

# Crystallization and Preliminary X-Ray Diffraction Analysis of PD-L4, a Ribosome Inactivating Protein from *Phytolacca dioica* L. leaves

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**Abstract:** PD-L4, a type 1 ribosome inactivating protein from *Phytolacca dioica* leaves, has been successfully crystallized using vapour diffusion methods and PEG 4000 as a precipitant agent. In addition, crystals of a PD-L4 mutant, which has been recently observed to have a lower polynucleotide-adenosine glycosidase activity on DNA, rRNA and poly (A) substrates, have been obtained. To gather information on PD-L4 reaction mechanism both forms have been co-crystallized with adenine, the major product of their catalytic reaction. Diffraction patterns extend to atomic resolution and crystals belong to the orthorhombic P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space group, with one molecule in the asymmetric unit. Structure determination has been achieved using molecular replacement; preliminary electron density maps have clearly given evidence of adenine binding.

**Keywords:** Ribosome inactivating protein, *Phytolacca dioica*, crystallization, x-ray.

## 1. INTRODUCTION

Ribosome-inactivating proteins (RIPs) are rRNA N- $\beta$ -glycosidases (EC number 3.2.2.22) widespread in plants, where they play a role in defence against external pathogens [1]. They are able to inhibit protein biosynthesis in pathogens by inactivating their ribosomes through a site-specific deadenylation of the large ribosomal RNA [2]. In recent years, their cytotoxicity has been exploited for the development of immunotoxins for tumor therapy [3-5] as well as for applications as abortifacients and anti-human immunodeficiency virus (HIV) agents [2]. RIPs target a universally conserved sequence of the ribosome alpha-sarcin loop, which is also involved in the binding of elongation factors to the ribosome [6]. Despite the large amount of structural information on the ribosome accumulated recently [7-9], no detailed information is still known on the mode of interaction of RIPs with ribosomes. RIPs are also capable of inactivating many nonribosomal nucleic acid substrates [10,11] and hence can be more generally considered as polynucleotide:adenosine glycosidases [12].

These enzymes are classified into three groups based on their chemical-physical properties. Type 1 RIPs, such as pokeweed antiviral protein (PAP) and saporin (from *Saponaria officinalis*) are monomeric enzymes [13-15]. They are basic proteins that share a number of well conserved active cleft residues and secondary structure within the active site region, although members of this class may show different posttranslational modifications and often low sequence identity [12]. Type 2 RIPs, like ricin and abrin, are heterodimeric proteins capable to penetrate cells and are therefore highly toxic [16]. Type 3 RIPs are synthesized as inactive

precursors (pro-RIPs) and mature through proteolytic processing events [16]. Given the interest of RIPs in pharmaceutical applications [2,12], efforts have been devoted to understand how these proteins act. Crystal structures of various RIPs have been so far determined [14,15,17,18]. However, the mechanism by which they inhibit cell growth or pathogen infection is not well understood. Different mechanisms of action have been proposed based on x-ray studies and extensive site-directed mutagenesis [19,20]. On the other hand, only a few studies have been focused on the possible catalytic role of residues which are not located in the adenine binding pocket. Therefore, we have started a site-directed mutagenesis study using PD-L4, a type 1 RIP from *Phytolacca dioica* leaves, as a model system [21]. Leaves of *Phytolacca dioica* produce several type 1 RIPs, named PD-L1, PD-L2, PDL3, and PD-L4. Of these, PD-L4 (AC: P84854) is the sole isoform which is not glycosylated [22]. In this framework, it has been evidenced that a role in catalysis is exerted by the invariant residue S211 [21]. The polynucleotide-adenosine glycosidase activity of the S211A mutant is significantly lower compared to the *wild type* protein, with the major extent of reduction for poly(A) substrates [21]. With the aim of gaining insights on the reaction mechanism of RIPs and to understand the structural bases of the reduced activity of the PD-L4 S211A mutant we undertook a crystallographic study. We here report the crystallization of the *wild type* PD-L4 RIP and of the S211A mutant [21] as well as the preparation and crystallization of complexes of these two forms with adenine, the major product of their enzymatic reaction.

