Acta Crystallographica Section D
**Biological Crystallography**
ISSN 0907-4449

# Purification, partial characterization and crystallization of acucetin, a protein containing both disintegrin-like and cysteine-rich domains released by auto-proteolysis of a P-III-type metalloproteinase AaH-IV from *Agkistrodon acutus* venom

**Jianye Zang,[a,b]† Zhiqiang Zhu,[a,b] Yang Yu,[a,b] Maikun Teng,[a,b] Liwen Niu,[a,b]* Qingqiu Huang,[c]† Qun Liu[c] and Quan Hao[c]**

[a]Key Laboratory of Structural Biology, Chinese Academy of Sciences, 96 Jinzhai Road, Hefei, Anhui 230026, People's Republic of China, [b]Department of Molecular and Cell Biology, School of Life Sciences, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, People's Republic of China, and [c]MacCHESS, Cornell High Energy Synchrotron Source, Cornell University, NY 14853, USA

† These authors made equivalent contributions.

Correspondence e-mail: lwniu@ustc.edu.cn

Received 5 July 2003
Accepted 17 September 2003

AaH-IV, a P-III-type metalloproteinase found in *Agkistrodon acutus* venom, readily cleaves itself to release a stable protein named acucetin at 310 K under neutral and weakly alkaline conditions. A partial amino-acid residue sequence of acucetin indicates that the protein has a high homology to snake-venom proteins containing both disintegrin-like and cysteine-rich domains. Acucetin has been crystallized in space group *R*32, with hexagonal unit-cell parameters $a = b = 155.98$, $c = 76.07$ Å. The $V_M$ value of about 2.97 Å$^3$ Da$^{-1}$ suggests the presence of only one molecule in the asymmetric unit.

## 1. Introduction

The most severe consequence of injection with viperid and crotalid venoms is local or systemic haemorrhage. Snake-venom metalloproteinases (SVMPs), which act synergistically with many other snake-venom toxins, are primarily responsible for bleeding (Bjarnason & Fox, 1994; Hite *et al.*, 1994). The members of the SVMP family can be classified into four types, P-I, P-II, P-III and P-IV, according to their domain organization. P-I-type SVMPs possess only a metalloproteinase domain, which is common to the four types. The metalloproteinase domain is followed sequentially by a disintegrin-like domain (in P-II-type SVMPs) or by a disintegrin-like domain and a cysteine-rich domain (in P-III-type SVMPs) at its C-terminus. In P-IV-type SVMPs, an additional C-lectin domain is attached by a disulfide bond (Hite *et al.*, 1994; Jia *et al.*, 1996).

Some P-III-type SVMPs, including HR1A, HR1B, HT-1, brevilysin H6 and acurhagin (Takeya *et al.*, 1993; Fujimura *et al.*, 2000; Wang & Huang, 2002), can be degraded autoproteolytically *in vitro*, releasing a stable fragment with a molecular weight of about 30 kDa composed of a pair of domains, one disintegrin-like and one cysteine-rich, which we will refer to as a D/C-motif. A few snake-venom D/C-motif-containing proteins, including jararhagin-C, jaracetin and catrocollastatin-C, have also been suggested to be autoproteolysis products of their precursors (Usami *et al.*, 1994; De-Luca *et al.*, 1995; Shimokawa *et al.*, 1997), although no related auto-degradation experiments have been carried out *in vitro*.

Naturally purified or recombinant D/C-motif-containing proteins, such as jararhagin-C, atrolysin A/DC, catrocollastatin-C and halysetin, can inhibit platelet aggregation induced by collagen, ADP or both (Usami *et al.*, 1994; Shimokawa *et al.*, 1997; Jia *et al.*, 1997; Liu *et al.*, 2000). The inhibition of collagen-induced platelet aggregation is mediated by the adhesion of the disintegrin-like domain to platelet membrane receptor integrin $\alpha_2\beta_1$. Although several D/C-motif peptides containing the sequence motif SECD have been synthesized, only cyclic versions with more restricted conformations are effective in inhibiting platelet aggregation (Shimokawa *et al.*, 1997; Jia *et al.*, 1997, 2000; Ito *et al.*, 2001), indicating the importance of correct conformation in conferring biological function in these proteins. To our knowledge, no three-dimensional structure has been experimentally determined of either a P-III-type SVMP or a D/C-motif-containing protein.

