Purification, N-terminal sequencing, partial characterization, crystallization and preliminary crystallographic analysis of two glycosylated serine proteinases from *Agkistrodon acutus* venom

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AaV-SP-I and AaV-SP-II, two glycosylated serine proteinases from *Agkistrodon acutus* venom with fibrinogenolysis and esterolysis activities, have been purified to homogeneity by three-step ion-exchange chromatography. Estimated by SDS–PAGE, the molecular weights of AaV-SP-I and AaV-SP-II are about 32 and 31 kDa under reducing conditions and 26 and 25 kDa under non-reducing conditions, respectively. The first 24 N-terminal amino-acid residues are the same in both sequences and display a high homology with those of several snake-venom serine proteinases. However, the proteins possess obviously distinct carbohydrate contents. Using the conventional hanging-drop vapour-diffusion method, single crystals of both enzymes were grown that were suitable for X-ray diffraction analysis. The crystals of AaV-SP-I and AaV-SP-II belong to space groups $P2_12_12_1$ and $C2$, respectively. In each case there is only one molecule in the asymmetric unit.

1. Introduction

Snake-venom serine proteinases (SV-SPs) often show functional properties that differ from those of non-snake-venom serine proteinases, even though SV-SPs are classified into the subfamily of trypsin/kallikrein-like serine proteinase (Matsui *et al.*, 2000; Parry *et al.*, 1998; Braud *et al.*, 2000; Pirkle, 1998). SV-SPs display a wide substrate specificity, interacting with many components existing in blood or possessing kallikrein-like activities. Some SV-SPs are referred as to fibrinogenases or thrombin-like enzymes (TLEs) because they specifically hydrolyze or clot fibrinogen. These include batroxobin from *Bothrops atrox moojeni* venom (Itoh *et al.*, 1987) and ancrod from *Calloselama rhodostoma* (Nolan *et al.*, 1976). SV-SPs from different species have functionally distinctive properties. For example, TSV-PA from *Trimeresurus stejnegeri* venom is a plasminogen activator (Zhang *et al.*, 1995), ACC-C from *Agkistrodon contortrix contortrix* venom functions as an anticoagulant protein C activator (Kisiel *et al.*, 1987) and PA-BJ and KN-BJ from *B. jararaca* venom are proteins with platelet-aggregation activating and kinin-releasing activities, respectively (Serrano *et al.*, 1995, 1998). RVV-V is a factor V activator existing in *Vipera russelli* venom (Kisiel *et al.*, 1976). Moreover, with different glycosylated sites and glycoforms, some SV-SPs contain carbohydrates, especially N-linked oligosaccharide chains (Pfeiffer *et al.*, 1992; Tanaka *et al.*, 1992; Lochnit & Geyer, 1995). The structural features accounting for these functional properties are not yet fully understood, although the general sequence alignment indicates that approximately six variable-sequence regions might account for the substrate specificities and other functional properties (Braud *et al.*, 2000).

The crystal structure of TSV-PA is the only SV-SP structure deposited in the Protein Data Bank (Parry *et al.*, 1998) to date. With the aim of obtaining further structural information, this paper reports the purification, N-terminal sequencing, partial characterization, crystallization and preliminary crystallographic analysis of AaV-SP-I and AaV-SP-II, two glycosylated serine proteinases from *Agkistrodon acutus* venom.

2. Materials and methods

2.1. Materials

Dried crude *A. acutus* venom was purchased from the Tunxi Snakebite Institute (Anhui Province, China). DEAE-Sepharose and CM-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). *p*-Toluenesulfonyl arginine methyl ester (TAME) and standard proteins for molecular-weight estimation were produced by Shanghai Dongfeng Biochemical Technology Co. (China). The following reagents were products from Sigma Co.: human and bovine fibrinogen, phenyl-methanesulfonyl fluoride (PMSF), heparin, aprotinin, leupeptin, hirudin, *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) and soybean trypsin inhibitor (SBTI) (type I-S). Endogly-

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cosidase F from *Flavobacterium meningosepticum* was obtained from Roche Co. Other reagents and chemicals were of analytical grade from commercial sources.