## 2. METHODS

### 2.1. Purification and Crystallization

Native PD-L4 was extracted from spring leaves of *P. dioica* and purified as described [22]. The recombinant

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S211A mutant was expressed in *Escherichia coli* BL21(DE3) strain as previously reported [21]. The purity and homogeneity were tested by SDS-PAGE. Proteins were concentrated to 10 mg mL<sup>-1</sup> using a YM10 Centricon (Amicon Corp.) and stored at 277 K.

Crystallization experiments were performed at 293 K using either the microbatch-under-oil or the hanging-drop vapour-diffusion methods. Preliminary crystallization trials were carried out using commercially available sparse-matrix screens (Crystal Screen and Wizard kits I and II from Hampton Research and deCode Genetics, respectively). Optimisation of the crystallization conditions was performed by fine-tuning the protein and precipitant concentrations.

Complexes of both wild type PD-L4 (<sub>wr</sub>PD-L4) and of its S211A mutant (<sub>S211A</sub>PD-L4) with adenine were prepared by co-crystallization in the presence of 5 mM concentration of adenine. Adenine was also added to the stabilizing and the cryo solutions.

## 2.2. Data Collection and Processing

Preliminary diffraction data were collected in-house at 100 K using a Rigaku Micromax 007 HF generator producing Cu K $\alpha$  radiation and equipped with a Saturn944 CCD detector. Crystals were flash-cooled after the addition of 14% (v/v) ethylene glycol to the crystallization buffer. A stepwise treatment of crystals with solutions prepared at growing ethylene glycol concentration was necessary to avoid deterioration of crystal quality. In particular, each crystal was transferred to 20  $\mu$ L of stabilising solution containing 24% (w/v) polyethylene glycol (PEG) 4000, 10% (v/v) iso-propanol, 0.1 M Hepes (pH 7.5). Various additions

to the droplet of small amounts (about 6  $\mu$ L) of cryo-solutions with increasing ethylene glycol concentration (4, 7 and 14 %) followed. High resolution data were collected at beamline BW7A of the DESY synchrotron (Hamburg, Germany) at 100 K. Complete datasets were registered for <sub>wr</sub>PD-L4 and <sub>S211A</sub>PD-L4 both in their unliganded forms and in the presence of adenine. Data processing and scaling was performed using the HKL2000 package [23].

## 2.3. Structure Determination

The solution of the structure has been obtained using molecular replacement and the program "il Milione" [24]. The structure of RIP from *Phytolacca americana* (PDB code 1qci) has been used as a starting model [25]. Model building using both automatic [26] and manual [27] approaches is in progress.

