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Structural and functional significance of disulfide bonds in saxatilin, a 7.7 kDa disintegrin☆

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Abstract

Saxatilin is a 7.7 kDa disintegrin that belongs to a family of homologous protein found in several snake venoms. Six disulfide bond locations of the disintegrin were determined by enzymatic cleavage and matrix-assisted-laser-desorption–ionization time-of-flight mass spectrometry (MALDI-TOF). Functional implications of the disulfide bonds related to the biological activity of saxatilin were investigated with recombinant protein species produced by site-directed mutagenesis of saxatilin. Several lines of experimental evidence indicated that three disulfide bonds, Cys21–Cys35, Cys29–Cys59, and Cys47–Cys67, of the disintegrin are closely associated with its biological function such as its ability to block the binding of integrin GPIIb–IIIa and $\alpha_v\beta_3$ with fibrinogen and extracellular matrix. Those disulfide linkages were also revealed to be important for maintaining the functional structure of the protein molecule. On the other hand, the disulfide bridges of Cys6–Cys15 and Cys8–Cys16 do not appear to be critical for the molecular structure and function of saxatilin.

Keywords: Disintegrin; Saxatilin; Arg–Gly–Asp; GPIIb–IIIa; $\alpha_v\beta_3$; Disulfide bonds; Structure

Disintegrins are a family of cysteine-rich, low molecular weight proteins found in various snake venoms [1]. These proteins bind selectively to integrins GPIIb–IIIa, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ expressed in platelets, vascular endothelial cells and some tumor cells [2,3]. Disintegrins inhibit fibrinogen-dependent platelet aggregation through the binding to integrin GPIIb–IIIa [4]. They also inhibit angiogenesis induced by basic fibroblast growth factor (bFGF), and suppress tumor growth and metastasis through selective blockade of $\alpha_v\beta_3$ on endothelial and tumor cells [5,6].

Most disintegrins contain the Arg–Gly–Asp (RGD) or Lys–Gly–Asp (KGD) sequence, which are the structural motif recognized by various kinds of integrins [7]. Synthetic RGD peptides block the function of GPIIb–IIIa on platelet and $\alpha_v\beta_3$ integrin on tumor cells and endothelial cells [4–6]. It has been reported that disintegrins inhibit platelet aggregation 500–1000 times more strongly than GRGDSP peptide [8]. It was also demonstrated that cyclo-GRGDSPA peptide is a much better inhibitor of the adhesion of B16 melanoma cells to vitronectin or to fibrinonectin than its linear counterpart, GRGDSPA [9]. These results indicate that other structural determinants in addition to the RGD sequence are important for the high affinity binding of disintegrin to integrin on the target cells. Covalent structures of disintegrins have a high content of disulfide bridges. Alkylation or reduction of the disulfide bridges significantly decreases their biological activity [10–12]. It has been proposed that the specific conformation of RGD sequence is essential for the attachment of adhesive proteins to integrins, and the specific RGD

☆ *Abbreviations:* HUVEC, human umbilical vein endothelial cell; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; PBS, phosphate-buffered saline; SMC, smooth muscle cell; MALDI-TOF MS, matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry; TACTS, 20 mM Tris–Cl, pH 7.5/0.02% NaN_3/2 mM $CaCl_2$/0.05% Tween20/150 mM NaCl; DTT, dithiothreitol; PCR, polymerase chain reaction; EBM, endothelial cell basal medium.

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conformation of disintegrin may be determined by appropriate pairings of cysteine residues [13].

Recently, we have purified and characterized a snake venom-derived disintegrin, saxatilin, that strongly inhibits human platelet aggregation, bFGF-induced proliferation of HUVEC and vitronectin-induced SMC migration [14]. Saxatilin is a single-chain polypeptide composed of 73 amino acids including 12 cysteines as well as a tripeptide sequence Arg–Gly–Asp.

This work demonstrates the structural and functional significance of disulfide bonds in saxatilin.