2.2. Purification, sequencing of N-terminal amino-acid residues, estimation of molecular weight and carbohydrate content

AaV-SP-I and AaV-SP-II were isolated and purified by three-step ion-exchange chromatography from the crude *A. acutus* venom (see Fig. 1). The molecular weight was determined from conventional SDS–PAGE and MALDI–TOF mass spectra. The carbohydrate contents were estimated according to the molecular-mass differential between the native and deglycosylated forms. The deglycosylated samples were

(a)

(b)

(c)

prepared using endoglycosidase F from *F. meningosepticum*.

The N-terminal amino-acid residue sequences of the two proteins were determined by the conventional Edman degradation method using a sequencer (ABI 475A) at the School of Life Sciences, Hunan Normal University, China.

2.3. Assays for activities of arginine esterolysis and fibrinogenolysis

Arginine esterase activity was detected according to the conventional method (Nolan *et al.*, 1976) with TAME as a substrate. The kinetic parameters (K_M and k_{cat}) were determined by a double-reciprocal Lineweaver–Burk plot (initial velocity *versus* substrate concentration).

The fibrinogenolytic activity was assayed by conventional SDS–PAGE (see Fig. 2). The hydrolysis of the three fibrinogen chains (Aα, Bβ, γ) was evaluated by the changes in the migrated distances of the three corresponding intact chains.

2.4. Crystallization

Both AaV-SP-I and AaV-SP-II were crystallized by the conventional hanging-drop vapour-diffusion method. 2 µl of AaV-SP-I protein solution (10 mg ml^{-1} in distilled water) was mixed with an equal volume of reservoir solution [30 m*M* sodium cacodylate pH 6.3, containing 27%(*w/v*) Li$_2$SO$_4$]

and equilibrated against the reservoir solution. A few microcrystals appeared in the hanging drops at room temperature (about 293–298 K) 4 d later. A larger crystal with dimensions of about $0.3 \times 0.3 \times 0.6$ mm was harvested one month later.

Crystal Screen I and II kits were utilized to search for the initial growth conditions of AaV-SP-II crystals. By improving the crystallization conditions at room temperature, an AaV-SP-II crystal with dimensions of about $0.8 \times 0.4 \times 0.4$ mm finally appeared in hanging drops made up by mixing 2 µl protein solution (12 mg ml^{-1} in distilled water) with an equal volume of reservoir solution [containing 0.2 *M* ammonium sulfate and 30%(*w/w*) PEG 4000] and equilibrated against the reservoir solution for two months.

