In addition, Glu^{64} around the region of SECD in halysetin is replaced by Ala in catrollastatin-C and Jararhagin-C, and Ala^{115} in cysteine-rich domain is replaced by Asp in those two proteins. Glu and Asp are negative-charged amino acids. It is very possible that these two amino acid residues play roles on biological activity and specificity.

A recently discovered gene family encoding membrane proteins with a disintegrin and metalloprotease domain (ADAM) has been found in a wide array of mammalian tissues as well as in lower eukaryotes (31). These proteins contain pro-, metalloprotease-like, disintegrin-like, cysteine-rich, endothelial growth factor (EGF)-like, transmembrane and cytoplasmic domains. These domains are similar to domains in SVMPs (22). It has been implicated that ADAMs were involved in sperm–egg membrane binding and fusion (32), and essential for the partitioning of neural and nonneural cells during the development of both the central and peripheral nerve systems in *Drosophila* (33). Studies on SVMPs, as analogues of ADAMs, will facilitate the understanding of ADAMs mechanism. We are expressing halysetin in *E. coli* now by gene engineering. The relationship between structure and function will be studied using site-directed mutation in the future.

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