Type 1 ribosome-inactivating proteins are the most abundant proteins in iris (Iris hollandica var. Professor Blaauw) bulbs: characterization and molecular cloning

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The most abundant protein of Iris bulbs has been identified as a type 1 ribosome-inactivating protein (RIP). Analysis of the purified proteins and molecular cloning of the corresponding cDNAs demonstrated that this type 1 RIP is a mixture of three isoforms that exhibit a high degree of sequence identity and have similar, though not identical, ribosome-inactivating and polynucleotide:adenosine glycosidase activities. The accumulation of large quantities of type 1 RIP in a vegetative storage organ suggests that this presumed defence-related protein also plays a role in the nitrogen-storage metabolism of the bulb.

INTRODUCTION

Numerous plant species contain proteins, which by virtue of their highly specific RNA N-glycosidase activity are capable of catalytically inactivating ribosomes [1]. According to the generally accepted ideas, the so-called ribosome-inactivating proteins (RIPs) remove a single adenine residue from a conserved loop present in the large ribosomal RNA [2]. Recent work, however, has demonstrated that some, and possibly all, RIPs remove adenine residues from different polynucleotides such as RNA, DNA and poly(A), and hence behave as polynucleotide:adenine glycosidases [3–6]. RIPs are usually divided in two subgroups with a different molecular structure. Type 1 RIPs consist of a single polypeptide chain of approx. 30 kDa. In contrast, type 2 RIPs contain catalytically active A-chains and carbohydrate-binding B-chains. Both chains are derived from a single precursor by the excision of a linker sequence between the A and B domain and are held together by a disulphide bridge between cysteine residues at the C-terminus of the A domain and the N-terminus of the B domain.

In the past numerous type 1 RIPs have been isolated and characterized in some detail. Type 1 RIPs are a homogeneous family of proteins with regard to their biochemical properties and primary structure but differ from each other with respect to their enzymic properties and biological activities. Since there is still a broad interest in type 1 RIPs because of their potential use as plant resistance factors against viruses and fungi, and their possible therapeutic applications as biomissiles, the search for novel type 1 RIPs with interesting properties continues. At present, the majority of type 1 RIPs have been isolated from dicotyledonous plants of the families Cucurbitaceae, Chenopodiaceae, Caryophyllaceae, Euphorbiaceae, Nyctaginaceae, Phytolaccaceae and Lauraceae. Until now, the occurrence of similar proteins in monocotyledonous species has only been documented for a few Gramineae species and for Asparagus officinalis (Asparagaceae, Liliales). Therefore the isolation and characterization of novel type 1 RIPs from other monocots will be of great interest to further corroborate the biological activities and molecular evolution of the whole group of type 1 RIPs.

In this paper we report the isolation, characterization and molecular cloning of three closely related type 1 RIPs from the bulbs of Iris hollandica (Iridaceae, Liliales). The identification of the most abundant bulb protein as a type 1 RIP not only demonstrates for the first time the occurrence of large quantities of such proteins in a typical vegetative storage organ, but also readdresses the question of their possible physiological role.

EXPERIMENTAL

Materials

Iris (Iris hollandica var. Professor Blaauw) bulbs were purchased from a local store. Oligo(dexothymidine)–cellulose was purchased from Sigma Chemical Co. Radioisotopes were obtained from ICN. A cDNA synthesis kit, restriction enzymes and DNA modifying enzymes were obtained from Pharmacia LKB Biotechnology Inc. Escherichia coli XL1 Blue competent cells were purchased from Stratagene.

