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NMR Structural Studies on Novel Disintegrin, Saxatilin from *Gloydius saxatilis* Venom

Joon Shin[1,2], Dong-Hee Lee[1,2], Sung-Yu Hong[1], Kwanghoe Chung[3], Doo-sik Kim[1] and Weontae Lee[1,2*]

[1]Department of Biochemistry and [2]HTSD-NMR Laboratory, College of Science, Yonsei University, Seoul 120-740 Korea, [3]Cardiovascular Research Institute and BK21 Project for Medical Sciences, College of Medicine, Yonsei University, Seoul 120-752, Korea

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Abstract : A new disintegrin protein named saxatilin was purified from Korean snake venom (*Gloydius saxatilis*). Saxatilin is a 73 residue small ploypeptide, which has a primary recognition motif in extracellular matrix, Arg-Gly-Asp (RGD) sequence. Data from inhibition activity assay for the $\alpha_v\beta_3$ integrin showed that saxatilin showed about 5000-fold higher activity than those of RGD peptides, suggesting that RGD sequence may not be sufficient to induce full cellular function of this site. The solution structures calculated from NMR data were well converged for backbone atoms except RGD loop. The structure revealed that most of tight turns are stabilized by medium range NOE contacts and the RGD motif is located far from the rigid core of the C-terminal domain. The three-dimensional fold and biological function of saxatilin are discussed with those of salmosin, which is a disintegrin protein derived from *Agkistrodon halys brevicaudus*.
Keywords : RGD, disintegrin, saxatilin, integrin, NMR

INTRODUCTION

Disintegrins are a member of protein family with low-molecular weight and cysteine rich, RGD-containing peptide which is a key structural motif of cell recognition. Disintegrin proteins inhibit fibrinogen by binding not only to glycoprotein complex but also to RGD-dependent integrins on the surface of other cells [1-3]. Disintegrins share a high level of seque-

* To whom correspondence should be addressed. E-mail : wlee@spin.yonsei.ac.kr

nce homology containing Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequence, having unique biological functions such as potent inhibitors of platelet aggregation and integrin dependent cell adhesion [2-9]. The RGD sequence was identified as the cell recognition site on fibronectin and it is present in a variety of proteins including fibrinogen, von Willebrand factor, thrombospondin, collagen, vitronectin, and osteopontin [10]. The particular tripeptide sequence is thought to mediate adhesive function of those proteins. The RGD sequence motif could also be found in many other proteins, however, the RGD sequence only may not be sufficient for activating its biological function. A conformation and structural organization of the RGD motif within native protein might render protein its full-activity. Structure-functional data of other disintergrins were reported and structural criteria would allow identifying its biological activity of RGD site in the molecular level [9].

Saxatilin from Gloydius saxatilis which consisted of 73 amino acid polypeptide are determined as a member of disintegrin proteins and it showed a high degree of homology with disintegrin proteins, too. The sequence alignment of other disintegrin proteins is shown in Fig. 1. Saxatilin purified from venom inhibits glycoprotein GPIIb-IIIa from binding to fibrinogen. Very recently, we isolated and structure determination of a new disintegrin, salmosin, from the venom of the Korean snake Agkistrodon halys brevicaudus, using inhibition assay, mass spectrometry and NMR spectroscopy. Though, the primary sequences of two proteins are nearly identical, the biological functions are quite different. Therefore, it will be also of interest to compare two structures related with their biological roles and receptor specificity.

In this report, NMR structure of the newly purified disintegrin, saxatilin has been determined by two-dimensional nuclear magnetic resonance (NMR) spectroscopy and simulated annealing calculations. We will also discuss the structure-functional relationship between two different disintegrins, saxatilin and salmosin protein.

