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Purification, crystallization and preliminary X-ray diffraction studies of disintegrin (schistatin) from saw-scaled viper (*Echis carinatus*)

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This is the first report of crystallographic data on a disintegrin molecule from any source. The heterodimeric disintegrin with a molecular weight of 14 kDa from *Echis carinatus* venom is a potent antagonist of α4 integrins. The intact disintegrin, containing two subunits *A* and *B*, was isolated and purified using affinity and ion-exchange columns. It has been crystallized using 1.6 *M* ammonium sulfate as a precipitating agent. The crystals grew to dimensions of $0.25 \times 0.20 \times 0.20$ mm and diffracted to 2.5 Å resolution. The crystals belong to space group $I4_122$, with unit-cell parameters $a = b = 91.7$, $c = 55.1$ Å. Assuming a molecular weight of 14 kDa, a V_M of 2.1 Å^3 Da^{-1} is obtained for one molecule of disintegrin in the asymmetric unit.

1. Introduction

Integrins are a family of cell-surface proteins that mediate cell–cell interactions and the adhesion of cells to extracellular matrix proteins and other ligands (Hynes, 1992). The integrins play important roles in many diverse biological processes such as platelet aggregation, tissue repair, angiogenesis, bone destruction, tumour invasion, and inflammatory and immune reactions (Hynes, 1996; Lobb & Hemler, 1994). Recently, intense studies have been initiated to obtain naturally occurring peptides or develop synthetic peptides that may selectively inhibit integrin–ligand interactions. The investigations on disintegrins, a family of low molecular-weight ($M_r \simeq 14\,000$), cysteine-rich peptides from viper venoms, have shown them to be potent antagonists of integrins (Gould *et al.*, 1990; Musial *et al.*, 1990). This integrin is a relatively selective antagonist of α4 integrins, which inhibits interaction with ligands in an RGD-independent manner (Marcinkiewicz, Calvete, Marcinkiewicz *et al.*, 1999). It is composed of two covalently linked subunits *A* and *B*, which show a high degree of homology including alignment of conserved cysteines. We have isolated this novel disintegrin molecule from the venom of *E. carinatus*, purified it and have determined its N-terminal sequence. It has been crystallized and preliminary X-ray crystallographic data have been obtained.

2. Materials and methods

2.1. Isolation and purification

The crude venom of *E. carinatus* was obtained from the Irula Cooperative snake farm in Tamilnadu, India. 250 mg of lyophilized venom was dissolved in 20 m*M* ammonium acetate buffer pH 5.0 to make the final concentration of venom 10 mg ml^{-1}. It was centrifuged at 20 000 rev min^{-1} for 15 min at 293 K. The insoluble material was removed and the supernatant was diluted three times with the same buffer. This was loaded onto a Blue Sepharose CL-6B column (15.0×2.5 cm) which was pre-equilibrated with 20 m*M* ammonium acetate buffer pH 5.0. The column was washed repeatedly with the same buffer to remove unbound proteins. The bound proteins

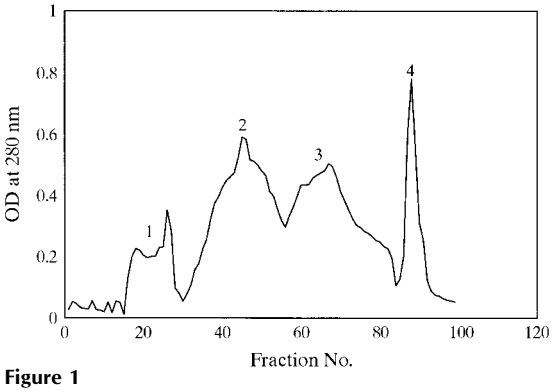

Figure 1
Elution profile of the purification of disintegrin by ion-exchange chromatography on SP Sephadex C-25 in 20 m*M* ammonium acetate pH 5.0 with NaCl gradient (0.0–0.5 *M*): peak 1, 0.07 *M*; peak 2, 0.15 *M*; peak 3, 0.26 *M*; peak 4, 0.38 *M*. Peak 4 corresponds to disintegrin.

Table 1
X-ray data-collection statistics.

Values in parentheses correspond to the last resolution shell.

Space group	$I4_122$
Unit-cell parameters (Å)	$a = b = 91.65$, $c = 55.05$
Data-collection resolution (Å)	20.0–2.5
Last resolution shell (Å)	2.65–2.50
No. of observed reflections	44217
No. of unique reflections	3684
R_{sym} (%)	12.2 (20.6)
$I/\sigma(I)$	9.2 (5.2)
Completeness (%)	99.9 (98.5)