Isolation and separation of three type 1 RIPs from Iris bulbs

Dry Iris bulbs (200 g) were stripped of their sclerotized outer layer and homogenized in 1 litre of 20 mM acetic acid using a Waring blender. The homogenate was squeezed through cheesecloth, centrifuged at 3000 g for 10 min and the supernatant adjusted to pH 3.0 with 1 M HCl. After standing overnight in the cold room (2 °C), the extract was recentrifuged (3000 g for 10 min). The supernatant was filtered through Whatman 3MM filter paper, diluted with an equal volume of distilled water and loaded on to a column (5 cm × 5 cm; 100 ml bed volume) of S Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM acetic acid. After loading the proteins, the column was washed with 20 mM sodium formate (pH 3.8) until the A280 fell below 0.01. The proteins were eluted with 0.2 M NaCl in 0.5 M

Abbreviations used: HCA, hydrophobic cluster analysis; RIP, ribosome-inactivating protein.

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The new nucleotide sequences reported in this paper have been submitted to Genbank/EMBL/DDBJ under the accession numbers U78039, U78040 and U78041.
Tris/HCl, pH 8.7, dialysed against water, freeze-dried and dissolved in 20 ml of PBS (1.5 mM KH$_2$PO$_4$/10 mM Na$_2$HPO$_4$/3 mM KCl/140 mM NaCl, pH 7.4). Insoluble material was precipitated by centrifugation at 12000 g for 10 min and the supernatant chromatographed on a column (40 cm × 5 cm; 800 ml bed volume) of Sephacryl 100 equilibrated with PBS. Fractions of the main peak were pooled, dialysed against water, freeze-dried and redissolved in 20 ml of 20 mM sodium acetate, pH 4.0, 100 mM KCl, 10 pmol of Iris protein and 20 µg of polynucleotideic substrate. Ribosomes (40 pmol) were incubated with 10 pmol of Iris protein in a final volume of 50 µl, for 40 min at 30 °C in 7 mM magnesium chloride, 20 mM Tris/HCl buffer, pH 7.8, 1 mM dithiothreitol. Controls were run without RIP, and a standard curve of adenine was run with each experiment.

**RNA isolation**

Young shoots were dissected from the inner part of the dry bulbs and finely ground in liquid N$_2$. Total cellular RNA was prepared essentially as described by Van Damme and Peumans [13]. Poly(A)-rich RNA was isolated by affinity chromatography on oligo(deoxythymidime)-cellulose.

**Construction and screening of cDNA library**

A cDNA library was constructed with poly(A)-rich mRNA from young Iris shoots using the cDNA synthesis kit from Pharmacia. cDNA fragments were inserted into the EcoRI site of PUC18. The library was propagated in E. coli XL1 Blue (Stratagene).

The cDNA library was initially screened with a 32P-labelled synthetic oligonucleotide derived from the N-terminal sequence of the most prominent type 1 RIP from Iris. In a later stage, cDNA clones encoding the type 1 RIP from Iris were used as probes to screen for more cDNA clones. Hybridization was carried out overnight as described previously [14]. After washing, filters were blotted dry, wrapped in Saran Wrap and exposed to Fuji film overnight at −70 °C. Positive colonies were selected and rescreeened at low density using the same conditions. Plasmids were isolated from purified single colonies on a miniprep scale using the alkaline lysis method as described by Mierendorf and Pfeffer [15] and sequenced by the dyeoxy method [16]. DNA sequences were analysed by using programs from PC Gene (Biomed, Geneva, Switzerland) and Genepro (Riverside Scientific, Seattle, WA, U.S.A.).
Figure 2 SDS/PAGE of a crude extract and purified type 1 RIPs from Iris bulbs

Reduced (with 2-mercaptoethanol) (lanes 1–3) and non-reduced (lanes 4–7) samples were run as follows: lanes 1 and 4, IrisRIP.A1; lanes 2 and 5, IrisRIP.A2; lanes 3 and 6, IrisRIP.A3; lane 7, crude extract from Iris bulbs. Molecular-mass reference proteins are shown in lane R; they are lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase b (96 kDa).