EXPERIMENTAL

Sample preparation for biological assay

Two RGD containing peptides, Ace-RGD-NH_2, Ace-ARGDD-NH_2 were synthesized

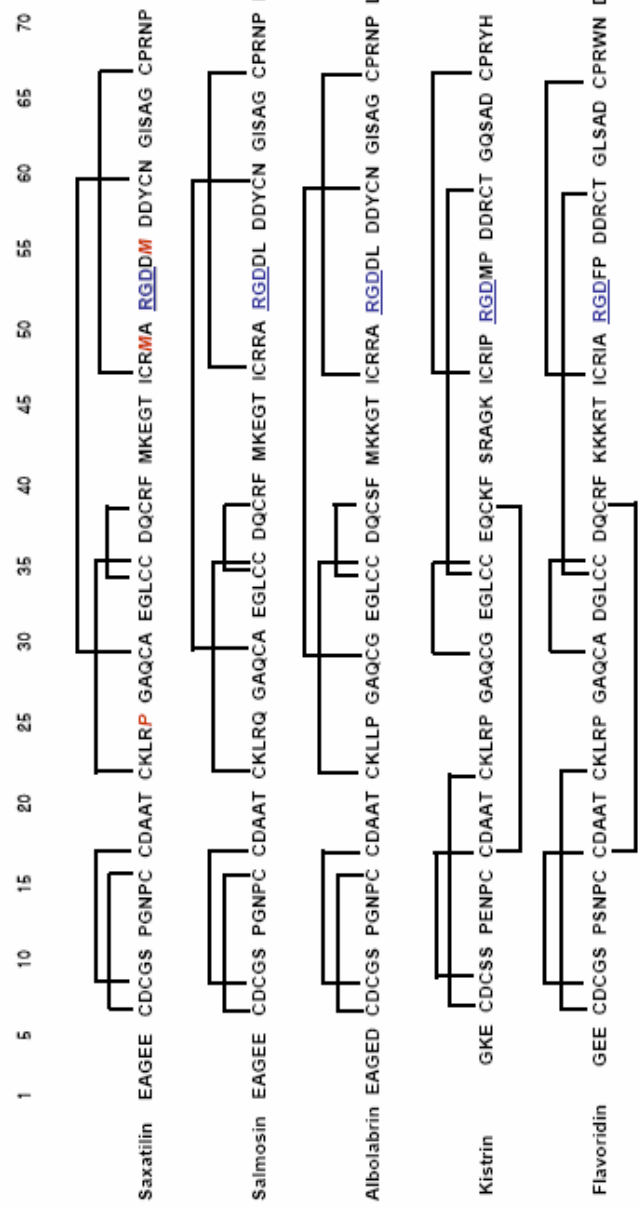

Fig. 1. Amino acid sequences of disintegrin from the venom of different snake. The alignment of the amino acids of different disintegrins has been arranged to maximize homology between them. The solid lines indicate the disulfide bridges determined for disintegrin peptide.

commercially (Anygen Inc, Kwangju, Korea). Synthetic peptides were purified by reverse-phase liquid chromatography using a Nova-Pak C18 column on a Waters Delta Prep 4000 system and further purification was achieved by equilibrating the column with 0.1% TFA in water and developing with a linear gradient of acetonitrile. Purified peptides were finally characterized by the combined use of high-pressure liquid chromatography and mass spectroscopy.

Sample preparations for NMR spectroscopy

Saxatilin was purified from Korean snake (*Gloydius saxatilis*) venom by previously published methods [11]. The sample for NMR measurements was prepared by dissolving the protein in 90% H_2O/10% D_2O or 99.9% D_2O at pH 7 with a PBS buffer. The concentration of NMR sample was approximately 2-4 mM.

Vitronectin/$\alpha_v\beta_3$ integrin binding assay

Microtiter plates were coated with purified vitronectin (1 g/well). After blocking with 1% bovine serum albumin in assay buffer (20 mM Tris, pH7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 % Triton X-100) for 1 hr, the plate was washed, and protein samples to be tested was added followed immediately by addition of purified $\alpha_v\beta_3$ integrin (1 g/ml) in assay buffer containing 0.5% bovine serum albumin. After 2 hr incubation, the plate was washed and mouse anti-$\alpha_v\beta_3$ integrin antibody was added. Following additional 1hr incubation and washing, goat anti-mouse IgG conjugated to horseradish peroxidase was added. A final wash was performed and developing substrate 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 absorbance measurement at 492 nm.