A P-III-type SVMP named AaH-IV was previously purified from *Agkistrodon acutus* venom and crystallized (Zhu *et al.*, 1996, 1997). The purpose of this paper is to report the purification, partial characterization and crystallization of acucetin, a stable D/C-motif-containing product from the auto-degradation of AaH-IV under relatively mild conditions.

## 2. Materials and methods

### 2.1. Materials

Lyophilized *A. acutus* venom was obtained from Tunxi Snakebite Institute (Anhui, China). DEAE-Sepharose was from Pharmacia (Sweden). Other reagents and chemicals were of analytical grade from commercial sources.

### 2.2. Preparation of acucetin and sequencing of N-terminal residues

AaH-IV from *A. acutus* venom was purified at 277 K according to the procedure described previously (Zhu *et al.*, 1997). Briefly, 1.5 g of crude venom was dissolved in 30 ml of loading buffer (0.02 *M* Tris–HCl pH 8.0) and centrifuged at 12 000 rev min$^{-1}$ for 20 min. The supernatant was applied to a DEAE-Sepharose Fast Flow column (1.6 × 40 cm) pre-equilibrated with loading buffer. The column was washed with loading buffer for 80 min at a flow rate of 180 ml h$^{-1}$ and then eluted with a linear NaCl gradient from 0.0 to 0.2 *M* (in 800 ml of loading buffer). Finally, the column was eluted with 100 ml of 0.02 *M* Tris–HCl buffer pH 8.0 containing 0.5 *M* NaCl. The fractions containing AaH-IV were pooled and stored in lyophilized form for further use.

Using single-step DEAE-Sepharose chromatography, acecetin could be purified from AaH-IV solution (1 mg ml$^{-1}$ in 0.1 *M* Tris–HCl pH 8.0) incubated for 20 h at 310 K (see Fig. 1).

N-terminal sequencing of acucetin took place using the Edman degradation method in a Procise 491 protein sequencer (ABI, USA).

### 2.3. Crystallization

Acucetin pooled from DEAE-Sepharose chromatography (Fig. 1) was desalted against 20 m*M* Tris–HCl buffer pH 7.2 and concentrated to approximately 2 mg ml$^{-1}$ in Tris–HCl buffer. Crystallization was performed using the hanging-drop vapour-diffusion method at room temperature (McPherson, 1982). 2 µl of protein solution without any additives was mixed with 2 µl of mother liquor (containing 1.2 *M* ammonium sulfate, 10 m*M* $CuSO_4$, 0.1 *M* sodium cacodylate pH 6.4) and then equilibrated against 300 µl of reservoir solution (containing 0.9 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.6). Microcrystals appeared after 24 h and had reached dimensions of approximately 0.3 × 0.3 × 0.25 mm two weeks later (Fig. 2).

### 2.4. Collection and reduction of X-ray diffraction data

The X-ray diffraction data were collected on Cornell High Energy Synchrotron Source MacCHESS beamline F1 at a wavelength of 0.9160 Å. A total of 180 imaging frames were recorded from one crystal cooled by liquid nitrogen to a temperature of about 100 K. The composition of the cryoprotectant was 1.2 *M* ammonium sulfate, 10 m*M* $CuSO_4$, 20% glycerol and 0.1 *M* sodium cacodylate pH 6.4. The crystal-to-detector distance, oscillation angle and exposure time for each image frame were set at 200 mm, 1° and 20 s, respectively. The diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The data-collection and processing statistics are listed in Table 1.

