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Crystallization and preliminary crystallographic analyses of pokeweed antiviral protein from seeds

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Abstract

Pokeweed antiviral protein from seeds (PAP-S) is a ribosome inactivating protein which has lowest toxicity and highest inhibition activity as opposed to other pokeweed antiviral proteins and its three potential glycosylation sites (10, 44, 255) were shown to bind to *N*-acetylglucosamine. Good quality crystals of PAP-S were grown at high protein concentration (100 mg ml^{-1}) and high temperature (306 K). The crystals have space group *I*222 and cell parameters $a = 78.7$, $b = 85.2$ and $c = 93.0$ Å. An X-ray diffraction data set with resolution up to 1.8 Å was collected. This high-resolution data will help to locate the sugars bound to the protein and provide accurate structural data for understanding structure–function relationships of PAP-S.

1. Introduction

Pokeweed antiviral proteins (PAP's) (Irvin, 1975; Irvin *et al.*, 1980; Barbieri *et al.*, 1982; Bolognesi *et al.*, 1990) are isozymes from different tissues of *Phytolacca americana* at different developing stages, noticed by their ability to inhibit the multiplication of virus. We now know that they are type I ribosome-inactivating proteins (RIP's) (Stirpe *et al.*, 1992); which act as RNA *N*-glycosidase, removing an invariant adenine base from a loop region of rRNA interrupting and the process of protein synthesis.

Several isozymes of PAP's have been isolated including PAP from spring leaves (Irvin, 1975), PAP II from summer leaves (Irvin *et al.*, 1980), PAP-S from seeds (Barbieri *et al.*, 1982), PAP-R from roots (Bolognesi *et al.*, 1990), and PAP-C from cell culture (Barbieri *et al.*, 1989). Although PAP's have high sequence homology (70–80%) (Kung *et al.*, 1990; Lin *et al.*, 1991; Kataoka *et al.*, 1992) and, as representatives of RIP's, are supposed to have a common catalytic mechanism, on cells or on intact ribosomes from animals, plants or bacteria, they have very different toxicities or inhibition activities. The PAP's and the ribosomes must have a mechanism to recognize each other. Among all PAP's, PAP-S has the lowest toxicity to mice and the highest inhibition activity in rabbit (Stirpe *et al.*, 1992). Therefore, PAP-S has its unique importance in structure–function relationship investigations. In addition, the sequence of PAP-S, which consists of 261 amino-acid residues with two disulfide bonds and a calculated molecular mass of 29 167 Da (Kung *et al.*, 1990), has three potential glycosylation sites (*i.e.* 10, 44 and 255), each of which had been shown to be connected to one *N*-acetylglucosamine (Islam *et al.*, 1991). A high-resolution X-ray structure of PAP-S will provide greater insight in understanding the function of this series of isozymes, as well as the effect of glycosylation on the catalytic mechanism of PAP-S.

The two isozymes, PAP and α-PAP (Kataoka *et al.*, 1992) (the latter is from a genomic clone of pokeweed DNA), have had their crystal structures solved at resolutions of 2.5 and 2.3 Å, respectively (Monzingo *et al.*, 1993; Ago *et al.*, 1994). To elucidate the X-ray structure of PAP-S, including the structure of sugars connected to it, we have successfully crystallized this protein and collected a diffraction data set with highest resolution to 1.8 Å.

2. Materials and methods

PAP-S was prepared from seeds of *Phytolacca americana* (Pokeweed) by the method first reported by Barbieri *et al.* (1982) and modified by Zhu & Hu (1989).

Mature seeds of pokeweed were harvested in autumn in the garden of Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China, and stored at 258 K. Then the dried seed powder was extracted with distilled water at 277 K for 12 h, and the extracted solution was applied to a CM-Sephadex C-50 column at pH 7.0. The protein adsorbed onto the column was eluted with a 0.6 mol l^{-1} NaCl buffered with 20 mmol l^{-1} phosphate buffer at pH 7.0. The eluted solution was dialyzed against 20 mmol l^{-1} phosphate buffer at pH 7.0, centrifuged and then loaded onto a Sephadex G-50 column for gel filtration. The first peak fraction was further separated on a CM-cellulose 52 column with a linear gradient elution of 0.1–0.4 mol l^{-1} NaCl at pH 7.0. The fraction of PAP-S (Barbieri *et al.*, 1982; Zhu & Hu, 1989) was then collected, desalted and lyophilized. The purity of the preparation, as well as the molecular weight, was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The isoelectric point was determined by IEF electrophoresis in a pH range of 3.5–9.5. The N-terminal amino-acid determination was carried out on an automated amino-acid analyzer (Hitachi 835). The antiviral potency was checked by a plaque-inhibition method (Irvin, 1983). The protein-synthesis inhibition effect was tested in rabbit reticulocyte lysate using the method described by Maniatis *et al.* (Maniatis *et al.*, 1982).

The lyophilized sample was used in the crystallization trials. Extensive crystallization experiments were carried out by making use of the hanging-drop vapour-diffusion method on Petri dishes.

3. Results

3.1. *Identification of PAP-S*

The lyophilized sample showed only one band on SDS–PAGE with molecular weight of 30 kDa in agreement with the result of gel filtration. In IEF analysis, an isoelectric point of 8.4 was found confirming that the sample is an alkaline protein. The N-terminal amino acid was determined as Ile.

