SHORT COMMUNICATIONS

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Crystallization and preliminary X-ray crystallographic study of ribosome-inactivating protein from barley seeds.

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Abstract

Ribosome-inactivating protein from barley seeds has been crystallized using polyethylene glycol as precipitant. The crystal belongs to the monoclinic space group $C2$, with unit-cell parameters $a = 88.36$, $b = 62.59$, $c = 53.18$ Å and $\beta = 108.62°$. The asymmetric unit contains one molecule of ribosome-inactivating protein with a corresponding crystal volume per protein mass (V_m) of 2.32 Å^3 Da^{-1} and a solvent content of 47% by volume. The crystal diffracts to about 2.3 Å with X-rays from a rotating-anode source and is very stable in the X-ray beam. X-ray data (nearly complete to 2.4 Å Bragg spacing) have been collected from a native crystal.

Introduction

Ribosome-inactivating proteins (RIP's), widely distributed in the plant kingdom (Stirpe & Barbieri, 1986; Gould & Richardson, 1987), inhibit protein synthesis by damaging the 60S subunit of the eukaryotic ribosome in a catalytic manner (Endo, Tsurugi & Lambert, 1988). RIP's are usually classified into two groups: type I and type II. Type I (or single-chain) RIP's are unable to enter intact cells and thus are only capable of intoxicating cell-free systems. However, type II RIP's, such as ricin and abrin, are highly toxic to intact cells. This is due to the presence of a *B*-chain, which binds to the cell-surface receptor containing terminal galactose residues, in addition to the catalytic *A*-chain (Stirpe & Barbieri, 1986; Gould & Richardson, 1987). RIP's have attracted considerable interest in recent years because of their potential applications in the development of immunotoxins, which can be selectively targeted to a particular cell type, such as cancer cells (Olsnes & Pihl, 1982; Vitetta & Uhr, 1985). Type I RIP's are thought to be better candidates than type II RIP's for constructing immunotoxins because they lack their own cell recognition and binding ability. The toxicity of type I RIP's can be greatly enhanced by their coupling to a cell-binding protein (such as antibody or lectin) or by incorporating them into liposomes or erythrocyte ghosts which can be fused to intact cells. Moreover, some type I RIP's have also been shown to have a potent activity against HIV-1-infected T-cells and macrophages: trichosanthin (McGrath *et al.*, 1989); *Momordica* anti-HIV protein, MAP 30 (Lee-Huang *et al.*, 1990); *Trichosanthes* anti-HIV protein, TAP 29 (Lee-Huang, Huang *et al.*, 1991); *Gelonium* anti-HIV protein, GAP 31; and *Dianthus* anti-HIV proteins, DAP's 30 and 32 (Lee-Huang, Kung *et al.*, 1991).

RIP from barley seeds, belonging to the type I category, inactivates eukaryotic ribosomes *via* a mechanism identical to that of ricin *A*-chain. That is, it hydrolyzes the N-glycosidic bond at A^{4324} of 28S rRNA of the 60S ribosomal subunit (Endo, Tsurugi & Ebert, 1988). Like many other type I RUP's, it is a basic protein with p*I* of above 9.0 (Hejgaard & Bjørn, 1985). Its amino-acid sequence has been deduced from the cDNA sequence. The mature polypeptide chain, consisting of 280 amino-acid residues, has a molecular mass of 29976 Da (Leah, Tommerup, Svendsen & Mundy, 1991).