**Table 1**
Statistics of diffraction data collection and reduction.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | *R*32 |
| Unit-cell parameters (Å) | $a = b = 155.98$, $c = 76.07$ |
| No. of observed reflections | 178793 |
| No. of independent reflections | 34460 |
| Resolution limits (Å) | 20–2.2 (2.24–2.2) |
| $I/\sigma(I)$ | 21.0 (7.7) |
| $R_{\rm merge}$† (%) | 6.8 (26.4) |
| Completeness‡ (%) | 98.5 (88.7) |

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

## 3. Results and discussion

AaH-IV, a P-III-type metalloproteinase naturally occurring in *A. acutus* venom, undergoes autoproteolysis easily at 310 K under natural or weakly alkaline conditions, leading to the release of a 30 kDa protein named acecetin. On alignment using the software package *CLUSTALW* (Thompson *et al.*, 1994), the N-terminal amino-acid residue sequence of acucetin (LGTDIISPPLCGNELLEVGEECDCGTPENCQ) shows a high homology to that of the spacer region in other P-III type SVMPs, indicating that acucetin has no N-terminal metalloproteinase domain. Furthermore, two trypsin-cleaved fragments of acecetin have also been purified (data not shown) and

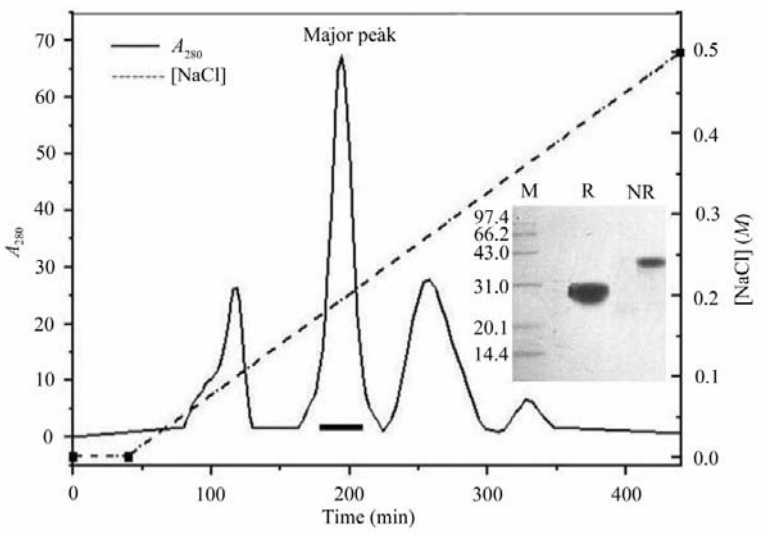


**Figure 1**
Preparation of acucetin. The mixture from autoproteolysis of AaH-IV was applied to a DEAE-Sepharose Fast Flow column (1.6 × 40 cm) pre-equilibrated with loading buffer (0.02 *M* Tris–HCl pH 8.0) and then sequentially eluted with loading buffer for 80 min at a flow rate of 180 ml h$^{-1}$ and with a 1000 ml linear NaCl gradient (0–0.5 *M* in loading buffer). Acucetin fractions in the major peak (indicated by the bar) were pooled and concentrated. Inset: a 12% SDS–PAGE analysis of the purified acucetin was carried out under non-reducing (NR) and reducing (R) conditions, respectively. 5 µl of acecetin (2 mg ml$^{-1}$) was mixed with 5 µl of loading buffer in the presence (R) or absence (NR) of 0.1 *M* β-mercaptoethanol. The left lane (M) contains standard protein molecular-weight markers (kDa).

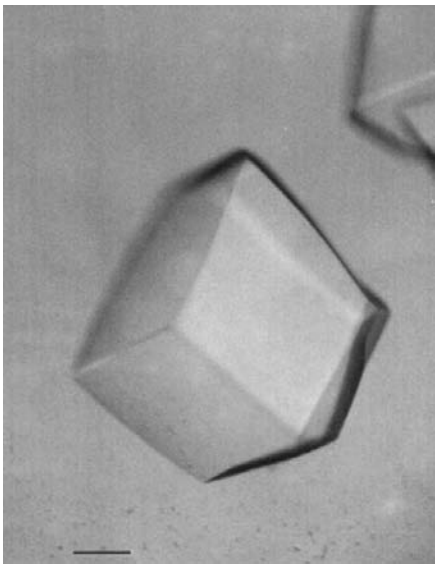

**Figure 2**
Photomicrograph of a crystal of acucetin. The bar at the bottom is 0.1 mm in length.

sequenced (LYCKDDSPGQNNPCKMFYS and MFYSNDDEHKGMVLPGTK) and suggest that acucetin is homologous to several snake-venom D/C-motif-containing proteins such as catrocollastatin-C, jararhagin-C, halysetin, HT-1-31K, HR1A-32K and atrolysin-A/DC (Shimokawa *et al.*, 1997; Usami *et al.*, 1994; Liu *et al.*, 2000; Takeya *et al.*, 1993; Jia *et al.*, 1997). Indeed, acucetin has no haemorrhagic and pH-dependent caseinolytic activities and instead exhibits significant inhibitory effects on ADP- and collagen-induced platelet aggregation (data to be published elsewhere).

