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Crystallization and preliminary X-ray crystallographic analysis of agkicetin-C from *Deinagkistrodon acutus* venom

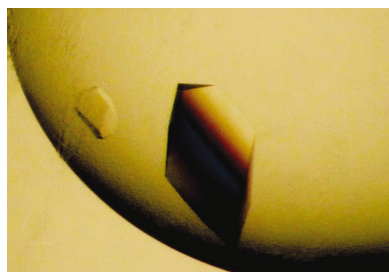
The crystallization and preliminary crystallographic analysis of agkicetin-C, a well known platelet glycoprotein Ib (GPIb) antagonist from the venom of *Deinagkistrodon acutus* found in Anhui Province, China is reported. Crystals of agkicetin-C suitable for structure determination were obtained from 1.8 M ammonium sulfate, 40 mM MES pH 6.5 with 2% (v/v) PEG 400. Interestingly, low buffer concentrations of MES seem to be necessary for crystal growth. The crystals of agkicetin-C belong to space group *C2*, with unit-cell parameters $a = 177.5$, $b = 97.7$, $c = 106.8$ Å, $\beta = 118.5^\circ$, and diffract to 2.4 Å resolution. Solution of the phase problem by the molecular-replacement method shows that there are four agkicetin-C molecules in the asymmetric unit, with a V_M value of 3.4 Å³ Da⁻¹, which corresponds to a high solvent content of approximately 64%. Self-rotation function calculations show a single well defined non-crystallographic twofold axis with features that may represent additional elements of non-crystallographic symmetry.

1. Introduction

Cardiovascular disease has been the most common cause of death in the developed world for many years. As currently used anti-thrombotic drugs such as aspirin, clopidogrel and platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor inhibitors (Coller, 1997) do not specifically inhibit shear-induced platelet aggregation, development of new products that could inhibit platelet aggregation by interfering with the GPIIb–von Willebrand Factor (VWF) interaction would represent a breakthrough in terms of mechanism of action and could possess potential clinical utility (Wu *et al.*, 2002).

Snake-venom components have received more and more attention in recent years because of their activity in coagulation processes. GPIIb-binding proteins have been purified from snake venoms of many different genera (Andrews & Berndt, 2000), including flavo-cetin-A from *Trimeresurus flavoviridis* (Taniuchi *et al.*, 1995), alboaggregin-B (AL-B) from *T. albolabris* (Peng *et al.*, 1991), echi-cetin from *Echis carinatus* (Peng *et al.*, 1993), agkicetin from *Deinagkistrodon acutus* (Chen & Tsai, 1995), jararaca GPIIb-binding protein (GPIIb-bp) from *Bothrops jararaca* (Fujimura *et al.*, 1995), tokaracetin from *T. tokarensis* (Kawasaki *et al.*, 1995), CHH-B from *Crotalus horridus horridus* (Andrews *et al.*, 1996), mamushigin from *Agkistrodon halys blomhoffii* (Sakurai *et al.*, 1998) *etc.* More recently, a traditional platelet glycoprotein VI (GPVI) binding protein, convulxin from *C. durissus terrificus*, has also been reported to bind to native human GPIIb α (Kanaji *et al.*, 2003). These venom proteins exhibit a variety of activities against platelets, suggesting that their exact binding regions and the binding mechanisms to platelet GPIIb are not identical. In-depth investigations of their inhibitory mechanisms are expected to contribute to the design of potent GPIIb antagonists for therapeutic use. As a result, the crystal structures of these proteins have recently attracted much attention (Fukuda *et al.*, 2000; Murakami *et al.*, 2003; Jasti *et al.*, 2004; Huang *et al.*, 2004).

Here, we report the crystallization and preliminary crystallographic analysis of agkicetin-C from the venom of *D. acutus*, a snake from the Anhui Province of China. Like many of these venom components, agkicetin-C is a heterodimer. Agkicetin-C strongly inhibits platelet aggregation induced by ristocetin (Chen & Tsai, 1995), botrocetin or low concentrations of α -thrombin and also



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completely blocks the adhesion of platelets to human type III collagen under high stress without causing thrombocytopenia *in vitro* (to be published elsewhere), all of which strongly suggest the potential value of this unique component for clinical utility.