Crystal structures of several RIP's have been reported: (i) type II ricin (Montfort *et al.*, 1987; Rutenber *et al.*, 1991) and abrin (Liaw, Tahirov, Chu, Lu & Lin, 1993); (ii) type I ricin *A*-chain (Monzingo & Robertus, 1992; Mlsna, Monzingo, Katzin, Ernst & Robertus, 1993; Weston, Tucker, Thatcher & Pauptit, 1993), pokeweed antiviral protein (Monzingo, Collins, Ernst, Irvin & Robertus, 1993), trichosanthin (Pan *et al.*, 1986; Xia, Zhang, Zhang, Wu & Dong, 1993; Xiong, Zhang, Xia & Wang, 1993; Ma, Jin, Gong & Wang, 1993) and momorcharin (Ren, Wang, Dong & Stuart, 1993). Folding patterns and active-site structures of pokeweed antiviral protein, trichosanthin, momorcharin and abrin *A*-chain are similar to those of ricin *A*-chain (Xia, Zhang, Zhang, Wu & Dong, 1993; Ben, Yaoping, Shizhi, Shen, Xingqi & Yicheng, 1993; Liaw, Tahirov, Chu, Lu & Lin, 1993). It has also been noticed that the RIP's, retroviral reverse transcriptases and RNase H share common structural elements (Ready, Katzin & Robertus, 1988). However, there are interesting sequence differences among RIP's. For example, the RIP from barley seeds has a four-residue insertion at the end of the key active-site helix of ricin *A*-chain (Ready, Katzin & Robertus, 1988). Therefore, it would be interesting to compare the structure of the RIP from barley seeds with other related structures. Crystallization of *Mirabilis* antiviral protein has been reported (Miyano *et al.*, 1992). In this paper crystallization and preliminary X-ray data of RIP from barley seeds are reported.

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Experimental procedure

Purification

RIP from unmilled barley seeds was purified by a modification of the previously reported procedure (Hejgaard & Bjørn, 1985; Roberts & Selitrennikoff, 1986; Leah, Mikkelsen, Mundy & Svendsen, 1987). Unmilled barley seeds were ground to flour. The flour was extracted with buffer *A* (50 m*M* sodium phosphate at pH 6.0). The suspension was centrifuged for 20 min at 8540*g* (8000 rev min^{-1} Sorvall GSA rotor). RIP was precipitated in the range of 50–80% saturation of ammonium sulfate. The precipitated protein was redissolved in buffer *A* and was subject to dialysis against the same buffer. An ion-exchange chromatographic step was performed on a carboxymethylcellulose column (2.5 × 38 cm), which was previously equilibrated with buffer *A*. The protein was eluted with a linear gradient of 50–300 m*M* sodium chloride in buffer *A* at a flow rate of 50 ml h^{-1}. Further purification of RIP was obtained by gel filtration on a Sephacryl S-200 HR column (2.5 × 76 cm), which was previously equilibrated with buffer *A* containing 50 m*M* sodium chloride. Elution was performed with the above buffer at flow rate of 9 ml h^{-1}, collecting 4.5 ml fractions. An activity assay of the purified protein was performed by gel electrophoresis of RNA's from toxin-treated rat-liver ribosomes (Endo, Tsurugi & Ebert, 1988). The homogeneity of purified ribosome-inactivating protein was judged by polyacrylamide gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970). This procedure yielded approximately 25 mg of homogeneous ribosome-inactivating protein from 1 kg of barley seeds.

Crystallization

The purified protein solution was concentrated to about 25 mg ml^{-1} by an Amicon YM 10 membrane (Amicon) and then dialyzed against 50 m*M* Tris–HCl buffer (pH 8.42) for crystallization. The protein concentration was estimated by measuring the absorbance at 280 nm, assuming a correspondence of 1.0 mg ml^{-1} concentration to the unit absorbance at 280 nm for 1.0 cm path length. Crystallization was performed by the hanging-drop vapor-diffusion method at room temperature (about 295–297 K) using 24-well tissue-culture plates (Flow laboratories). The initial survey for crystallization condition was undertaken by the incomplete factorial method (Carter & Carter, 1979) and sparse matrix sampling (Jancarik & Kim, 1991). Preliminary results indicated that the addition of adenosine 5′-monophosphate (AMP) was beneficial in growing good crystals. The optimized crystallization conditions are as follows. The reservoir solution [29.5%(*w*/*v*) PEG 1500, 100 m*M* Tris–HCl, final pH 8.41] was prepared by mixing appropriate volumes of 1.0 *M* Tris–HCl buffer (pH 8.50) and 50%(*w*/*v*) PEG 1500 (Merck) and adjusting the final volume with water. The RIP protein solution containing 9 m*M* 1,4-dithiothreitol (DTT), 0.9 m*M* dihydronicotinamide adenine dinucleotide (NADH) and 10 m*M* AMP (final protein concentration being 22.5 mg ml^{-1}] was prepared by mixing appropriate volumes of the protein solution, 300 m*M* DTT, 30 m*M* NADH and 320 m*M* AMP. The hanging drop was prepared on a siliconized coverslip by mixing equal volumes of the above protein solution and the reservoir solution.