NMR spectroscopy:

NMR experiments were performed on Bruker DRX600 and DRX500 spectrometers equipped with a triple resonance probe head with triple-axis gradient coils. All NMR data were collected at 298K. The strong solvent resonance was suppressed by water-gated pulse sequence combined with pulsed-field-gradient (PFG) pulses. Nuclear Overhauser effect spectroscopy (NOESY) experiments were performed with mixing times of 50-400ms to ex-

tract NOE information. Total correlation spectroscopy (TOCSY)[12] data were recorded in both H_2O and D_2O solutions with mixing times of 77.5ms using MLEV17 spin lock pulses[13]. Double quantum-filtered (DQF) COSY[14] spectra were collected in H_2O solution to determine vicinal coupling constants. All data were recorded in the phase sensitive mode using the time proportional phase increment (TPPI) method [15] with 2048 data points in the t_2 domain and 256 points in the t_1 domain. Slowly exchanging amide protons were identified by lyophilization of a fully protonated sample in H_2O to dryness, redissolution in a 99.99% D_2O, and immediate acquisition of a series of 2D-NOESY spectra [16]. The DQF-COSY data were processed to 8192 x 1024 data matrices to obtain maximum digital resolution for coupling constant measurements. All NMR data were processed using Bruker XWIN-NMR (Bruker Instruments) software on a SGI Indigo II workstation and analyzed using Sparky 3.1 software. The proton chemical shifts were referenced using internal sodium 2, 2-dimethyl-4-silapentane 1-sulfonate (DSS).

Structural constraints and simulated annealing calculations:

Structure calculations were performed using CNS 1.1[17] on a SGI Indigo^{II} workstation. Solution structures were calculated by hybrid distance geometry and simulated annealing (SA) calculations[18-20]. The potential energy function consisted of covalent, repulsion, NOE and torsion angle terms. The target functions used for NOE and torsion angle restraints were the same as those used by Driscoll et al.[21]. A whole of 960 distances and 21 torsion angle constraints were used for structure calculations. For structural constraints, the structural and spectral information from salmosin have been used for analysis of NMR spectrum. All NOE distance constraints were classified as strong (1.8-2.7 Å), medium (1.8-3.3 Å), and weak (1.8-4.0 Å) based on intensities derived from NOESY spectrum at 25℃, pH 7. Corrections for pseudo-atom representations were used for non-stereo specifically assigned methylene, methyl group, and tyrosine ring protons [15]. Backbone dihedral angle restraints were derived from $^3J_{HN\text{-}H\alpha}$ coupling constants in DQF-COSY spectra in H_2O solution [22]. Backbone dihedral restraints were used as -55°± 45° ($^3J_{HN\alpha} < 6$ Hz) and -120°± 50° ($^3J_{HN\alpha} > 8$ Hz). Each of the six disulfide bonds was represented by three distance constraints. Final structures were analyzed using PROCHECK and displayed using Insight II (Biosym/Molecular Simulations, Inc) and MOLMOL program.

RESULTS AND DISCUSSION

Binding affinities of disintegrins and RGD peptides

Cell adhesion process normally involves molecular interactions between integrins and extracellular proteins, facilitated by the RGD motif. Eventhough the RGD motif is essential for disintegrin function, it may not fulfill all requirements for the full biological activity by binding to integrin. Out of 3819 proteins, about 100 proteins contained RGD sequences and only 25% among proteins containing RGD sequence demonstrate their activity through their RGD motif [9]. The conformation of RGD loop has also been determined by the NMR analysis. We synthesized the RGD peptides to compare with inhibition activity of saxatilin for the $\alpha_v\beta_3$ integrin (Table 1). The assay result showed that a peptide with ARGDD sequence demonstrated 4-fold higher activity than tri-peptide with RGD sequence. In addtion, saxatilin showed about 5000-fold higher activity than that of RGD peptide, suggesting that RGD sequence is not sufficient to induce full cellular function of this site, thus it migh be important to have proper tertiary structure of disintegrin protein.

