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Isolation and characterization of heterotepalins, type 1 ribosome-inactivating proteins from *Phytolacca heterotepala* leaves

Antimo Di Maro [a,*], Angela Chambery [a], Addolorata Daniele [a], Paolo Casoria [b], Augusto Parente [a]

[a] *Dipartimento di Scienze della Vita, Seconda Università di Napoli, Via Vivaldi 43, I-81100 Caserta, Italy*
[b] *Dipartimento di Scienze per l'Ambiente, Università di Napoli Parthenope, Viale Amm. Acton 38, I-80133 Napoli, Italy*

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Abstract

Leaves from *Phytolacca heterotepala* H. Walter (Mexican pokeweed) contain at least 10 type 1 RIP isoforms, named heterotepalins. Their Mr values are included in the range 28,000–36,000, as shown by SDS-PAGE performed under reduced conditions and the pI values in the pH range 8.50–9.50. Some heterotepalins are glycosylated. ESI-QTOF mass spectrometry provides the accurate Mr of heterotepalin 4 (29,326.00) and heterotepalin 5b (30,477.00), two isoforms purified to homogeneity by conventional chromatographic techniques. The N-terminal sequences up to residue 35, show that heterotepalins exhibit an high percentage identity with other type 1 RIPs isolated from Phytolaccaceae. Some heterotepalins cross-react with antisera raised against RIPs isolated from *Phytolacca dioica* leaves.

The complete amino acid sequence of heterotepalin 4 matches that of *Phytolacca heterotepala* anti-viral protein PAP (RIP1), deduced from the cDNA sequence of PhRIP1 gene (AC: AY327475), with one exception concerning residue 245 which, in the native protein, is Ile instead of Met. This substitution, found by mass spectrometry mapping, has been directly confirmed by Edman degradation sequencing of the C-terminal tryptic peptide 242–262. The results show the high potential of mass spectrometry and Edman degradation to verify and to uncover possible amino acid substitutions between native proteins and their cDNA deduced sequences.
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1. Introduction

Ribosome-inactivating proteins (RIPs; rRNA *N*-β-glycosidases; EC 3.2.2.22) are cytotoxic enzymes which selectively remove a single adenine from the universally conserved sarcin/ricin domain (S/R domain) of the largest rRNA, thus irreversibly inactivating ribosomes for protein synthesis (Stirpe, 2004). They are present in a number of plants and have been found also in fungi, algae and bacteria (Girbes et al., 2004).

RIPs are currently divided into three groups: type 1 RIPs consist of a single polypeptide chain of about 30 kDa; type 2 RIPs are composed of two chains linked by disulfide bridge(s); type 3 RIPs consist of an amino-terminal domain, resembling type 1 RIPs, linked to a carboxyl-terminal domain with unknown function (Peumans et al., 2001).

It has been reported that, besides the rRNA *N*-glycosidase activity, some RIPs have RNase, DNase, DNA glycosylase activity and can cleave supercoiled dsDNA (Roncuzzi and Gasperi-Campani, 1996; Nicolas et al., 1998; Aceto et al., 2005). In addition, certain type 1 RIPs display a variety of antimicrobial activities, including antifungal, antibacterial (Nielsen et al., 2001), and broad-spectrum antiviral effects against different plant and animal

* Corresponding author. Tel.: +39 0823274535; fax: +39 0823274571.
E-mail address: antimo.dimaro@unina2.it (A. Di Maro).

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viruses (Picard et al., 2005), including a human immunodeficiency virus (Stebbing et al., 2003; Wang et al., 2005). Despite extensive enzymatic and antimicrobial characterization, the significance of RIPs for plant biology remains largely unknown. According to several hypotheses, they could play a major role in the antipathogenic action (against viruses and fungi), in stress and senescence responses. Therefore, widening the knowledge on the occurrence, structural properties and biological functions of RIPs may contribute to the understanding of their role *in vivo* (Stirpe and Battelli, 2006).

Plants commonly produce several RIP isoforms encoded by multi-gene families that could posses adaptations related to their role in tissues. The highest number of RIPs has been found in Caryophyllaceae, Sambucaceae, Cucurbitaceae, Euphorbiaceae, Phytolaccaceae and Poaceae (Girbes et al., 2004). Indeed, a large number of type 1 RIPs were purified from Phytolaccaceae: *Phytolacca americana* (Irvin, 1975; Irvin et al., 1980; Park et al., 2002); *Phytolacca dioica* (Parente et al., 1993; Di Maro et al., 1999) and *Phytolacca insularis* (Song et al., 2000).