2. Materials and methods

2.1. Purification, N-terminal sequencing and mass-spectrometric peptide analysis of agkicetin-C

1 g of crude *D. acutus* venom (purchased from Huangshan Institute of Snakes, Anhui, China) was first fractioned by ion-exchange chromatography on DEAE-Sepharose (Amersham-Pharmacia, Uppsala, Sweden) and agkicetin-C was then purified by a minor modification of Chen's method (Chen & Tsai, 1995) (Fig. 1). Fractions containing agkicetin-C were identified in a ristocetin-dependent aggregation assay.

The purity of this agkicetin-C preparation was verified by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining of the protein bands; native polyacrylamide gel electrophoresis was also

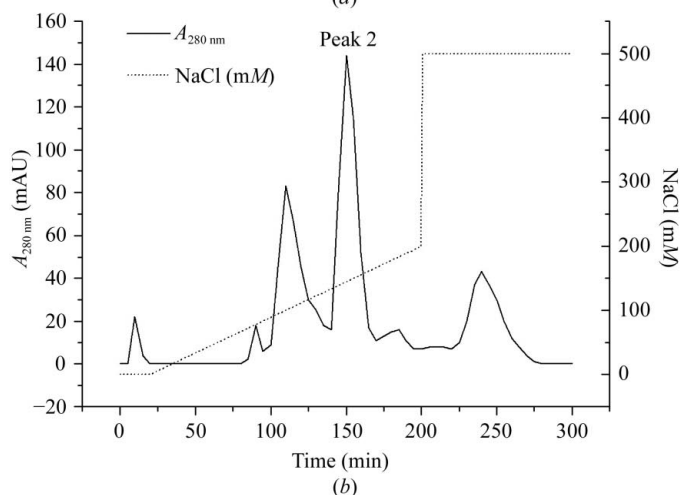
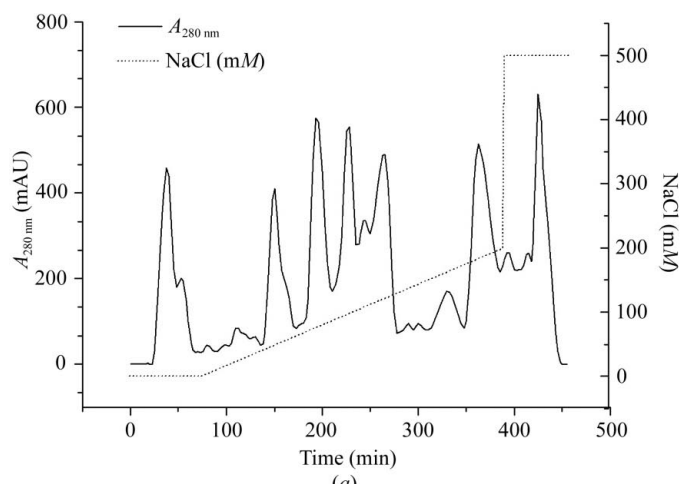


Table 1

Comparison of the experimentally determined molecular weights of trypsin-cleaved fragments of agkicetin-C (MW_T) with the corresponding calculated molecular weights (MW_C) from cDNA sequences.

	MW_C	MW_T	Corresponding fragment sequence
α Subunit	1003.027	1003.329	61–70
	1244.751	1244.399	61–73
	1260.658	1260.376	19–28
	1411.710	1411.397	88–99
	1491.769	1491.190	47–60†
	1952.892	1953.029	29–46†
β Subunit	1106.523	1106.367	101–109
	1225.603	1225.284	114–123
	1353.640	1353.225	110–120
	1360.692	1360.279	89–100
	1580.723	1580.156	101–113
	1654.764	1654.103	22–34
	2448.197	2448.066	89–109

† Consistent with the expectation that the amino acid at position 46 of our protein is Arg.

performed to test the homogeneity under non-denaturing conditions. Purified agkicetin-C was then reduced and *S*-pyridylethylated according to the method of Atoda *et al.* (1991) (Fig. 2) and N-terminal sequencing was performed with a Procise 491 protein sequencer (Applied Biosystems, USA).

