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Research paper

Structural characterization and comparative modeling of PD-Ls 1–3, type 1 ribosome-inactivating proteins from summer leaves of *Phytolacca dioica* L.

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Abstract

The amino acid sequence and glycan structure of PD-L1, PD-L2 and PD-L3, type 1 ribosome-inactivating proteins isolated from *Phytolacca dioica* L. leaves, were determined using a combined approach based on peptide mapping, Edman degradation and ESI-Q-TOF MS in precursor ion discovery mode. The comparative analysis of the 261 amino acid residue sequences showed that PD-L1 and PD-L2 have identical primary structure, as it is the case of PD-L3 and PD-L4. Furthermore, the primary structure of PD-Ls 1–2 and PD-Ls 3–4 have 81.6% identity (85.1% similarity). The ESI-Q-TOF MS analysis confirmed that PD-Ls 1–3 were glycosylated at different sites. In particular, PD-L1 contained three glycidic chains with the well known paucidomannosidic structure $(Man)_3$ $(GlcNAc)_2$ $(Fuc)_1$ $(Xyl)_1$ linked to Asn10, Asn43 and Asn255. PD-L2 was glycosylated at Asn10 and Asn43, and PD-L3 was glycosylated only at Asn10. PD-L4 was confirmed to be not glycosylated. Despite an overall high structural similarity, the comparative modeling of PD-L1, PD-L2, PD-L3 and PD-L4 has shown potential influences of the glycidic chains on their adenine polynucleotide glycosylase activity on different substrates.

Keywords: Ribosome-inactivating proteins; *Phytolacca dioica*; Edman degradation; ESI-Q-TOF mass spectrometry; Comparative modeling

1. Introduction

Ribosome-inactivating proteins (RIPs) are widely distributed plant proteins present in various organs, in some cases, even in large amounts [1,2]. Moreover, although most of them await further confirmation, previous reports provide evidences of the presence of proteins of the RIP family also in fungi, bacteria and at least one alga [3].

RIPs are RNA *N*-β-glycosidases (EC 3.2.2.22) [4] and remove a site-specific single adenine residue (A_{4324} in the case of rat liver ribosomes) from the highly conserved sarcin/ricin loop of the 28S rRNA, thus arresting protein synthesis [5]. As further results indicated that RIPs can depurinate DNA, RNA and poly(A), they were named polynucleotide:adenosine glycosidases (PAG; [6]), in turn renamed adenine polynucleotide glycosylases (APGs; [7]). RIPs are conventionally classified into three types: types 1, 2 and 3 on the basis of number and type of the constituting polypeptide chain(s) [8]. Type 1 RIPs are single-chain proteins endowed with glycosidase activity, while type 2 RIPs consist of a catalytic chain

Abbreviations: RIPs, ribosome-inactivating proteins; PD-Ls, RIPs isolated from leaves of *Phytolacca dioica* L; PD-S2, RIP (major form) isolated from seeds of *Phytolacca dioica* L; CNBr, cyanogen bromide; Lys-C and Asp-N, endoproteinase Lys-C and Asp-N, respectively; PE-Cys, pyridylethylcysteine; Hse, homoserine; Hse>, homoserine lactone; hsDNA, herring sperm DNA; HPAEC-PAD, high performance anion-exchange chromatography with pulsed amperometric detection; Nbs2, 5,5′-[dithiobis (2-nitrobenzoic acid)]. For the amino acids the standard one or three-letter code has been used.

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(A chain) linked to a sugar binding chain (B chain). Type 3 RIPs, found in maize and barley, consist of an N-terminal active chain linked to an unrelated C-terminal domain with unknown function [1,9].

Interest in RIPs is 3-fold: (i) their substrate is structurally complex as they act on native prokaryotic and eukaryotic ribosomes, by removing a specific adenine [10]; (ii) they have potential use in crop plant biotechnology with the aim of increasing resistance to fungal and virus pathogens [1], and (iii) if properly targeted they could be used as therapeutic agents [11,12].

The genus *Phytolacca* is traditionally known as suitable source of several highly conserved RIPs, firstly discovered on the basis of their antiviral properties [1]. Indeed, several members of this genus have been found to contain type 1 RIPs, such as PAP forms from *Phytolacca americana* [13–15], dodecandrin from *Phytolacca dodecandra* [16], insularin from *Phytolacca insularis* [17], heterotepalins from *Phytolacca heterotepala* [18], PD-Ss and PD-Ls from seeds and leaves of *Phytolacca dioica*, respectively [19,20]. The ability of PAP, isolated from *Phytolacca americana* leaves, to inhibit protein synthesis by enzymatically inactivating ribosomes was initially reported in 1973 [21].

