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

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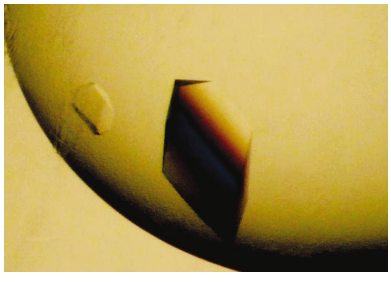

The crystallization and preliminary crystallographic analysis of agkicetin-C, a well known platelet glycoprotein Ib (GPIb) antagonist from the venom of *Deinagkistrodon acutus* found in Anhui Province, China is reported. Crystals of agkicetin-C suitable for structure determination were obtained from 1.8 *M* ammonium sulfate, 40 m*M* 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 *C*2, with unit-cell parameters a = 177.5, b = 97.7, c = 106.8 Å, β = 118.5°, 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 Å^3 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 GPIb–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. GPIb-binding proteins have been purified from snake venoms of many different genera (Andrews & Berndt, 2000), including flavocetin-A from *Trimeresurus flavoviridis* (Taniuchi *et al.*, 1995), alboaggregin-B (AL-B) from *T. albolabris* (Peng *et al.*, 1991), echicetin from *Echis carinatus* (Peng *et al.*, 1993), agkicetin from *Deinagkistrodon acutus* (Chen & Tsai, 1995), jararaca GPIb-binding protein (GPIb-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 GPIbα (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 GPIb are not identical. In-depth investigations of their inhibitory mechanisms are expected to contribute to the design of potent GPIb 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

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 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).

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.

For further analysis, protein bands in the SDS–PAGE were excized from the gel and washed with 50 m*M* $(NH_4)_2CO_3$/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 µl protein