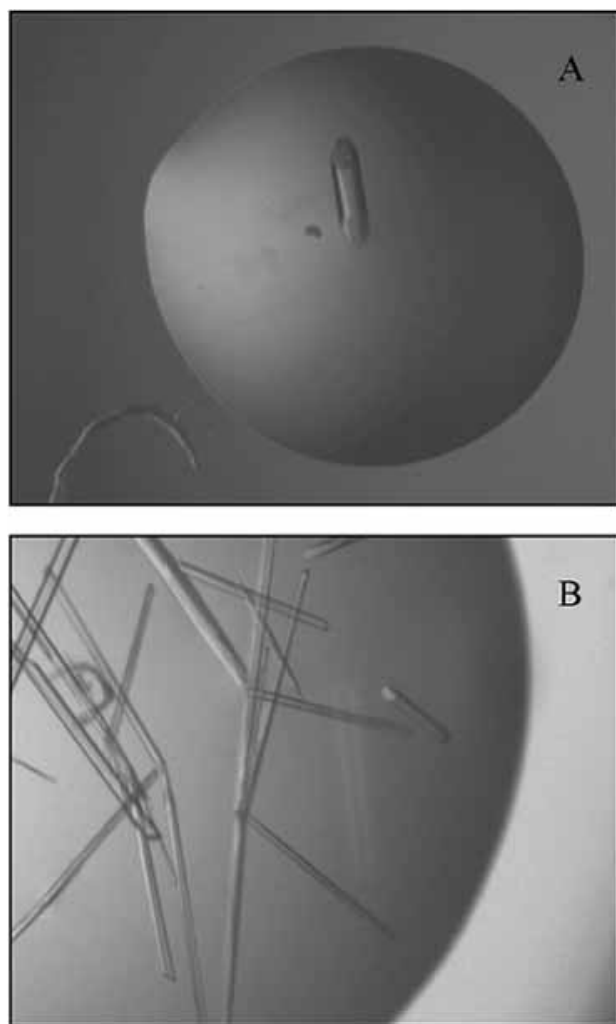
## 3. RESULTS AND DISCUSSION

The screenings carried out using commercially available solutions indicated several promising conditions for the crystallization of PD-L4. All favourable conditions were characterised by the presence of PEG as a precipitating agent (crystals with a similar morphology were obtained using PEG 4000, PEG 8000, PEG 1500, PEG 10000). The quality of the crystals was improved by fine-tuning the concentration of the protein and of the precipitants. Best crystals of <sub>wr</sub>PD-L4, of size 0.1x0.1x0.4 mm, were obtained in about four days using a protein concentration of 8 mg mL<sup>-1</sup> and in the presence of 20% (w/v) PEG 4000, 10% (v/v) iso-propanol, 0.1 M Hepes (pH 7.5). Similar conditions, with a protein concentration of 10 mg mL<sup>-1</sup>, produced high quality crystals of

**Table 1. Data Collection Statistics.** Values in Parentheses Refer to the Highest Resolution Shell

	Wild type PD-L4		S211A PD-L4	
		Complex with adenine		Complex with adenine
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Unit-cell parameters (Å)				
<i>a</i>	43.521	43.507	43.619	43.601
<i>b</i>	58.813	58.751	61.541	61.651
<i>c</i>	98.774	99.056	91.626	91.695
Resolution (Å)	1.10	1.24	1.29	1.24
Wavelength (Å)	0.9800	0.9800	0.9800	0.9800
N. of unique reflections	102812	72193	61843	68965
Total oscillation range – oscillation step (°)	250 - 0.5	180 - 0.3	180 - 0.5	270 - 0.5
Average redundancy	9.5 (8.1)	7.0 (6.6)	5.2 (3.2)	9.4 (4.5)
R <sub>merge</sub> (%)	3.9 (29.0)	5.3 (38.0)	6.9 (39.9)	5.7 (38.4)
Completeness (%)	99.3 (97.8)	99.9 (99.9)	96.9 (92.3)	95.6 (90.9)
Mean I/ $\sigma$ (I)	48.9 (5.9)	24.9 (2.9)	19.8 (2.0)	30.4 (2.4)

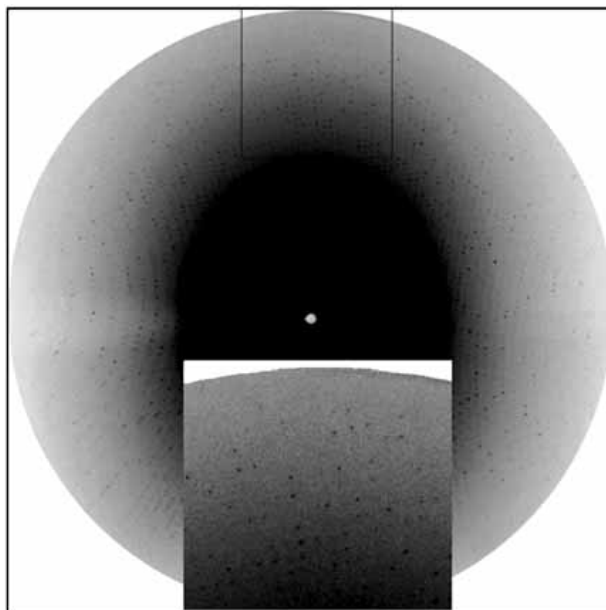
*S211A*PD-L4 with a slightly different morphology than those of *w<sub>t</sub>*PD-L4 in about one week (Fig. 1). Both types of crystals diffracted at a resolution of 1.4 Å using in-house Rigaku Micromax 007 HF generator equipped with a Saturn944 CCD detector. Prompted by the good quality of these crystals we also crystallized the two forms of PD-L4 in the same conditions and in the presence of 5 mM adenine. Attempts to obtain complexes of PD-L4 with adenosyl-monophosphate (AMP) were also performed by soaking crystals in a solution containing 24% (w/v) PEG 4000, 10% (v/v) iso-propanol, 0.1 M Hepes (pH 7.5), 5mM AMP. As a result, crystals rapidly cracked. This indicates that a significant protein conformational change, which is not compatible with crystal packing, occurs upon AMP binding.