Materials and methods

Materials. HUVECs were purchased from Biowhittacker. Quick Change site directed mutagenesis kit was from Stratagene. pPIC9 was purchased from Invitrogen. Delta pak C18 HPLC column was from Waters Co. All other reagents were of the highest purity available from commercial source.

Identification of disulfide bonds of saxatilin. Saxatilin (2 mg/ml in 100 mM NH_4HCO_3, pH 8.0) was digested with 500 U of TPCK-trypsin (Sigma–Aldrich) for 18 h at 37 °C followed by heat-inactivation at 100 °C for 5 min. The reaction products were further digested with 0.4 U of porcine pancreatic elastase (Sigma–Aldrich) for 12 h at 37 °C. The salt in the digested product was removed by a vacuum dryer. The molecular masses of digested products were determined by MALDI-TOF mass spectrometry to identify the disulfide pairings of saxatilin.

Pichia pastoris expression construct for saxatilin. Saxatilin was cloned from cDNA library of the *Gloydius saxatilis* venom gland using the sense primer 5′-GAGGCCGGAGAAGAATGTGACTGTGGC-3′ and the anti-sense primer 5′-GGCATGGAAGGGATTTCTGGGAC A-3′ in PCR. The amplified PCR fragment was cloned into pBluescript KS (+) and sequenced. This cDNA was modified by PCR for *P. pastoris* expression vector pPIC9. The N-terminal primer 5′-CCGCTCGA GAAAAGAGAGGCCGGAGAAGAATGT-3′ contains a *Xho*I recognition and a *KEX2* endopeptidase cleavage site. The C-terminal primer 5′-CGGAATTCTCATTAGGCATGGAAGGGA-3′ contains two stop codons and an *Eco*RI restriction site. After the PCR reaction, the amplified fragment was subcloned into pBluescript KS (+) *Eco*RV site, and its nucleotide sequence was analyzed. This modified saxatilin cDNA was inserted into *Xho*I and *Eco*RI sites of *P. pastoris* expression vector pPIC9, which forms a fusion of the prepro signal of α mating factor to the mature form of saxatilin. The pPIC-saxatilin vector was digested with *Sal*I restriction enzyme. This linearized plasmid was transformed into electro-competent GS115 cells. Methanol utilization ability of the His^+ pPIC-saxatilin transformants was analyzed by replica selection on minimal dextrose and on minimal methanol plate. Finally, His^+ and Mut^+ cells were collected for saxatilin expression.

Site-directed mutagenesis of saxatilin. The site directed mutagenesis of saxatilin was performed by the PCR-based double primer protocol of Quick Change site directed mutagenesis kit. The oligonucleotide primers for target sites, each complementary to opposite strands of a vector, were extended during temperature cycling by *Pfu* Turbo DNA polymerase. Upon extension of the oligo-primers, a mutated plasmid containing staggered nick is generated. After the PCR, the product was treated with *Dpn*I restriction enzyme and then transformed into *E. coli* TOP10 (Invitrogen). Mutagenic primers are 5′-GCCGGAGAAGAA TCTGACTGTGGCGCTCCT-3′, 5′-AGGAGCGCCACAGTCAGA TTCTTCTCCGGC-3′, 5′-GAAGAATGTGACTCTGGCGCTCCTG CAAAT-3′, 5′-ATTTGCAGGAGCGCCAGAGTCACATTCTTC-3′, 5′-CCTGCAAATCCGTCCTGCGATGCTGCAACC-3′, 5′-GGTTG CAGCATCGCAGGACGGATTTGCAGG-3′, 5′-GCAAATCCGTG CTCCGATGCTGCAACCTGT-3′, 5′-ACAGGTTGCAGCATCGG AGCACGGATTTGC-3′, 5′-GATGCTGCAACCTCTAAACTGAG ACCAGGG-3′, 5′-CCCTGGTCTCAGTTTAGAGGTTGCAGCAT C-3′, 5′-CCAGGGGCGCAGTCTGCAGAAGGACTGTGT-3′, 5′-A TGGATGATTACTCCAA TGGCATATCTGCT-3′, 5′-AGCAGAT ATGCC ATTGGAGTAATCATCCAT-3′, 5′-GAAGGAACAATAT CCCGGATGGCAAGG-3′, 5′-CCTTGCCATCCGGGATATTGTT CCTTC-3′, 5′-GGCATATCTGCTGGCTCTCCCAGAAATCCCTT C-3′, 5′-GAAGGGATTTCTGGGAGAGCCAGCAGATATGCC-3′, 5′-GAAAGGCTGTGTTCTGACCAGTGCAGATTT-3′, 5′-AAATC TGCACTGGTCAGAACACAGTCCTTC-3′.