Resonance assignments

The spin system identification was performed using the data of DQF-COSY, TOCSY, and it was confirmed by NOESY data. Only three residues are different between saxatilin and salmosin. Two residues M49 and M55 of the saxatilin are near the flexible RGD loop and the whole structure may be similar. As the result of NMR experiment data of saxatilin and salmosin spectra is almost same. Determination of strong $d_{NN}(i,i+1)$ NOEs, we assigned backbone structure of saxatilin. Fig. 2 shows some example of the NOE sequential assignment for backbone atoms. The NOE connectivity is summarized in Fig. 3.

Table 1. Binding activity of low molecular weight analogs of saxatilin with integrin $\alpha_v\beta_3$

Analog	IC50 against $\alpha_v\beta_3$ integrin (μM)
Ace-RGD-NH2	368
Ace-ARGDD-NH2	91
saxatilin	0.074

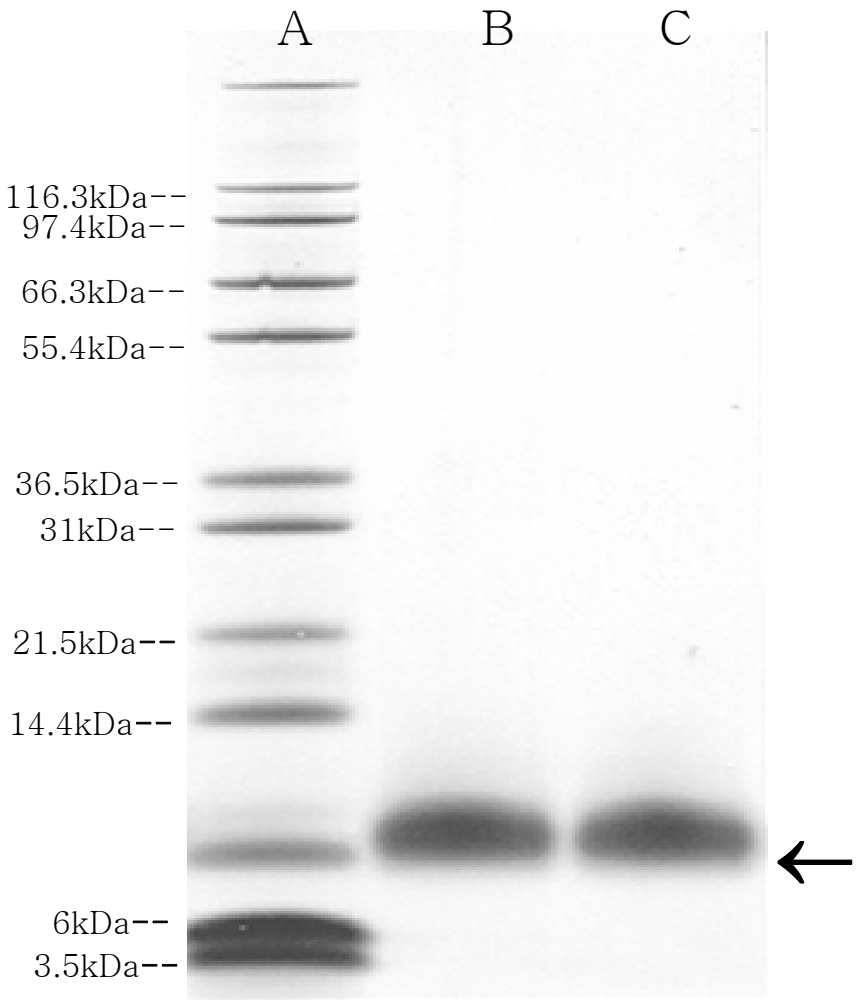

Fig. 2. SDS-PAGE analyses of purified saxatilin and salmosin. Lane B is saxatilin, Lane C is salmosin with molecular weight markers. They are resolved on an 18% SDS-PAGE containing β-mercaptoethanol.