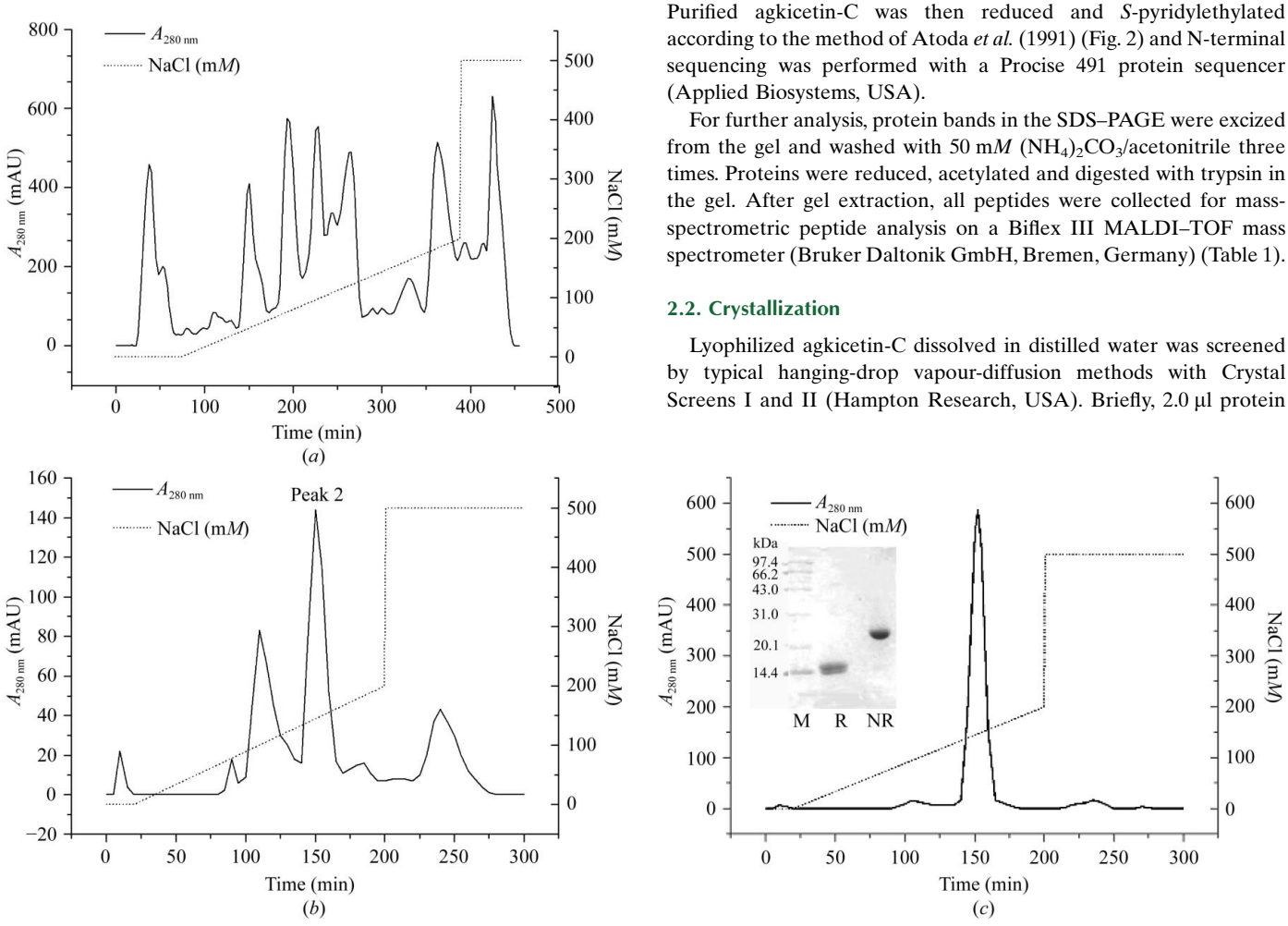

Figure 1
Purification of agkicetin-C. (*a*) 1 g crude venom from *D. acutus* was dissolved in 20 ml buffer *A* (20 m*M* Tris–HCl pH 8.0) and centrifuged at 12 000 rev min^{-1} for 15 min. The supernatant was applied onto a DEAE-Sepharose Fast Flow column (1.6 × 40 cm) pre-equilibrated with buffer *A*. 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 m*M* in buffer *A*). Finally, the column was exhaustively eluted with a high concentration of NaCl (500 m*M* in buffer *A*). Peak 4 containing agkicetin-C was pooled. (*b*) Peak 4 mentioned above was condensed and dialyzed against buffer *B* (50 m*M* sodium citrate pH 5.0) overnight at 277 K and then applied onto a CM-Sepharose Fast Flow column (1.6 × 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 × 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 m*M* in buffer *A*). Finally, the column was eluted with a high concentration of NaCl (500 m*M* 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	*C*2
Unit-cell parameters	
a (Å)	177.5
b (Å)	97.7
c (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)
$R_{\rm merge}$‡ (%)	7.2 (26.4)

† The completeness is the ratio of number of observed reflections to that of possible reflections. ‡ $R_{\rm merge} = \sum_h \sum_j |\langle I(h)\rangle - I(h)_j| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the jth 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 µl of the screening agent and equilibrated against 400 µl 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 × 0.3 × 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 m*M* MES pH 6.5 at 293 K after three weeks.

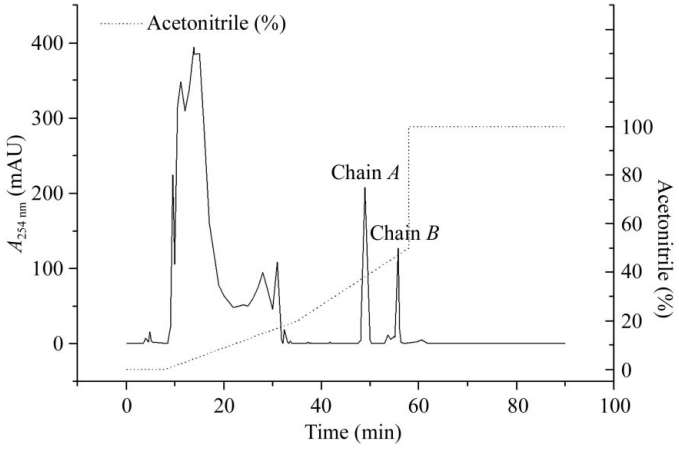

Figure 2
Preparation of reduced and *S*-pyridylethylated agkicetin-C. 3 mg agkicetin-C was dissolved in 0.5 ml 0.5 *M* Tris–HCl buffer pH 8.0 containing 6 *M* guanidine hydrochloride and 2 m*M* EDTA. 7 mg dithiothreitol was added to the protein solution, mixed and then incubated for 3 h at 323 K. After 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 × 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 (λ = 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 DCPPDWSSY 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 *C*2 and diffracted to 2.4 Å resolution. Molecular-replacement analysis showed that four agkicetin-C molecules were located in the asymmetric unit; the $V_{\rm M}$ value was 3.4 Å^3 Da^{-1}, which is within the expected range (Matthews, 1968). This $V_{\rm M}$ 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 *αβ* heterodimer of the structure of flavocetin-A (PDB code

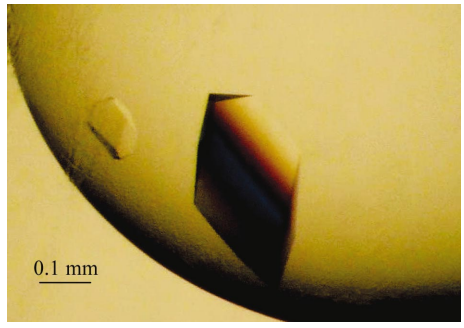

Figure 3
Crystals of agkicetin-C from 1.8 *M* ammonium sulfate, 2% PEG 400, 40 m*M* MES pH 6.5.

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 was 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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