Expression and purification of saxatilin mutants. Stock cells were grown at 30 °C in 250 ml of glycerol minimal medium (1.34% yeast nitrogen base with ammonium sulfate and without amino acids, 4×10^{-5} biotin and 1% glycerol). After 24 h, cells were collected by centrifugation and then transferred into 500 ml of methanol minimal medium (1.34% yeast nitrogen base with ammonium sulfate and without amino acids, 4×10^{-5} biotin and 1% methanol) in 100 mM potassium phosphate (pH 6.0) at 30 °C. A total of 1% methanol was added every 24 h to induce saxatilin mutants expression for 2 days.

The supernatant was collected by centrifugation. Ammonium sulfate was added to the supernatant to reach 2.0 M and then the supernatant was loaded to a phenyl–sepharose column (Amersham-Pharmacia Biotech) followed by protein elution with 1 M ammonium sulfate solution. The saxatilin mutant protein expressed by *P. pastoris* was further purified by the reverse phase C18 HPLC with acetonitrile gradient.

Mass spectrometry. MALDI-MS for the purified recombinant saxatilin and its mutants were obtained by a Kratos Kompact model II mass spectrometer.

GPIIB–IIIa binding inhibition assay. Fibrinogen/GPIIb–IIIa ELISA was performed by a modified method of Nachman and Leung [15]. Microtiter plates were coated with purified human fibrinogen (10 μg/ml). After blocking with 1% bovine serum albumin in TACTS for 1 h, the plate was washed, and each protein sample to be tested was added followed immediately by the addition of purified GPIIb–IIIa (40 μg/ml (Calbiochem) in TACTS containing 0.5% bovine serum albumin. After 2 h incubation, the plate was washed and mouse anti-human GPIIIa anti-body (CHEMICON) was added. Following an additional 1 h incubation and washing, goat anti-mouse IgG conjugated to horseradish peroxidase (BIO-RAD) was added. A final wash was performed and developing substrate (1-Step ABTS, PIERCE) solution was added. Then, the plate was incubated for about 10 min until color developed. The reaction was stopped with 3 M HCl followed by the absorbance measurement at 492 nm.

HUVEC adhesion assay. Ninety six-well plates were coated with vitronectin (0.5 μg/well) in PBS for 16 h at 4 °C. The plates were washed and incubated for 1 h with 10 mg/ml heat-denatured bovine serum albumin to block remaining protein binding sites. The plates were washed with PBS before use. HUVEC were dissociated by treatment with trypsin–EDTA, washed three times in PBS, and resuspended in serum-free EBM. Prior to the addition of the cells to each well, the cells (5×10^4) were preincubated with saxatilin and its analogs for 20 min at 37 °C. After the incubation, the cells were transferred to each well and incubated for 1 h at 37 °C in 5% CO_2 and 95% air. Unattached cells were removed by washing with PBS. Attached cells were fixed and stained with Coomassie blue. Absorbance at 540 nm of the individual well was measured to determine the relative cell number.

CD spectra analyses. CD spectra of saxatilin and its mutants were measured at 27 °C on Jasco J-720 spectropolarimeter using a quartz cell of 1-mm pathlength in the wavelength range from 195 to 245 nm. The sample concentration was 1 mg/ml in PBS (pH 7.4). Five scans were averaged for each protein with a scan speed of 10 nm/min. The secondary structure of a sample protein was analyzed by the method of Chen et al. [16].