**Figure 1.** Image of typical crystals of wild type PD-L4 (A) and of the S211A mutant (B) grown using PEG 4000 as a precipitating agent (see text for details).

Atomic resolution diffraction data were collected for *w<sub>t</sub>*PD-L4 and *S211A*PD-L4 both in their unliganded state and in

complex with adenine using the DESY synchrotron radiation (BW7A beamline). Data sets were indexed and processed with the HKL2000 suite of programs [23]. Statistics of data processing are reported in Table 1.



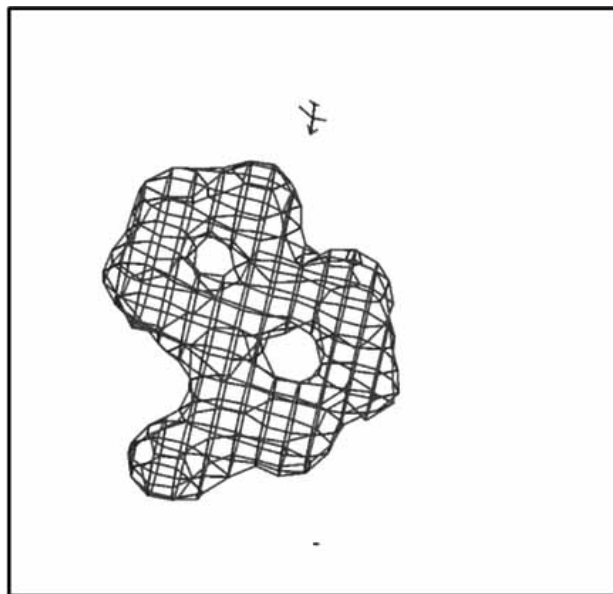
**Figure 2.** Diffraction pattern of a RIP *w<sub>t</sub>*PD-L4 crystal. Diffraction data are detectable to 1.07 Å resolution.

Manual and automatic model-building sessions, aimed at defining the complete structures, are in progress using the programs ARP/wARP [28] and O [27]. Refinement of the four crystal structures, in progress, will provide the highest resolution description for a RIP ever achieved and will add new insights into the role played by the conserved S211 in the catalytic mechanism of this enzyme.

Crystals belong to the  $P2_12_12_1$  space group. A slight difference in the unit cell of crystals of *w<sub>t</sub>*PD-L4 and *S211A*PD-L4, larger than what expected due to crystal flash cooling, could be detected (Table 1). However, this difference in the unit cell is not to be ascribed to the S211A mutation, since other crystals of *w<sub>t</sub>*PD-L4 obtained in the same conditions presented the same unit cell of *S211A*PD-L4 (data not shown).

Matthews coefficient calculations (Matthews, 1968) suggested the presence of one molecule in the asymmetric unit.  $V_M$  values and solvent content for *w<sub>t</sub>*PD-L4 were  $2.17 \text{ \AA}^3 \text{ Da}^{-1}$  and 43.2 %. Similar values ( $V_M=2.11$  and solvent content 41.6%) were observed for *S211A*PD-L4.

Molecular replacement using the program “Il Milione” [24] and the structure of the ribosome inactivating protein from *Phytolacca americana* (PDB code 1qci) as a starting model [25] resulted in a clear solution, with one molecule in the asymmetric unit and an R-factor of 0.39. Preliminary electron density maps are of an excellent quality and clearly show the adenine location in both *w<sub>t</sub>*PD-L4 and *S211A*PD-L4 adenine complexes (Fig. 3).



**Figure 3.** Omit (Fo-Fc) electron density of adenine, contoured at  $3.0 \sigma$ , in the complex with  $w_t$ PD-L4.

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#### ABBREVIATIONS

RIP	=	Ribosome inactivating protein
$w_t$ PD-L4	=	Wild type PD-L4 from <i>Phytolacca dioica</i>
S211A PD-L4	=	S211A mutant of PD-L4 from <i>Phytolacca dioica</i>
AMP	=	Adenosyl-mono-phosphate

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