Furthermore, for several RIPs from the Phytolaccaceae family a differential seasonal expression has been demonstrated: (i) PAP and PAP-II from *P. americana*, isolated from spring and summer leaves, respectively [22] and (ii) RIPs from *P. dioica*, expressed all over the year [23].

In particular, *P. dioica* leaves of adult plants contain at least four type 1 RIPs (PD-Ls), named PD-L1, PD-L2, PD-L3 and PD-L4 [20]. On the basis of their preliminary biochemical characterization it was demonstrated that: (i) they have molecular weights in the range 28–32 kDa and p$I \geq 8.5$; (ii) they are all glycosylated proteins with the exception of PD-L4; (iii) PD-L1 shares the same N-terminal sequence with PD-L2, as well as PD-L3 with PD-L4; (iv) they have different enzymatic activity on substrates such as DNA, rRNA, poly(A) [20]; and (v) some of them are endowed with a DNase activity on supercoiled DNA [24] and antiviral activity against tobacco mosaic virus (TMV; unpublished data).

In the present work, we report the complete primary structure, the glycosylation pattern and the comparative three-dimensional modeling of PD-L1, PD-L2 and PD-L3, isolated from *P. dioica* summer leaves, with the aim to unravel the structural basis responsible of the different biological activities.

2. Materials and methods

2.1. Materials

Phytolacca dioica L. leaves were harvested at the end of June from a single plant in the garden of the Biological Sciences of the Second University of Naples (Caserta). The HPLC system was a Waters BREEZE™ apparatus (Milan, Italy); HPLC-grade solvents and reagents were obtained from Carlo Erba (Milan, Italy). Endoproteinase Lys-C and Asp-N (sequencing grade) were purchased from Promega (Monza, Italy). Herring sperm DNA (hsDNA) and cyanogen bromide were obtained from Sigma-Aldrich/Fluka (Milan, Italy). Reagents for automated Edman degradation were supplied by Applera (Monza, Italy). Trypsin and monosaccharides (fucose, Fuc; glucosamine, GlcN; mannose, Man; xylose, Xyl) were from Sigma (Milan, Italy), while trifluoroacetic acid (TFA) was from Pierce (Rockford, IL, USA).

Solvents were: solvent A, 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA; solvent C, 5% CH_3CN, containing 0.1% formic acid; solvent D, 2% CH_3CN in Milli-Q water; solvent E, 95% CH_3CN in Milli-Q water.

2.2. Purification of native PD-Ls 1–4

Native PD-Ls 1–4 were purified from *P. dioica* summer leaves as described elsewhere using a general protocol for the preparation of basic RIPs [20].

2.3. Determination of free sulfhydryl groups

Determination of SH groups was performed with Nbs2 [25] on the protein denatured in the presence of 6 M Gdn.Cl.

2.4. Reduction and S-pyridylethylation

Prior to enzymatic digestion, Edman degradation or after CNBr cleavage (see later) native proteins were *S*-pyridylethylated with 4-vinylpyridine as previously reported [26]. Modified proteins were desalted by RP-HPLC using a C4 column (0.46 × 15 cm; Alltech, Italy), by eluting with a linear gradient of solvent A and solvent B, from 5 to 65%, over 60 min, at a flow rate of 1 mL/min, monitoring at 214 nm.

2.5. Analytical procedures

Experimental procedures for automated Edman degradation, chemical (with CNBr) or enzymatic (with endoproteinases Lys-C and Asp-N) cleavages on protein aliquots were performed as previously described [26,27]. Tryptic hydrolysis was performed in 0.1 M Tris–Cl, 20 mM $CaCl_2$, pH 8.5, containing 10% acetonitrile. The enzyme was added in three steps with a final enzyme-to-substrate ratio of 1:50 (w/w) and the reaction was carried out at 37 °C for 24 h.

2.6. Peptide separation

Separation of Lys-C, Asp-N and tryptic peptides by RP-HPLC was obtained on a Waters Breeze instrument, using a C18 Symmetry column (0.46 × 15 cm, 5 μm particle size; Waters, Milford, MA, USA) at a flow-rate of 1 mL/min. Peptide elution was obtained using a linear gradient of solvent A and solvent B from 5 to 55% of solvent B over 150 min. Peptides were monitored at 214 nm. Insoluble material after endoproteinase digestion, when present, was solubilized with formic acid and analyzed as described.