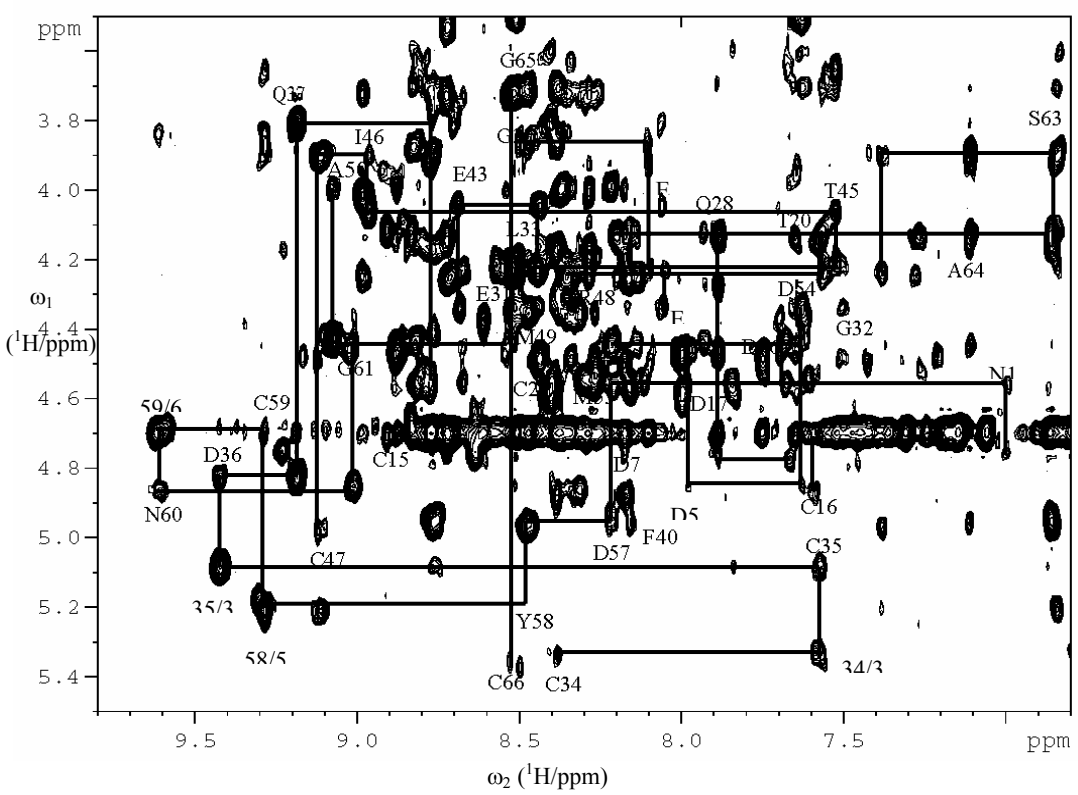

Fig. 3. The spectrum demonstrates sequential NOEs in 500 MHz 2D-NOESY proton spectrum of saxatilin with mixing time of 150 ms in H2O at 298 K.

Tertiary structure of saxatilin:

Since the primary sequence of saxatilin is nearly identical except RGD loop region (P25, R49, L55), the solution structures of saxatilin were calculated using the combined use of NOE constraints derived from saxatilin and salmosin. The 980 distance constraints, 9 hydrogen bonds and 21 torsion angle restraints were used in structure calculation. The 20 lowest energy structure ($<SA>_k$) with no constraint violations greater than 0.5 Å for the distance and 5℃ for torsion angle were selected for further structure analysis (Fig. 4A). The average structure ($<SA>_k$) was calculated from the geometrical average of 20 ($<SA>_k$) structure coordinates and subjected to restraint energy minimization (REM) to correct covalent bond and angle distortion. Fig. 4(B) is superposition of the REM average structure

of saxatilin (black) with that of salmosin (grey). The RGD loop is displayed with circle.