X-ray crystallographic studies

A crystal was mounted in a thin-walled glass capillary and the capillary was sealed with wax after filling both ends with the mother liquor. X-ray data were collected at 290 K on a FAST area-detector system (Enraf–Nonius) using the *MADNES* software (Messerschmidt & Pflugrath, 1987). Graphite-monochromatized Cu $K\alpha$ X-rays from a rotating-anode generator (Rigaku RU-200BH), running at 40 kV and 70 mA with a 0.3 mm focus cup, were used. The unit-cell dimensions were determined by the autoindexing and parameter-refinement procedure of *MADNES* software. The reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled by the Fourier scaling program (Weissman, 1982).

Results and discussion

Rod crystals of RIP from barley seeds were obtained when the reservoir solution contained 29.5%(*w*/*v*) PEG 1500, 100 m*M* Tris–HCl, final pH 8.41. They grew to typical dimensions of 1.3 × 0.3 × 0.15 mm within a week (Fig. 1). An examination of the intensity distribution of the X-ray data indicates that the crystal belongs to the monoclinic space group *C*2, with unit-cell parameters of $a = 88.36$, $b = 62.59$, $c = 53.18$ Å and $\beta = 108.62°$. The asymmetric unit contains one molecule of ribosome-inactivating protein with a corresponding crystal volume per protein mass (V_M) of 2.32 $Å^3$ Da^{-1} and a solvent content of 47% by volume. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). The crystal diffracts to about 2.3 Å with X-rays from a rotating-anode source and is very stable in the X-ray beam. X-ray data have been collected roughly to 2.4 Å Bragg spacing from a native crystal. The final merged native data set consists of 38896 measurements of 10057 unique reflections with an R_{merge} (on intensity) of 5.3% (rejecting 2.1% outliers). The completeness of the data set is 90.7% for the 29–2.4 Å range (see Table 1 for more details). Attempts are being made to solve the structure by a combination of molecular replacement and multiple isomorphous replacement.

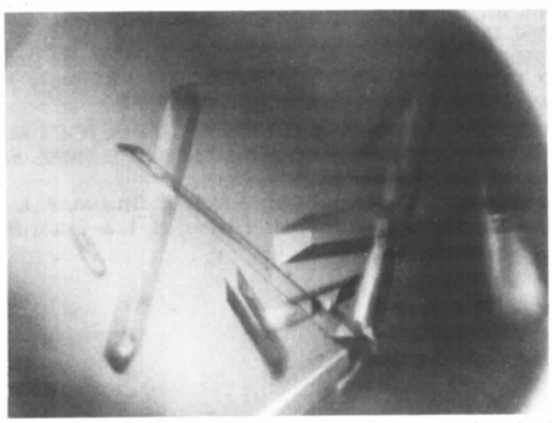

Fig. 1. Photograph of monoclinic crystals of ribosome-inactivating protein from barley seeds. Their dimensions are 1.3 × 0.3 × 0.15 mm. See the text for detailed crystallization conditions.

Table 1. *Data collection statistics*

Resolution shell (Å)	Number of unique reflections	Completeness (%)	R_{merge}* (%)
29.0–10.0	161	92.6	5.1
10.0–6.00	551	98.7	3.9
6.00–4.00	1672	99.2	3.8
4.00–3.30	1822	99.5	4.7
3.30–2.90	1964	99.4	7.1
2.90–2.60	2264	96.3	9.9
2.60–2.40	1140	74.3	9.5
2.40–2.34	187	22.1	8.1
29.0–2.40	9870	90.7	5.3
29.0–2.34	10057	85.7	5.3

* $R_{merge} = \sum_{h,i}|I_{h,i} - \langle I_h\rangle|/\sum_{h,i}I_{h,i}$, where $\langle I_h\rangle$ is the average intensity of the i observations of reflection h.

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