Results

Identification of disulfide bonds

In order to assign the disulfide bridges in the polypeptide sequence of saxatilin that was previously identified by cDNA cloning [14], MALDI-TOF mass analyses were carried out for the peptide fragments digested by trypsin and elastase. The measured molecular weights of saxatilin fragments were compared with the calculated molecular mass of each disulfide-bonded fragment sequence (Table 1). Combinations of disulfide bridges are illustrated in Table 2. Two disulfide bridges, Cys29–Cys59 and Cys47–Cys67, were directly identified by MALDI-TOF mass spectrometric data. Data analysis results indicated that each of the other two peptide fragments contains cysteines; 6, 8, 15, and 16 for fragment 1 (F1) and 21, 34, 35, and 38 for fragment 2 (F2), respectively. On the basis of disulfide pairings of albolabrin [12] and flavoridin [17], it is possible to assume that the four cysteines of each peptide are involved in the formation of two disulfide bridges between themselves. There are two possible sets of disulfide combinations in F1 peptide: Cys6–Cys15 and Cys8–Cys16, or Cys6–Cys16 and Cys8–Cys15. Similarly, the sets of Cys21–Cys35 and Cys34–Cys38, or Cys21–Cys34 and Cys35–Cys38 can be assumed for F2 peptide. Although the pairings in the substructures containing the disulfide positions of F1 and F2 were not experimentally confirmed, we were able to deduce that the correct disulfide

Table 1
Disulfide bond patterns in proteolytic fragments of saxatilin

Fragment	Fragment sequence	Calculated mass (Da)	Measured mass (Da)
F1	EAGEC^{6}DC^{8}G PANC^{15}C^{16}DA	1785.6	1787.4
F2	TC^{21}KLRPGA EGLC^{34}C^{35}DQC^{38}RFMKEGTI	2673.1	2673.7
F3	AQC^{29}AEG GDDMDDYC^{59}NI	1806.5	1805.5
F4	C^{47}R ISAGC^{67}PR	977.9	976.4

Cysteine residue numbers are marked with superscripts.

Table 2
Disulfide bond patterns of saxatilin and its mutants

Protein	Sequence and disulfide linkages
Saxatilin	EAGEECDCGAPANPCCDAATCKLRPGAQCAEGLCCDQCRFMKEGTICRMARGDDMDDYCNGISAGCPRNPFHA
	1 11 21 31 41 51 61 71
SaxAB	EAGEE**S**D**S**GAPANP**SS**DAATCKLRPGAQCAEGLCCDQCRFMKEGTICRMARGDDMDDYCNGISAGCPRNPFHA
	1 11 21 31 41 51 61 71
SaxC	EAGEECDCGAPANPCCDAAT**S**KLRPGAQCAEGLC**S**DQCRFMKEGTICRMARGDDMDDYCNGISAGCPRNPFHA
	1 11 21 31 41 51 61 71
SaxD	EAGEECDCGAPANPCCDAATCKLRPGAQ**S**AEGLCCDQCRFMKEGTICRMARGDDMDDY**S**NGISAGCPRNPFHA
	1 11 21 31 41 51 61 71
SaxF	EAGEECDCGAPANPCCDAATCKLRPGAQCAEGLCCDQCRFMKEGTI**S**RMARGDDMDDYCNGISAG**S**PRNPFHA
	1 11 21 31 41 51 61 71

sets are Cys6–Cys15 and Cys8–Cys16 for F1, and Cys21–Cys35 and Cys34–Cys38 for F2 because those combinations are commonly found disulfide patterns in disintegrins which have a highly homologous polypeptide sequence with saxatilin [12].

Site-directed mutagenesis

Cysteines of saxatilin which form a disulfide bond were replaced by serines to selectively remove the disulfide bridge of the disintegrin. TGT or TGC sequence encoding cysteine in cDNA of saxatilin was changed to TCT or TCC by point mutation. The polypeptide sequence and disulfide bond pattern of saxatilin mutants are shown in Table 2. SaxAB has four disulfide bonds, and SaxC, SaxD, or SaxF contains five disulfide bonds while all of the cysteines in native saxatilin are connected by six disulfide bridges.