In the present work we report: (i) the purification and chemical–physical properties of type 1 RIPs (heterotepalins) from leaves of *Phytolacca heterotepala* H. Walter (Mexican pokeweed); (ii) the N-terminal amino acid sequences up to residue 35 of some native heterotepalins and, (iii) the complete primary structure of heterotepalin 4, one of the major isoforms. A protein databank search has shown that the native heterotepalin 4 sequence matches that deduced from a cDNA (PhRIP1; AC: AY327475), obtained from the same plant and cloned in tobacco (Corrado et al., 2005), with Ile instead of Met in position 245.

2. Results and discussion

2.1. Isolation of heterotepalins and general properties

Crude extracts from *P. heterotepala* leaves inhibited protein synthesis by a rabbit reticulocyte lysate system and showed depurination activity on hsDNA substrates (data not shown). To ascertain whether the activity was due to the presence of RIPs, a purification procedure employed for basic type 1 RIPs was undertaken. Total proteins were extracted from *P. heterotepala* leaves in phosphate-buffered saline and acid precipitated with acetic acid (pH 4.0). Soluble proteins were fractionated by cation exchange chromatography on SP-Sepharose, gel filtration on Sephacryl S-100 HR and finally cation exchange chromatography on CM-52. Ten protein peaks (numbered from 1 to 10) were obtained from the last purification step (Fig. 1a), all with *N*-β-glycosidase activity, as they catalysed the release of adenine both from hsDNA and from purified yeast ribosomes (Massiah and Hartley, 1995; see later).

SDS-PAGE analysis (Fig. 2a) showed that peak 1 of Fig. 1a contained at least three protein bands, with mobilities corresponding to 35, 28 and 23 kDa, respectively (lane 1); peaks 2, 4, 5, 6, 7 and 8 (see the corresponding lanes), in

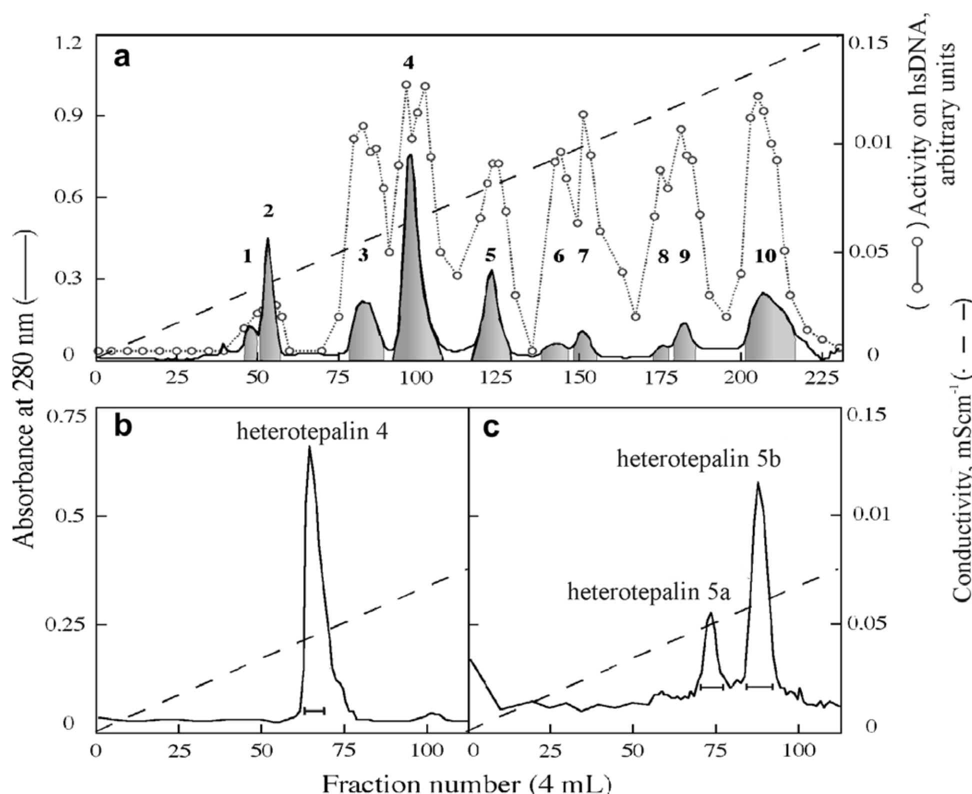

Fig. 1. Purification of RIPs from *P. heterotepala* leaves. (a) Elution profile from the CM-52 chromatography (from Corrado et al., 2005); (b) S-Sepharose rechromatography of peak 4 from a; (c) S-Sepharose rechromatography of peak 5 from (a).

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