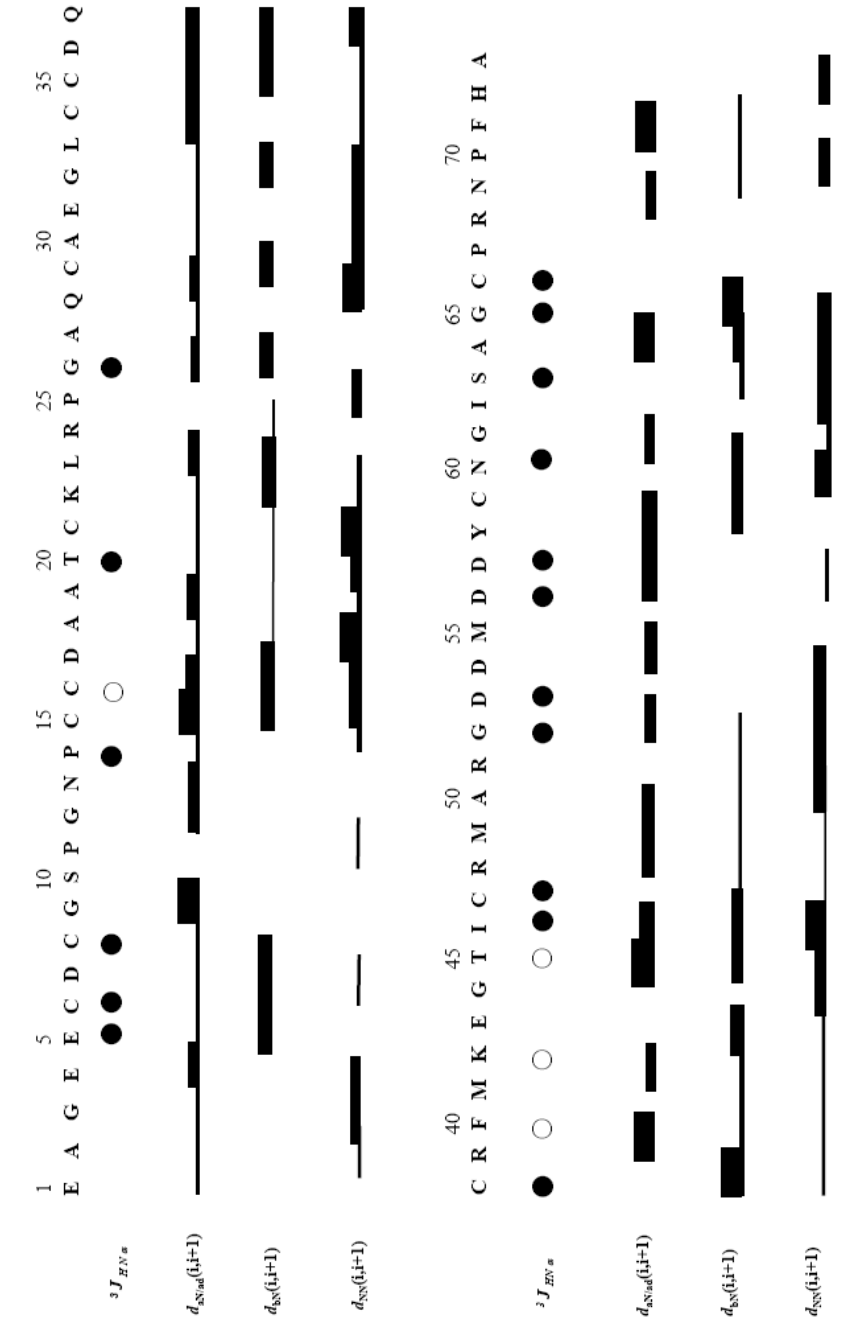

Fig. 4. Summary of sequential NOE connectivity and amide proton exchange data of saxatilin. The strength of the observed NOEs is classified by the thickness of the lines.