For further analysis, protein bands in the SDS-PAGE were excised from the gel and washed with 50 mM $(NH_4)_2CO_3$ /acetonitrile three times. Proteins were reduced, acetylated and digested with trypsin in the gel. After gel extraction, all peptides were collected for mass-spectrometric peptide analysis on a Biflex III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) (Table 1).

2.2. Crystallization

Lyophilized agkicetin-C dissolved in distilled water was screened by typical hanging-drop vapour-diffusion methods with Crystal Screens I and II (Hampton Research, USA). Briefly, 2.0 μ l protein

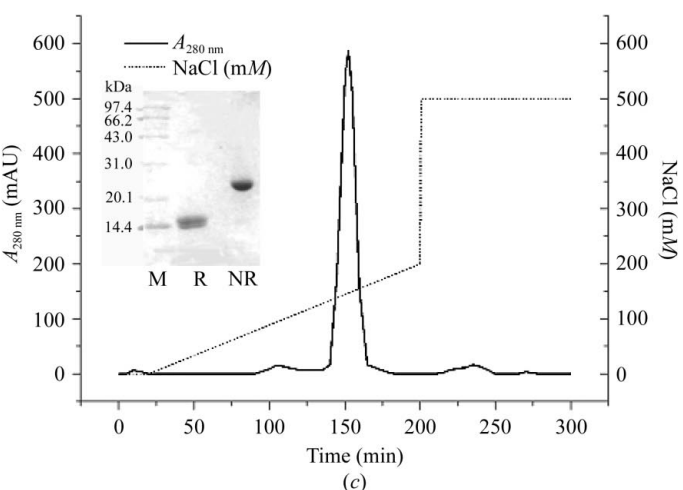


Figure 1

Purification of agkicetin-C. (a) 1 g crude venom from *D. acutus* was dissolved in 20 ml buffer A (20 mM Tris-HCl pH 8.0) and centrifuged at 12 000 rev min^{-1} for 15 min. The supernatant was applied onto a DEAE-Sepharose Fast Flow column (1.6 \times 40 cm) pre-equilibrated with buffer A. The column was washed at a flow rate of 120 ml h^{-1} initially with buffer A until peaks 1 and 2 were eluted and then with an 800 ml linear NaCl gradient (0–200 mM in buffer A). Finally, the column was exhaustively eluted with a high concentration of NaCl (500 mM in buffer A). Peak 4 containing agkicetin-C was pooled. (b) Peak 4 mentioned above was condensed and dialyzed against buffer B (50 mM sodium citrate pH 5.0) overnight at 277 K and then applied onto a CM-Sepharose Fast Flow column (1.6 \times 40 cm) pre-equilibrated with buffer B. A similar strategy to the linear NaCl gradient used above was applied with buffer B and peak 2 was pooled. (c) Peak 2 mentioned above was condensed and dialyzed against buffer A overnight at 277 K and then applied onto a Mono Q HR 5/5 column (3.6 \times 100 mm) pre-equilibrated with buffer A. The column was washed at a flow rate of 60 ml h^{-1} initially with 5 ml buffer A and then with a linear NaCl gradient (0–200 mM in buffer A). Finally, the column was eluted with a high concentration of NaCl (500 mM in buffer A) as mentioned above. The main fraction collected proved to be agkicetin-C.