Acucetin has been crystallized in space group *R*32, with hexagonal unit-cell parameters $a = b = 155.98$, $c = 76.07$ Å. The $V_M$ value (Matthews, 1968) is estimated to be 2.97 Å$^3$ Da$^{-1}$, suggesting the presence of only one protein molecule per asymmetric unit. The preparation of acucetin crystals suitable for X-ray diffraction is a good start towards structure determination. Both structure determination and sequencing of the entire acucetin fragment are being carried out in our laboratory.

Financial support for this project to LN and MT was provided by research grants from China National Natural Science Foundation (grant Nos. 39870108, 30025012 and 30121001), 973 Plan of China Ministry of Science and Technology (grant No. G1999075603) and Chinese Academy of Sciences (grant Nos. STZ-2-07 and STZ98-2-12). The authors acknowledge with appreciation the kind help of Mr Zhi-qiang Yu in the sequencing of protein samples.

## References

Bjarnason, J. B. & Fox, J. W. (1994). *J. Pharmacol. Exp. Ther.* **62**, 321–372.
De-Luca, M., Ward, C. M., Ohmori, K., Andrews, R. K. & Berndt, M. C. (1995). *Biochem. Biophys. Res. Commun.* **206**, 570–576.
Fujimura, S., Oshikawa, K., Terada, S. & Kimoto, E. (2000). *J. Biochem.* **128**, 167–173.
Hite, L. A., Jia, L.-G., Bjarnason, J. B. & Fox, J. W. (1994). *Arch. Biochem. Biophys.* **308**, 182–191.
Ito, M., Hamako, J., Sakurai, Y., Matsumoto, M., Fujimura, Y., Suzuki, M., Hashimoto, K., Titani, K. & Matsui, T. (2001). *Biochemistry*, **40**, 4503–4511.
Jia, L.-G., Shimokawa, K.-I., Bjarnason, J. B. & Fox, J. W. (1996). *Toxicon*, **34**, 1269–1276.
Jia, L.-G., Wang, X.-M., Shannon, J. D., Bjarnason, J. B. & Fox, J. W. (1997). *J. Biol. Chem.* **272**, 13094–13102.
Jia, L.-G., Wang, X.-M., Shannon, J. D., Bjarnason, J. B. & Fox, J. W. (2000). *Arch. Biochem. Biophys.* **373**, 281–286.
Liu, J. W., Du, X. Y., Liu, P., Chen, X., Xu, J. M., Wu, X. F. & Zhou, Y. C. (2000). *Biochem. Biophys. Res. Commun.* **278**, 112–118.
McPherson, A. (1982). *Preparation and Analysis of Protein Crystals.* New York: Wiley.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Shimokawa, K.-I., Shannon, J. D., Jia, L.-G. & Fox, J. W. (1997). *Arch. Biochem. Biophys.* **343**, 35–43.
Takeya, H., Nishida, S., Nishino, N., Makinose, Y., Omori-Satoh, T., Nikai, T., Sugihara, H. & Iwanaga, S. (1993). *J. Biochem.* **113**, 473–483.
Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). *Nucleic Acids Res.* **22**, 4673–4680.
Usami, Y., Fujimura, Y., Miura, S., Shima, H., Yoshida, E., Yoshioka, A., Hirano, K., Suzuki, M. & Titani, K. (1994). *Biochem. Biophys. Res. Commun.* **201**, 331–339.
Wang, W.-J. & Huang, T.-F. (2002). *Thromb. Haemost.* **87**, 641–650.
Zhu, Z., Gong, W., Niu, L., Teng, M. & He, H. (1996). *Acta Cryst.* D**52**, 407–408.
Zhu, Z., Gong, W., Zhu, X., Teng, M. & Niu, L. (1997). *Toxicon*, **35**, 283–292.