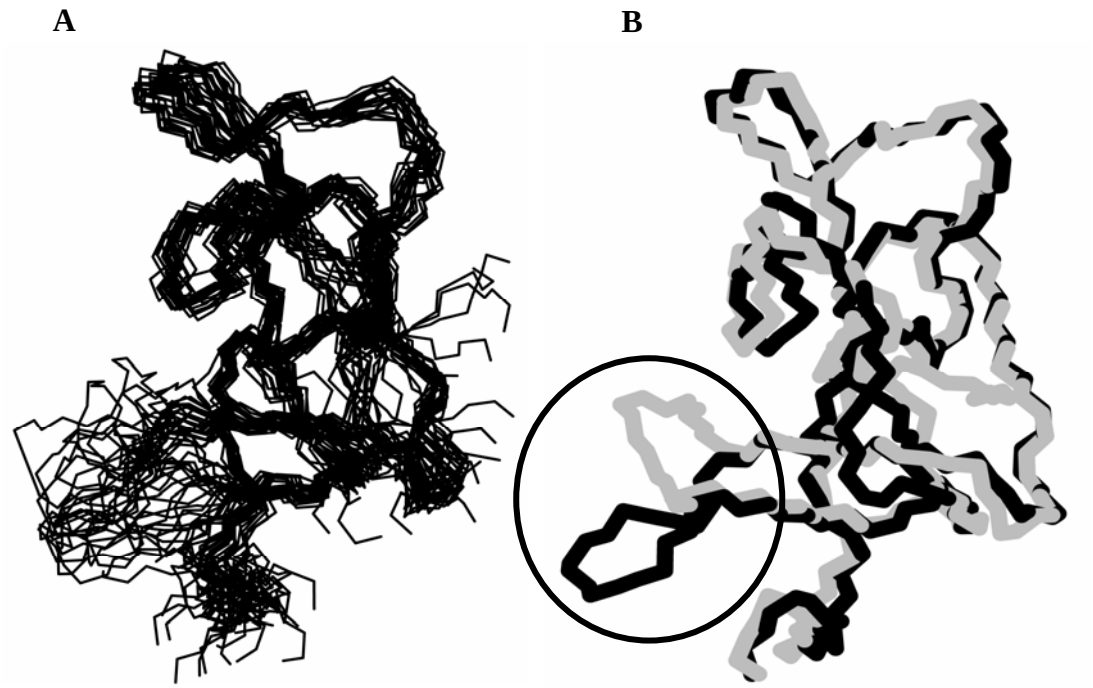

Fig. 5. Backbone traces of saxatilin and salmosin. (A) Superposition of backbone atoms of 20 accepted structures of saxatilin. RGD loop is very flexible. (B) Superimposition of backbone atoms of rem structure of saxatilin and salmosin. Black is saxatilin and grey is salmosin.