Table 2

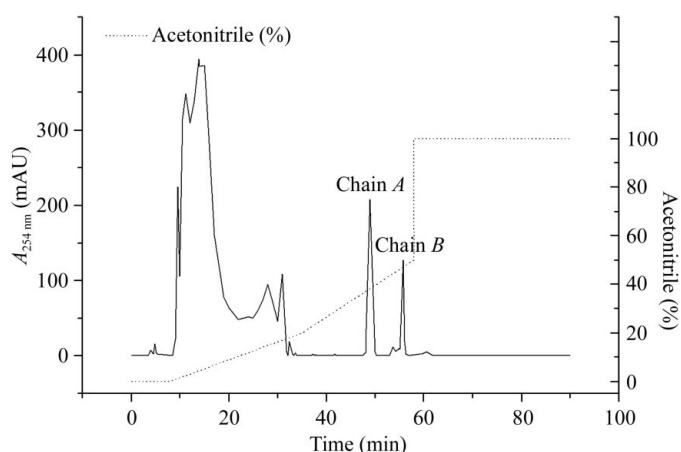
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	C2
Unit-cell parameters	
<i>a</i> (Å)	177.5
<i>b</i> (Å)	97.7
<i>c</i> (Å)	106.8
β (°)	118.5
Temperature (K)	100
Wavelength (Å)	1.1
Resolution (Å)	30.0–2.40
Total No. reflections	209024
No. unique reflections	73801
Completeness† (%)	95.6 (86.4)
$\langle I/\sigma(I) \rangle$	6.7 (2.1)
$R_{\text{merge}}^{\ddagger}$ (%)	7.2 (26.4)

† The completeness is the ratio of number of observed reflections to that of possible reflections. $\ddagger R_{\text{merge}} = \sum_h \sum_j |I(h) - I(h)_j| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the j th observed reflection intensity and $\langle I(h) \rangle$ is the mean intensity of reflection h .

solution (30 mg ml⁻¹) was mixed with 2.0 μ l of the screening agent and equilibrated against 400 μ l reservoir solution at room temperature. Crystals of agkicetin-C appeared after one week under condition No. 38 (1.4 M sodium citrate, 0.1 M Na HEPES pH 7.5) of Crystal Screen I. Unfortunately, these good-looking crystals failed to respond to optimization trials and could not be used for data collection. Only after nearly a year was a small trapezoid-shaped crystal of agkicetin-C found under condition No. 39 (2% PEG 400, 0.1 M Na HEPES pH 7.5, 2.0 M ammonium sulfate) of Crystal Screen I. After carefully searching using various pH values, additives, ions, reservoir solution concentrations *etc.*, we ultimately discovered the optimum condition for crystal growth by the accidental use of a lower buffer concentration. On comparing the crystals grown using different buffer concentrations, we found that different MES concentrations strongly affected crystal growth (data not shown). The best crystals of agkicetin-C (0.3 \times 0.3 \times 0.08 mm; Fig. 3) were finally obtained using 40 mg ml⁻¹ protein mixed with 1.8 M ammonium sulfate, 2% PEG 400, 40 mM MES pH 6.5 at 293 K after three weeks.


Figure 2

Preparation of reduced and *S*-pyridylethylated agkicetin-C. 3 mg agkicetin-C was dissolved in 0.5 ml 0.5 M Tris-HCl buffer pH 8.0 containing 6 M guanidine hydrochloride and 2 mM EDTA. 7 mg dithiothreitol was added to the protein solution, mixed and then incubated for 3 h at 323 K. After the addition of 4-vinylpyridine with a 3:1 molar ratio of 4-vinylpyridine:dithiothreitol, the mixture was further incubated for another 3 h at 323 K and then dialyzed against distilled water and 0.1% TFA solution. Finally, the mixture was applied onto a Delta-Pak C4 column (3.9 \times 150 mm) and sequentially eluted at room temperature with two linear gradients of acetonitrile solution (0–20% and 20–50% containing 0.1% TFA).

2.3. Collection and reduction of X-ray diffraction data

Diffraction data were collected from crystals of agkicetin-C at beamline 3wla, Beijing Synchrotron Radiation Facility, Institute of High Energy Physics, China ($\lambda = 1.1$ Å). Agkicetin-C crystals were mounted in loops after a soaking step in the crystallization solution augmented with 25% (v/v) glycerol as a cryoprotectant. The crystal used in data collection was flash-frozen in a stream of liquid nitrogen at 100 K. Data sets were collected on a MAR CCD detector with a 120 mm crystal-to-detector distance in 1.0° oscillation steps over a range of 140°. Diffraction data were processed with the *autoMAR* program v.1.4.2 (Bartels & Klein, 2003). The statistics of data collection and reduction are listed in Table 2.