Expression, purification, and identification of saxatilin mutants

Saxatilin mutants expressed in *P. pastoris* were purified from expression culture media in two steps. Culture media of *P. pastoris* was fractionated by phenyl–sepharose chromatograpy. The fractions eluted with 1 M ammonium sulfate solution were then pooled and applied on a reverse-phase HPLC column equilibrated with 0.1% (v/v) TFA, washed with the TFA solution and 5% (v/v) acetonitrile in a serial manner, and eluted with a linear gradient of acetonitrile as described in Materials and methods. Point mutations of expressed saxatilin were confirmed by MALDI-TOF mass spectrometry. Excellent correlations were found between measured and calculated molecular mass of saxatilin mutants that are devoid of disulfide bonds (data not shown).

GPIIb–IIIa binding inhibition assay

Inhibition of GPIIb–IIIa binding to immobilized fibrinogen by saxatilin or its mutant was measured by the solid-phase fibrinogen/GPIIb–IIIa ELISA. Their typical concentration-response curves are shown in Fig. 1. IC_{50} values of saxatilin and its mutants were determined on the basis of the curves in Fig. 1. As summarized in Table 3, IC_{50} of saxatilin was 2.7 nM. Reduction of disulfide bonds of saxatilin by DTT treatment decreased the activity by more than 250 times (IC_{50} of DTT-Sax = 670 μM). These experimental results indicate that disulfide bonds of disintegrins are an important covalent structure for its binding to GPIIb–IIIa receptor with high affinity. Removal of the disulfide bridges, Cys29–Cys59 and Cys47–Cys67, located at C-terminal region of saxatilin containing RGD sequence, dramatically decreased the activity more than 200-fold (IC_{50} of SaxD = 602 nM and IC_{50} of SaxF = 655 nM), which meant that

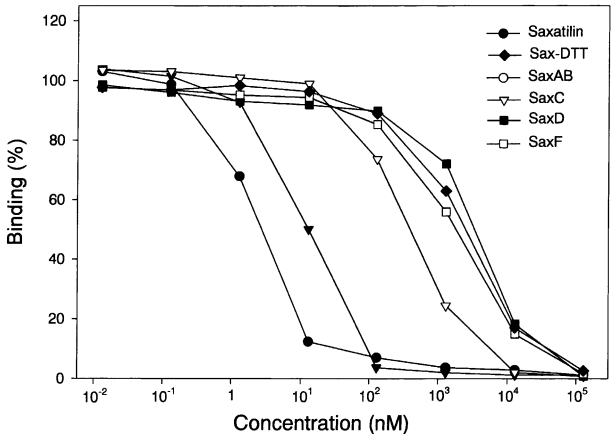

Fig. 1. Inhibitions of GPIIb–IIIa binding to immobilized fibrinogen by saxatilin and its mutants. The inhibitions were measured by the solid-phase ELISA assay as described in Materials and methods.

Table 3
IC_{50} of saxatilin and its mutants against GPIIb–IIIa binding to fibrinogen

Protein	IC_{50} (nM)
Saxatilin	2.7
Sax-DTT	670
SaxAB	8.7
SaxC	138
SaxD	602
SaxF	655

the conformation of RGD loop formed by disulfide bonds has a critical role in the activity of disintegrin. However, loss of two disulfide bonds, Cys6–Cys15 and Cys8–Cys16, located at N-terminal of saxatilin decreased the activity by threefold (IC_{50} of SaxAB = 8.7 nM). These results imply that disulfide bonds at the N-terminal segment of saxatilin are much less important than those containing the RGD sequence. The activity of saxatilin was decreased by approximately 50-fold (IC_{50} of SaxC = 138 nM) when the disulfide bond, Cys21–Cys35 in the middle of the polypeptide sequence that does not include RGD motif, was absent.