were eluted using a continuous NaCl gradient (0.0–0.6 *M*). The collected fraction was examined on SDS–PAGE. The fractions containing protein corresponding to 14 kDa were pooled and dialyzed against 20 m*M* ammonium acetate pH 5.0 to remove NaCl. The dialyzed samples were loaded onto a SP Sephadex column C-25 (8.0 × 2.5 cm). After washing out the unbound proteins, a linear NaCl gradient (0.0–0.5 *M*) was run. The elution profile is given in Fig. 1. The peaks were analyzed on SDS–PAGE. Peak 4 indicated the presence of a single band corresponding to 14 kDa. The sequence of 19 N-terminal residues of peak 4 determined using the PPSQ-10 (Shimadzu) protein sequencer was Asn-Ser-Val-His-Pro-Cys-Cys-Pro-Pro-Val-Ile-Cys-Glu-Pro-Arg-Glu-Gly-Glu-His. It matched well with the N-terminal sequences of disintegrins (Marcinkiewicz, Calvete, Vijay-Kumar *et al.*, 1999) and showed a sequence identity of more than 90% with the N-terminal sequence of disintegrin EC3 from *E. carinatus suchoreki* venom (Marcinkiewicz, Calvete, Marcinkiewicz *et al.*, 1999). The presence of two chains in disintegrin was analyzed with SDS–PAGE using 2-mercaptoethanol. As seen in Fig. 2, it gave a band at about 7 kDa, suggesting that it contained two subunits linked by a disulfide bond.

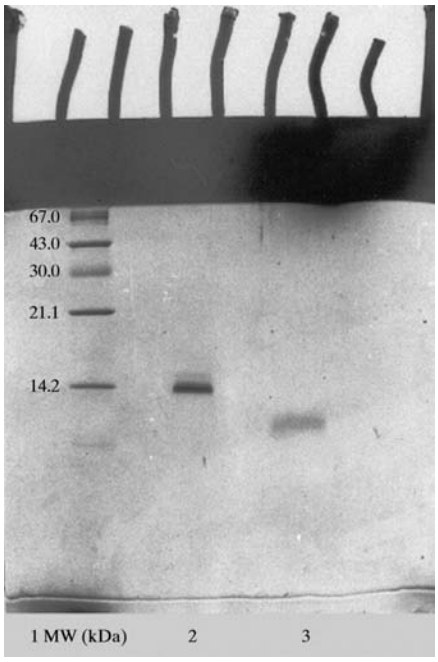

Figure 2
SDS–PAGE (13%) of disintegrin from *E. carinatus*. Lane 1, molecular-weight markers; lane 2, native disintegrin; lane 3, mercaptoethanol-treated disintegrin.

2.2. Crystallization

The purified samples of the heterodimeric disintegrin were crystallized using the sitting-drop vapour-diffusion technique by mixing equal volumes of a 3 mg ml^{-1} protein solution in 10 m*M* sodium cacodylate buffer at pH 6.0 with a reservoir solution that contained 1.6 *M* ammonium sulfate as the precipitating agent in the same buffer. Crystals appeared after 10 d and grew to maximum dimensions of 0.25 × 0.20 × 0.20 mm (Fig. 3).

2.3. Data collection and analysis

Diffraction data were collected at 288 K from a crystal of dimensions 0.25 × 0.20 × 0.20 mm mounted in a glass capillary with a small amount of mother liquor using a 300 mm MAR Research imaging-plate scanner and Cu $K\alpha$ radiation generated from a RU-200 rotating-anode generator (Rigaku) operating at 40 kV and 100 mA. 44 217 reflections were measured to 2.5 Å resolution and reduced to a set of 3684 unique reflections with 99.9% completeness characterized by R_{sym} = 12.2% and $\langle I/\sigma(I)\rangle$ = 9.2. Indexing and integration of the images were performed using *DENZO* and scaling of intensity data was carried out using *SCALEPACK*. Both *DENZO* and *SCALEPACK* are from the *HKL* program package (Otwinowski & Minor, 1997). The detailed data-collection statistics are given in Table 1. The Matthews coefficient calculated in the $I4_122$ unit cell with one molecule in the asymmetric unit was $V_M = 2.1$ Å^3 Da^{-1}, corresponding to a crystal solvent content of 41%, which is within the range normally observed in protein crystals (Matthews, 1968).

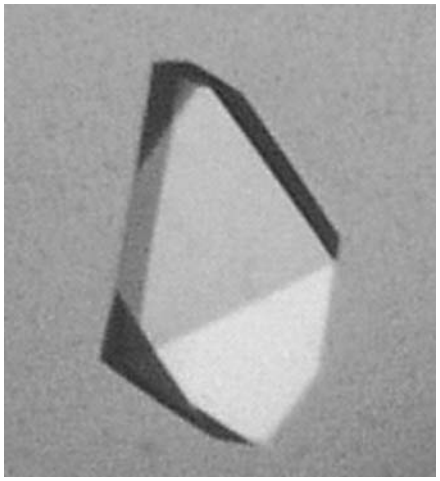

Figure 3
A crystal of disintegrin from the venom of *E. carinatus*. The crystals grow as prisms of maximum dimensions 0.25 × 0.20 × 0.20 mm.

This is the first disintegrin molecule which has been crystallized. Currently, no structural model is available in the database which could be used for the molecular replacement. Though an NMR structure of a much smaller disintegrin (echistatin) from the same source is available (Chen *et al.*, 1994), it did not work as a molecular-replacement model. Therefore, attempts are under way to solve the structure of this protein using the multiple isomorphous replacement technique.

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