2.5. Collection and processing of X-ray diffraction data

X-ray diffraction data from AaV-SP-I and AaV-SP-II crystals were collected at room temperature using a MAR Research image plate (diameter 300 mm) mounted on an X-ray generator with a graphite monochromator and sealed copper-target tube in our laboratory. The operating tube voltage and tube current were 40 kV and 50 mA, respectively. A data set of 180 imaging frames from one AaV-SP-I crystal was recorded at a 135 mm crystal-to-detector distance with a 1° oscillation angle and 560 s exposure time per imaging frame. Similarly, a total of 180 imaging frames from one AaV-SP-II crystal were collected using a 150 mm crystal-to-detector distance with a
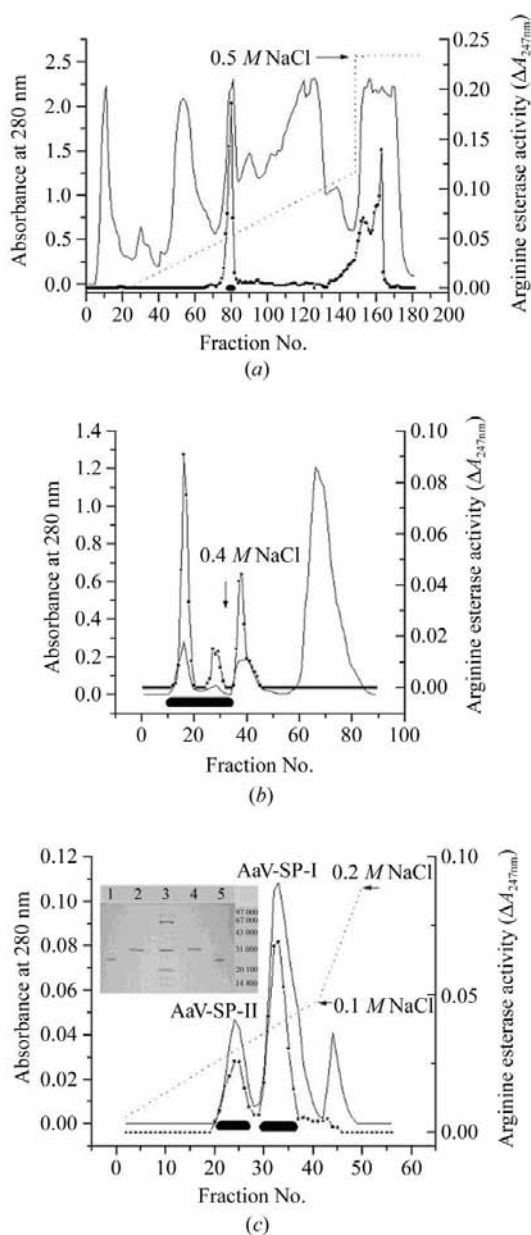
Figure 1
Purification of AaV-SP-I and AaV-SP-II from *A. acutus* venom. (*a*) 1.5 g of crude venom was dissolved in 50 ml buffer *A* (20 m*M* Tris–HCl pH 8.2) and left for 2 h at 277 K. The insoluble materials were removed by centrifugation (4500*g*) at 277 K for 20 min. The supernatant was then applied to a DEAE-Sepharose column (1.6 × 40 cm) pre-equilibrated with buffer *A*. The effluent was monitored at 280 nm and adjusted to a flow rate of 134 ml h^{-1}. After the first peak was washed out, the column was sequentially eluted with a linear gradient made by mixing 400 ml of buffer *A* with an equal volume of buffer *B* (20 m*M* Tris–HCl pH 8.2 containing 0.25 *M* NaCl) and 0.5 *M* NaCl solution. The effluent was collected for 3 min per glass tube and investigated for arginine esterase activity. Three major peaks with arginine esterase activity were observed; the first peak with arginine esterase activity was pooled, ultra-filtered and desalted to a volume of 20 ml. The absorbance at 280 nm is indicated by a solid line, the salt gradient by a dashed line, arginine esterase activity by squares and the collected fractions by a solid bar. (*b*) The protein fraction pooled from previous chromatography was loaded onto a CM-Sepharose column (1.8 × 20 cm) pre-equilibrated with a solution containing 50 m*M* sodium acetate buffered at pH 5.0 and then eluted with same solution at a flow rate of 94 ml h^{-1}. The first two elution peaks (indicated by a solid bar) possessing arginine esterase activity were collected, ultra-filtered and desalted to a volume of 10 ml for further purification. The other materials bound on the column were eluted with the solution of 0.4 *M* NaCl (shown by an arrow). (*c*) The protein fraction pooled from the second step was applied to another DEAE-Sepharose column (1.0 × 20 cm) pre-equilibrated with buffer *A*. Using a flow rate of 74 ml h^{-1}, the column was eluted with a linear gradient made by mixing 100 ml of buffer *A* with an equal volume of buffer *C* (20 m*M* Tris–HCl pH 8.2 containing 0.10 *M* NaCl). Two protein peaks were found to possess arginine esterase activity (indicated by solid bars); the major (on the right) and minor (on the left) components were designated AaV-SP-I and AaV-SP-II, respectively. Inset in (*c*): SDS–PAGE of AaV-SP-I (lanes 1 and 2) and AaV-SP-II (lanes 4 and 5). Lanes 1 and 5 are under non-reducing conditions. Lanes 2 and 4 are under reducing conditions containing β-mercaptoethanol. Lane 3: markers for molecular-weight estimation.