Inhibition of HUVEC binding

In a previous study, we have shown that saxatilin inhibits HUVEC binding to immobilized vitronectin by blocking $\alpha_v\beta_3$ integrin on the cell surface [14]. To examine the effect of disulfide bonds on the molecular function of saxatilin, the binding of HUVEC to immobilized vitronectin was investigated in the presence of saxatilin or its mutant. As shown in Fig. 2, saxatilin, SaxAB, and SaxC were able to inhibit the HUVEC binding to immobilized vitronectin in a dose-dependent manner, while SaxD and SaxF did not affect the binding up to their concentrations of 500 μg/ml. These binding assay results are in good agreement with those

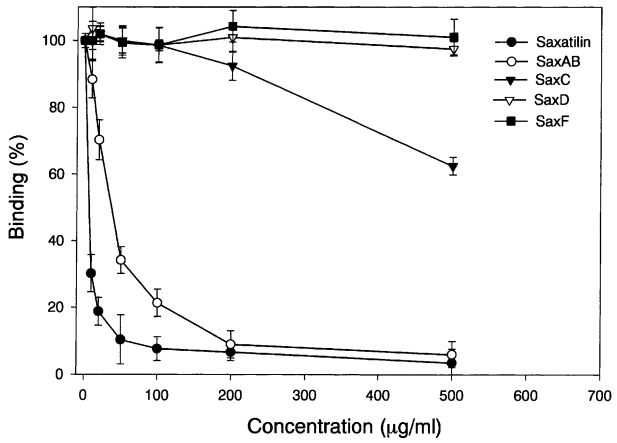

Fig. 2. Inhibitions of HUVEC adhesion to immobilized vitronectin by saxatilin and its mutants. HUVECs were preincubated in a 96 well plate with each protein for 30 min before the addition of the cells into vitronectin coated wells. The wells were rinsed after 1 h incubation, and then adhesion was determined.

obtained with the GPIIb–IIIa binding assay. Saxatilin was the most potent inhibitor followed by SaxAB and SaxC. These experimental observations indicate that disulfide bonds containing RGD sequence are critical for the activity of the disintegrin, while disulfide bridges at the N-terminal region are less important for its function.

CD spectra analyses of saxatilin and its mutants

CD spectroscopy was employed to examine the role of disulfides bond in maintaining the structure of saxatilin. Far-ultraviolet CD spectra of saxatilin and its mutants in PBS are shown in Fig. 3. The lack of a strong negative band at 222 nm revealed that saxatilin has little or no α-helical structure, while the presence of a negative band around 200 nm indicated that the disintegrin contains a large amount of random coil structure. Analyses of the CD spectra suggested that the secondary structure of saxatilin consists of β-turn, β-sheet, and random coil. The CD spectrum of saxatilin showed a strong positive band between 220 and 230 nm, which is commonly found in proteins containing multiple disulfide bridges. The position and the intensity of this band are sensitive to the environmental and the structural change of proteins [18,19]. Therefore, the changes of both intensity and position of 220–230 nm CD band suggest conformational alteration of the protein. When disulfide bonds including RGD sequence were removed by point mutations, the positive band around 225 nm was dramatically decreased. These results demonstrate that the disulfide bonds are critical elements for the functional structure of saxatilin. CD spectrum of Sax-AB indicated that the loss of the two disulfide bonds located in the N-terminal segment of saxatilin had little effect on the structure of the protein. As shown in CD spectrum of Sax-C, it appears that the disulfide bond located in the middle of saxatilin sequence is also essential for the overall structure, although this disulfide bond does not include the RGD loop.