Table 1 shows the purified sample has a strong ability to inhibit the multiplication of virus. At the protein density 4.17 ×

Table 1. *Plaque inhibition of poliovirus multiplication by the sample used in this paper*

	Protein content (mol l^{-1})	Plaque number	log	PFU ml^{-1}†	Inhibition (%)
	4.17×10^{-8}	0	<5.0	1.0×10^5	>98
	4.17×10^{-9}	1	5.7	5.0×10^5	90
	4.17×10^{-10}	2	6.0	1.0×10^6	83
	4.17×10^{-11}	4	6.3	2.0×10^6	66
Controls					
Virus		12	6.8	6.0×10^6	
Cell		0	0	0	

† PFU = plaque-forming units.

Table 2. *Inhibition of protein synthesis by PAP-S in a rabbit reticulocyte lysate*

	Protein content (mol l^{-1})	CPM†	Incorporated fold‡	ID_{50} (mol^{-1})
	1.12×10^{-10}	46080	18	8.36×10^{-11}
Control	0	138240	54	

† CPM before reaction = 2560. ‡ Incorporated fold = CPM/CPM before reaction.

10^{-8} mol l^{-1}, nearly full inhibition resulted. The density for half inhibition is less than 10^{-11} mol l^{-1}. For a cell-free protein synthesis system, in rabbit recticulocyte lysate, the sample has ID_{50} 2.6 ng ml^{-1} (8.6×10^{-11} mol l^{-1}, Table 2), consistent with the previously reported value (Barbieri *et al.*, 1982). All these data are in agreement with that of the known PAP-S (Barbieri *et al.*, 1982).

3.2. *Crystallization*

The crystallization conditions were extensively tested, and the best recipe is as follows. The drop contains one volume of protein (100 mg protein dissolved in 1 ml 0.03 *N* NaCl) plus one volume of 30%(*w*/*v*) PEG 6000 and the two volumes of 0.2 *M* NaH_2PO_4 pH 5.0–6.0. The reservoir contains 1 ml 30% PEG 6000. After two months of incubation at 306 K the crystals grew to about 0.8 × 0.2 × 0.1 mm in size (Fig. 1).

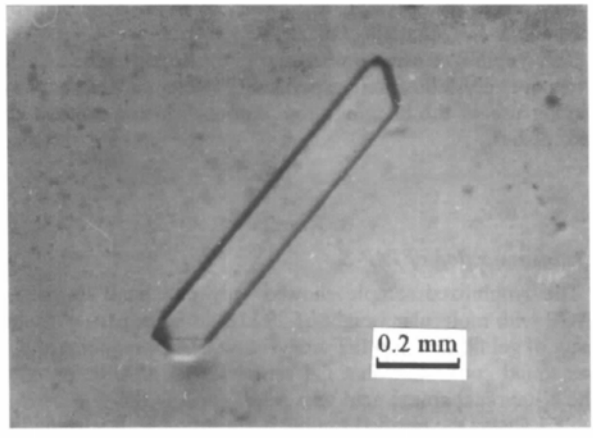

Fig. 1. Crystal of PAP-S used for diffraction data collection.

Interestingly, the best crystals appear only at higher temperature.

The crystal has space group *I*222 with cell parameters a = 78.8, b = 85.2, c = 93.0 Å, as determined by CAD-4 four-circle diffractometer. The crystal is mechanically strong, and diffracted to more than 2 Å. From the V_m value, which is 2.6 $Å^3$ Da^{-1}, the solvent content is about 53%, with one molecule in the asymmetric unit, as calculated by the method of Matthews (Matthews, 1968).

3.3. *Data collection*

Data collection was performed on beamline BL6A2 of the Photon Factory in KEK, Tsukuba, Japan, using a screenless Weissenberg Camera (Sakabe, 1983) with radius 429.7 mm. Data were recorded on image plates and read out by a Fuji film BA100 Scanner System, and processed using the program *WEIS* (Higashi, 1989). A wavelength of 1.0 Å and a rotation of 9° were used in the experiment. The diffraction reached a resolution limit beyond 1.8 Å. In the resolution range up to 1.8 Å a total of 58 568 reflections were observed, which were merged into 19 809 unique reflections with an R_{merge} of 6.2% and a completeness of 70%. The completeness in the highest resolution shell (1.94–1.80 Å) is 53%.

This high-resolution data set is now available for structure determination of PAP-S by the molecular replacement method and the process is under way. We hope that the high-resolution structure will provide more information on the structure. Especially since PAP-S not only has about 60 residues from a total of 261 residues different to the two previously determined structures (Lin *et al.*, 1991; Kataoka *et al.*, 1992), PAP and α-PAP (Monzingo *et al.*, 1993; Ago *et al.*, 1994), its biological behavior is also different from other isozymes in the PAP family (Stirpe *et al.*, 1992). Furthermore, among the three glycosylation sites, two of them are located not far from the binding cleft. In view of the fact that efforts to locate the sugars connected to ricin A chain have failed in the 2.5 Å crystal structure of ricin (Rutenber *et al.*, 1991), it is hoped that the high-resolution structure of PAP-S will allow us to determine the structure of sugars which might have an effect on the binding of PAP-S with ribosomes.

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