3. Results

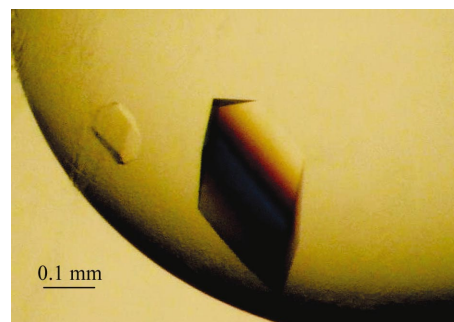
3.1. Purification and determination of agkicetin-C

Agkicetin-C was purified from the venom of *D. acutus* from Anhui Province, China. As a sequence has been reported from the same origin (GenBank accession No. AY091762) that is identical to the α subunit of agkicetin-C except for two substitutions at residues 8 (Ser/Cys) and 46 (Arg/Gly), possible evidence of polymorphism, we further investigated our component. The results of N-terminal sequencing gave DCLPGWSSY for the α subunit and DCPDWSSY for the β subunit, respectively, which proves that the amino acid at position 8 of our protein is Ser. For the other substitution site, we turned to the results of the mass-spectrometric peptide analysis. The molecular weights of the peptide fragments match those calculated from the cDNA sequences well (Chen *et al.*, 2000), but position 46 is at a break between fragments, indicating that there is in fact a trypsin-cleavage site after position 46. The molecular weights of the peptides on either side of the cleavage site closely match the values calculated from the cDNA sequence of agkicetin-C, so that the weight of the evidence is consistent with the amino acid at position 46 of our protein being Arg.

3.2. Preliminary X-ray crystallographic analysis and refinement

The crystals of agkicetin-C belonged to the monoclinic space group C2 and diffracted to 2.4 Å resolution. Molecular-replacement analysis showed that four agkicetin-C molecules were located in the asymmetric unit; the V_M value was 3.4 Å³ Da⁻¹, which is within the expected range (Matthews, 1968). This V_M value corresponds to a solvent content of approximately 64%.

The molecular-replacement analysis was performed with the program *AMoRe* (Navaza, 1994). After eliminating all water molecules, one $\alpha\beta$ heterodimer of the structure of flavocetin-A (PDB code


Figure 3

Crystals of agkicetin-C from 1.8 M ammonium sulfate, 2% PEG 400, 40 mM MES pH 6.5.

1c3a) was chosen as the search model (57 and 66% identity between the α and β subunits of the two proteins, respectively). The rotation search was performed with a radius of 30 Å and an angular step size of 2.5° within the resolution range 15.0–3.0 Å. The correlation coefficient (44.7%) and *R* factor (47.3%) of the initial solution for four agkicetin-C molecules in an asymmetric unit are reasonable and values for the next best solutions were far from these. One obvious self-rotation peak at about a third of the height of the proper crystallographic axes was identified in the Patterson map, suggesting a possible non-crystallographic twofold axis in the asymmetric unit. Results of initial model building with the program *O* (Jones *et al.*, 1991) and refinement with the program package *CNS* v.1.1 (Brünger *et al.*, 1998) confirm a correct solution and intensive crystallographic refinement of the model is under way.

4. Discussion

Agkicetin-C was first reported to have been isolated from *D. acutus* by Chen & Tsai (1995). It was also found to bind to the platelet glycoprotein Ib–IX–V (GPIb–IX–V) complex and to inhibit VWF-induced platelet agglutination when bound. Later, the complete amino-acid sequence was resolved by cDNA cloning and a putative three-dimensional model was constructed based on the crystal structure of the homologous habu coagulation factor IX/X-binding protein (Chen *et al.*, 2000), which was the first three-dimensional structural model of a snake-venom GPIb-binding protein to our knowledge.

Determination of the crystal structure of agkicetin-C has been prevented until now because of difficulties in its crystallization. In this communication, we report the crystallization of agkicetin-C. This is the first crystallization of a snake-venom GPIb-binding protein that only has an inhibitory effect on platelet aggregation to be reported. When compared with the structures of other snake-venom GPIb-binding protein, which can either inhibit or induce platelet aggregation (*e.g.* flavocetin-A and echicetin) or target more than one platelet receptor (*e.g.* convulxin), it is hoped that the crystal structure of agkicetin-C will provide new details of GPIb binding by these snake-venom proteins.

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