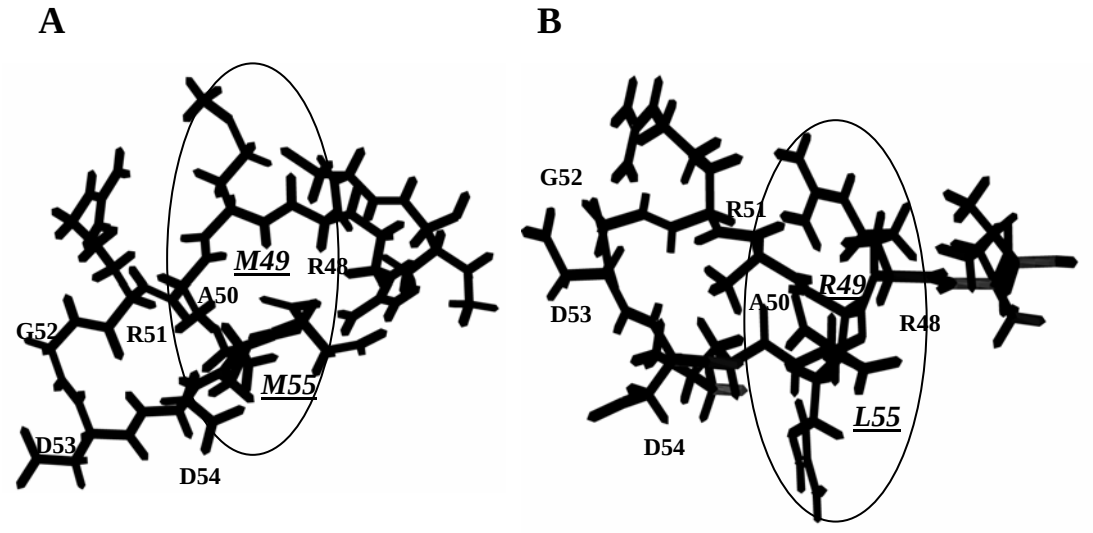

Fig. 6. RGD loop traces of saxatilin and salmosin. (A) is saxatilin and (B) is salmosin. Saxatilin is substituted R49M, L55M of salmosin.

Structure-function of saxatilin:

The three-dimensional fold of saxatilin is similar to that of salmosin except the orientation of RGD loop. Eventhough, saxatilin has three mutational points near to RGD loop (L25→P25, R49→M49, L55→M55) (Fig. 5), saxatilin demonstrates similar biological activity with that of salmosin. According to the previously study, the crooked RGD loop decreases cell recognition function by 10 fold.

Our previous report suggested that saxatilin inhibits glycoprotein (GP) IIb-IIIa binding to immobilized fibrinogen [11]. IC_{50} is the concentration of saxatilin that inhibit the activity of glycoprotein GPIIb-IIIa by 50%. Salmosin inhibits the activity of GPIIb-IIIa with IC_{50} of 2.2 nM, saxatilin does with IC_{50} of 2.0 nM more better than salmosin. IC_{50} of synthesized GRGDSP is 4.2 μM that decreases 2000-fold inhibition. Dose-dependent inhibitions of human platelet aggregation by saxatilin and salmosin, and the synthetic peptide GRGDSP were performed in ADP-induced platelet aggregation assay in the previous paper [11]. IC_{50} values of saxatilin, salmosin, and the synthetic peptide GRGDSP for the intact platelet aggregation were 127 nM, 131 nM, and 270 μM, respectively. Saxatilin has more effective glycoprotein GPIIb-IIIa inhibition and ADP-induced platelet aggregation

activity, compared with salmosin. Synthesized GRGDSP decreases these activity about 2000-fold. Inhibition activity of saxatilin for the function of GPIIb-IIIa and integrin $\alpha_v\beta_3$ is higher than that of salmosin, presumably due to the amino acid substitutions (R49→M49, L55→M55) near RGD motif. Sulfur atom of the methionine residue has non-covalent electron pairs (high electro-negativity) so that methionine residue may prevent oxidation (donating electron) process of the RGD loop. The crystal structure of human $\alpha_v\beta_3$ integrin showed that it is a α/β hetero-dimeric protein that connects cell-cell, cell-matrix adhesion controlling interaction between ligand and receptor. The binding site of $\alpha_v\beta_3$ integrin was determined by photo-affinity cross-linking measurement[6], indicating that RGD loop binds to D119, S121, S123 residues which are located at A-domain of $\alpha_v\beta_3$ integrin. Electro-potential distribution of saxatilin and salmosin (Fig. 6) showed that RGD loop is surrounded by a group of strong negative charges; therefore, the binding site of $\alpha_v\beta_3$ integrin might have a group of positive charges.

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