$1°$ oscillation angle and 300 s exposure time per imaging frame. The diffraction data were processed using *DENZO* and *SCALE-PACK* (Otwinowski & Minor, 1997). The data-collection and processing statistics are listed in Table 1.

3. Results and discussions

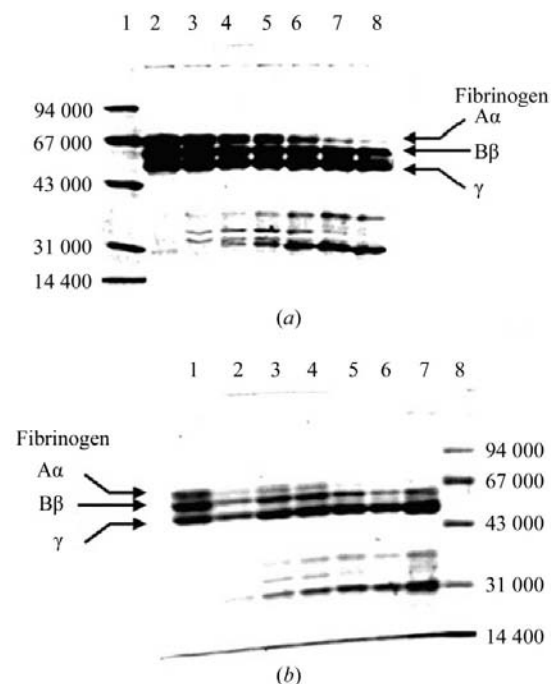Using three-step ion-exchange chromatography with arginine esterase activity tracing

Figure 2
SDS–PAGE analysis of the fibrinogenolytic activity of AaV-SP-I and AaV-SP-II. 10 μl of bovine fibrinogen solution (4 mg ml^{-1} in 50 mM^{-1} Tris–HCl pH 7.6 containing 0.15 M NaCl) was incubated with 10 μl of AaV-SP-I or AaV-SP-II solution (100 μg ml^{-1}) at 310 K for 30, 60, 90, 180, 360 and 480 min (lanes 3–8 in Fig. 2a for AaV-SP-I; lanes 2–7 in Fig. 2b for AaV-SP-II). Lane 2 in Fig. 2(a) and lane 1 in Fig. 2(b) are bovine fibrinogen solution incubated alone for 480 min; lane 1 in Fig. 2(a) and lane 8 in Fig. 2(b) are protein markers for molecular-weight estimation.
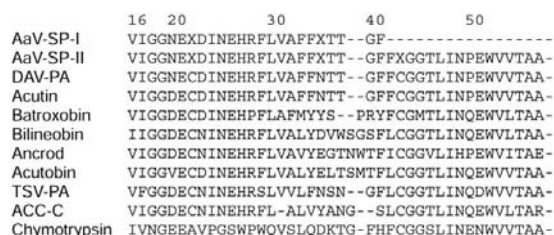
Figure 3
N-terminal sequence alignment of AaV-SP-I, AaV-SP-II and several other snake-venom serine proteinases. The amino-acid residue sequences of bovine chymotrypsin (Wang *et al.*, 1985) and other snake-venom serine proteinases, including ancrod (Burkhart *et al.*, 1992), bilineobin (Nikai *et al.*, 1995), batroxobin (Itoh *et al.*, 1987), ACC-C (McMullen *et al.*, 1989), TSV-PA (Zhang *et al.*, 1995), acutin (Pan *et al.*, 1999), acutobin and DAV-PA (Wang *et al.*, 2001), were found in the references. The numbering of the residue positions is according to the numbering system for chymotrypsinogen.

