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Purification, crystallization and preliminary X-ray diffraction analysis of haemorrhagin IV from the snake venom of *Agkistrodon acutus*

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Abstract

Haemorrhagin IV, a medium molecular weight haemorrhagin from the snake venom of *Agkistrodon acutus* (AaHIV), has been purified and crystallized. The molecular weight and isoelectric point of AaHIV are 44 kDa and p*I* 5.0, respectively. The crystal belongs to space group $C222_1$ with unit-cell dimensions of $a = 124.2$, $b = 114.5$, $c = 98.4$ Å, and could diffract X-rays to 3.0 Å, resolution. There are one or two molecules in the crystallographic asymmetric unit.

Haemorrhagins are metalloproteinases which exist widely in many kinds of snake venoms (Bjarnasson & Fox, 1994) and can cause local haemorrhage after injection into experimental animals. They can be divided into three classes based on their sizes: class I, the small toxins, having molecular weights of 20–30 kDa; class II, the medium-size toxins, with molecular masses of 30–60 kDa; and class III, the large and potent haemorrhagic toxins, having molecular masses of 60–100 kDa (Bjarnasson & Fox, 1989). The amino-acid sequences of haemorrhagic proteins are highly conserved (Gomis-Rüth *et al.*, 1994). Each haemorrhagin has a similar low molecular weight metalloproteinase domain. Three other domains, disintegrin, Cys-rich and lectin domains, are appended in the medium- and large-sized haemorrhagins (Bjarnasson & Fox, 1994). Two crystal structures of low molecular weight snake venom metalloproteinases, adamalysin II (Gomis-Rüth, Kress & Bode, 1993; Gomis-Rüth *et al.*, 1994) and atrolysin C (Zhang *et al.*, 1994) were solved recently. No medium or high molecular weight haemorrhagins crystals have been reported. It has been assumed that the additional domains could enhance haemorrhagic activities, but details are unknown.

Three low molecular weight haemorrhagins, haemorrhagin I, II and III, were isolated from the snake venom of *Agkistrodon acutus* (Xu, Wang, Liu & Lu, 1981). We report here the purification, crystallization and preliminary X-ray diffraction studies of a medium size haemorrhagin, AaHIV, from the same venom.

The crude venom powder was dissolved in Tris–HCl buffer (0.02 *M*, pH 8.0). The venom solution was applied to a DEAE–Sepharose Fast Flow (Pharmacia) column which had been equilibrated with the same buffer, and then eluted with a linear gradient from Tris–HCl buffer (0.02 *M*, pH 8.0) to Tris–HCl buffer (0.02 *M*, pH 6.0) containing 0.5 *M* NaCl. The last fraction, which contained AaHIV was collected and further purified by gel filtration on an S-200 Sephacryl (Pharmacia) column equilibrated and eluted with 0.15 *M* NaCl. The major eluting fraction was collected, dialyzed against distilled water and concentrated. All purification was carried out at room temperature (about 288 K). The purified AaHIV migrated as a single band on polyacrylamide gel electrophoresis. The molecular weight was determined to be 44 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The isoelectric point of 5.0 was obtained by isoelectric focusing on polyacrylamide gel plates.

Haemorrhagic and lethal activities were assayed according to the methods of Kondo, Kondo, Ikezawa, Murata & Ohsaka, (1960) and Litchfield &Wilcoxon (1949), respectively, which showed that AaHIV possessed high haemorrhagic activity with a minimum haemorrhagic dose of 0.4 µg but no lethal activity.

AaHIV was crystallized using the hanging-drop vapor-diffusion method (McPherson, 1982) at room temperature. Either polyethylene glycol (PEG) 4000 or 2-methyl-2,4-pentanediol (MPD) could be used to grow the crystals. In the PEG 4000 experiments, 5 µl of the precipitating solution containing 20%(*w*/*v*) PEG 4000 was mixed with 5 µl of the 20 mg ml^{-1} AaHIV solution and then equilibrated against 0.5 ml of precipitating solution. Both the precipitating and protein solution were buffered with 0.01 *M* citric acid–sodium citrate at pH 5.6. In the MPD experiments, the MPD concentration in the precipitating solution was 50%(*v*/*v*) and the buffer was 0.01 *M* acetic acid–sodium acetate at pH 6.0.

One week after diffusion, cuboid crystals with largest size 0.7 × 0.4 × 0.2 mm appeared in both PEG and MPD. Diffraction data from a crystal from PEG were collected using a Siemens X200B area detector mounted on a Rigaku rotating-anode X-ray generator at National Laboratory of Biomacromolecules, Peking. Cu $K\alpha$ radiation was generated at 10 kW. The crystal-to-detector distance was set to 150 mm. The detector 2θ angle was set to 15°. Total of 600 ω-scanning oscillation exposure frames in steps of 0.25° were recorded. The exposure time for each frame was 120 s.

Data reduction was accomplished using the *XENGEN* package (Howard *et al.*, 1987). The crystal diffracted X-rays to 3.0 Å. The unit-cell dimensions were given by *XENGEN* automatically. The unit cell belongs to the orthorhombic system with dimensions $a = 124.2$, $b = 114.5$, $c = 98.4$ Å. After efforts to search for the shortest reciprocal vector, it was clear that this was the reduced cell. Processing with space group $P222$ showed that there were systematic absences of $h + k \neq 2n$ for hkl reflections and $l \neq 2n$ for $00l$ reflections, which implied the space group $C222_1$. The final scaling was completed in space group $C222_1$ and the results are listed in Table 1. On basis of the molecular weight and cell dimensions, one or two molecules in the asymmetric unit are both possibilities, corresponding to V_m values of 4.0 and 2.0 Å^3 Da^{-1}, respectively, (Matthews, 1968).

Table 1. *Data collection and processing of AaHIV crystals*

Space group used in data processing	$C222_1$
Unit-cell dimensions	
a (Å)	124.2
b (Å)	114.5
c (Å)	98.4
Resolution limit* (Å)	3.05
No. of unique reflections	11569
Completeness† (%)	84.2
Last shell (Å) (completeness in %)	3.19–3.05 (72.1)
Reflections with $I/\sigma > 2.0$ (%)	
Total	80.3
Last shell (3.19–3.05 Å)	41.2
R_{merge}‡ (%)	6.60

* This represents the limit of the data we have collected. † Completeness is the ratio of the observed to the possible number of unique reflections. ‡ $R_{\text{merge}} = \sum_h \sum_i |I(h)_i - \langle I(h)_i \rangle| / \sum_h \sum_i I(h)_i$.

Diffraction data from a crystal from MPD were also collected as described above, but the crystal diffracted X-rays only to 5.0 Å spacings. The crystals grown from MPD appear to be isomorphous with those grown from PEG.

AaHIV is the first medium-sized snake venom haemorrhagin crystallized. There are no models of similar proteins at present. A search for heavy-atom derivatives is in progress.

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