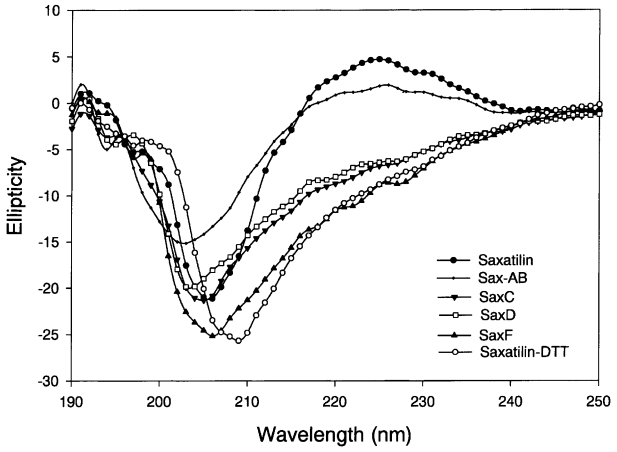

Fig. 3. CD spectra of saxatilin and its mutants. CD spectra were obtained with 1 mg/ml of each sample protein at 27 °C in the wavelength range 195–245 nm. Five scans were averaged for each protein with a scan speed of 10 nm/min.

Discussion

Based on the polypeptide length and number of cysteine residues, disintegrins can be divided into three classes; short disintegrins of about 50 residues with eight cysteines, medium disintegrins of about 70 residues with 12 cysteines, and long disintegrins of about 80 residues with 14 cysteines. All of the cysteine residues in these disintegrins are involved in disulfide bonds. In this report, we have investigated the role of each disulfide bond of saxatilin in its structure and biological function.

The disulfide bond patterns of several disintegrins have been reported [12,17,20]. In these disintegrins, two disulfide bridges form an interesting motif with respect to the RGD sequence. A structural domain containing the RGD sequence is defined by a combination of two disulfide bonds. One is formed between the cysteine closest to the RGD sequence on the N-terminal end of the peptide and the last cysteine. This disulfide connection is highly conserved. Another one is formed between the cysteine closest to the RGD sequence on the C-terminal end of the molecule and one cysteine of the N-terminal segment. This bond is semi-conserved. Like those disintegrins, saxatilin also has the conserved and semi-conserved disulfide bonds, Cys47–Cys66 and Cys 29–Cys59, containing the RGD sequence. As evidenced by the experimental results obtained with site-directed mutagenesis and CD spectroscopy, it is clear that Cys 47–Cys66 disulfide bond plays critical roles in functional structure and biological activity of saxatilin. NMR studies of disintegrins [21–25] suggested that the

RGD loop is formed by two anti-parallel β-strands held by a disulfide bond corresponding to cysteine-47 and cysteine-66 of saxatilin. In the absence of this disulfide bond, the RGD loop of disintegrins undergoes significant disruption of its conformation. Similarly, it is also evident that Cys29–Cys59 is closely associated with functional structure and biological activity of saxatilin. According to the NMR 3-D structure of albolabrin [21], the disulfide bond corresponding to Cys29–Cys59 of saxatilin is not involved in the RGD loop but it links the β-sheet strand of RGD loop to a neighboring β-sheet strand. Therefore, it is possible to speculate that Cys29–Cys59 contributes to the precise arrangement of the RGD loop in spatial structure of saxatilin. Interestingly, Cys21–Cys35 in the middle of saxatilin sequence that does not contain the RGD loop also greatly affects the activity and the structure. NMR structure of a disintegrin [21] exhibited that the densely packed protein core region in the middle of its sequence is stabilized by the disulfide bond which is fairly well matched with Cys21–Cys35 of saxatilin. It can be postulated that the loss of this disulfide bond destabilizes the core structure of saxatilin resulting in the disruption of the overall conformation including the functional arrangement of RGD loop. The saxatilin mutant that is devoid of two disulfide bonds in the N-terminal region has similar activity and CD spectrum to native saxatilin. NMR study of albolabrin demonstrated that the segment of 20 residues in its N-terminal has a high degree of flexibility, and is apart from protein core region in the middle of sequence as well as from the RGD loop. Removal of those two corresponding disulfide linkages of saxatilin may further increase the flexibility of its N-terminal region without affecting the conformation of RGD loop, which is the recognition site of integrin.

Recent studies of RGD-containing disintegrins have revealed new applications of those small proteins [26, 27]. In this report, we have shown the structural and functional significance of disulfide bridges in saxatilin that was found and characterized in our laboratory. Further studies with experimental evidence obtained in this work will provide useful information in developing new drugs based on disintegrins.

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