(see Fig. 1), about 2.54 mg of AaV-SP-I (with a 32-fold purification factor and 0.17% yield) and 0.93 mg of AaV-SP-II (with a 38-fold purification factor and 0.062% yield) could be obtained from 1.5 g crude of *A. acutus* venom, indicating that they are very rare in natural sources. The two proteins appear as a single band on SDS–PAGE under both reducing and non-reducing conditions (see inset in Fig. 1c). Estimated by SDS–PAGE, the molecular weights of AaV-SP-I and AaV-SP-II are about 32 and 31 kDa under reducing conditions and 26 and 25 kDa under non-reducing conditions, respectively. Surprisingly, the N-terminal amino-acid residue sequences of AaV-SP-I and AaV-SP-II are not only the same as each other, but are also similar to those of several other SV-SPs (Fig. 3). AaV-SP-I and AaV-SP-II display N-terminal sequences that are particularly highly homologous to those of acutin and DAV-PA from the same species (Pan *et al.*, 1999; Wang *et al.*, 2001; Fig. 3). The relationships between these proteins need to be investigated. Moreover, during the sequencing, the lack of information at site 35 (for comparison, the residue position numbering used is that of chymotrypsinogen) of both proteins possibly results from the glycosylation of the residues. In comparison, sites 35–37 of acutin and DAV-PA possess the typical glycosylation motif N-*X*-T and sites 36–37 of AaV-SP-I and AaV-SP-II are identical to those of acutin and DAV-PA (Fig. 3). In fact, based on MALDI–TOF mass spectra, both of the proteins should be glycosylated serine proteinases, but contain distinctive carbohydrate contents, *i.e.* about 9%(*w/w*) and 4%(*w/w*) for AaV-SP-I and AaV-SP-II, respectively (data not shown).

Using TAME (*p*-toluenesulfonyl arginine methyl ester) as a substrate, the specific arginine esterase activities and K_M values of AaV-SP-I and AaV-SP-II are 10.54 and 13.1 U mg^{-1} and 16.8×10^{-5} and 15.19×10^{-5} M, respectively. The catalytic processes could be inhibited by NPGB, leupeptin, aprotinin and

Table 1
Statistics of diffraction data-collection and reduction.

Values in parentheses are for the highest resolution shell.

	AaV-SP-I	AaV-SP-II
Space group	$P2_12_12_1$	$C2$
Unit-cell parameters		
a (Å)	49.4	119.21
b (Å)	74.5	42.76
c (Å)	82.4	44.80
α (°)	90	90
β (°)	90	99.67
γ (°)	90	90
No. of molecules in asymmetric unit	1	1
V_M (Å^3 Da^{-1})	3.03	2.25
Resolution limits (Å)	10–2.3	10–2.1
	(2.34–2.30)	(2.14–2.10)
No. of independent reflections	13967	12041
Completeness† (%)	99.4 (99.5)	92.2 (73.9)
R_{merge}‡ (%)	10.7 (41.0)	8.3 (20.8)

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

PMSF, but not by EDTA, SBTI, heparin and hirudin (data not shown). Meanwhile, similar to some other SV-SPs, both AaV-SP-I and AaV-SP-II show fibrinogenase activity (see Fig. 2). These properties of the two proteins indicate that both proteins are SV-SPs.

To our knowledge, only one SV-SP crystal structure, that of TSV-PA, has been solved so far, although a second SV-SP has been crystallized (Parry *et al.*, 1998; Watanabe *et al.*, 2002). However, the protein used for the crystallization of TSV-PA was obtained from an expression product. This paper reports the crystallization and preliminary crystallographic analysis of two glycosylated SV-SPs purified from natural sources. AaV-SP-I and AaV-SP-II crystals with different crystallographic symmetries diffracted X-rays to near 2 Å resolution in-house, perhaps providing a basis for further understanding of the structure–function relationship and of the structural features accounting for carbohydrate–protein interactions. The sequencing of all amino-acid residues and crystal structural analysis of the two enzymes are being carried out in our laboratory.

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