Table 1 Enzymic activity of type 1 RIPs from Iris

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Substrate</th>
<th>hsDNA</th>
<th>rRNA</th>
<th>Poly(A)</th>
<th>Rat liver ribosomes</th>
<th>IC_{50} (nM)</th>
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<td>IrisRIP.A1</td>
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<td>2</td>
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<td>0.16</td>
<td></td>
</tr>
<tr>
<td>IrisRIP.A2</td>
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<td>5</td>
<td>5</td>
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</tr>
<tr>
<td>IrisRIP.A3</td>
<td>139</td>
<td>9</td>
<td>3</td>
<td>0.86</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

Iris

RESULTS

Isolation and characterization of three isoforms of a type 1 RIP from Iris bulbs

Using a combination of extraction at low pH, ion-exchange chromatography and gel filtration, a pure preparation of a type 1 RIP was isolated from resting Iris bulbs. Using ion-exchange chromatography on a Neobar CS15/4 column, the total preparation was further resolved into three isoforms designated IrisRIP.A1, IrisRIP.A2 and IrisRIP.A3 following the elution position from the column (Figure 1). All three isoforms migrated as single polypeptide bands of 29 kDa upon SDS/PAGE (Figure 2) and were eluted with an apparent molecular mass of approx. 30 kDa upon gel filtration on a Superose 12 column (results not shown), indicating that they are single-chain proteins. No covalently bound carbohydrate could be detected in any of the three RIP isoforms by the phenol/sulphuric acid method.

N-terminal amino acid sequencing of the three isoforms revealed highly similar amino acid sequences (Figure 1). In addition, the sequences clearly exhibit sequence similarity to other type 1 and type 2 RIPs.

SDS/PAGE further indicated that the most abundant bulb protein co-migrated with the type 1 RIP (Figure 2). Sequencing of the 29 kDa polypeptide from the crude extract yielded a sequence identical with those of the purified RIPs (results not shown), which confirmed that RIPs are the most abundant proteins in the Iris bulb. The abundance of RIPs in a bulb extract can explain the high yield of purified proteins, which was approx. 250 mg of a mixture of the three isoforms from 100 g of bulbs.

Ribosome-inactivating activity of the Iris RIPs

All three isoforms inhibited protein synthesis by a reticulocyte lysate. Their activity was similar, the IC_{50} ranging from 0.10 to 0.16 nM (Table 1).

The three isoforms also released adenine, not only from whole...
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Figure 3 Comparison of the deduced amino acid sequences of the cDNA clones encoding the type 1 RIPs from Iris

The arrowhead indicates the processing site for the cleavage of the signal peptide. Dots denote gaps introduced to get maximal alignment of the sequences. Determined N-terminal amino acid sequences are underlined. Since the first methionine is most probably used as the translation initiation site, the amino acids preceding this methionine are shown in lower case letters. Amino acids that are conserved between the different Iris sequences are indicated by asterisks and chemically similar amino acids are indicated by dots on the bottom line.

Figure 4 Comparison of the HCA plots of the A-chain of ricin (A) and IrisRIP.A3 (B)

Helices (α) and strands of β-sheet (β) delineated on the HCA plot of ricin A-chain were reported on the HCA plot of IrisRIP.A3. These delineations were used to recognize the structurally conserved regions between the ricin A-chain and IrisRIP.A3.

Molecular cloning of cDNAs encoding the three RIPs

Screening of a cDNA library constructed with RNA from young Iris shoots resulted in the isolation of three groups of cDNAs. Each of these groups could be assigned to one of the three RIP isoforms on the basis of a perfect match with the N-terminal amino acid sequences of the purified proteins. Sequence analysis indicated that RIPIriHol.A1 (cDNA encoding the type 1 RIP from Iris) contains an open reading frame of 908 bp encoding a 303-amino-acid precursor with one putative initiation codon at position 4 of the deduced amino acid sequence (Figure 3). Translation starting with this methionine residue results in a protein of 300 amino acids with a calculated molecular mass of 33.4 kDa. According to the rules of von Heijne [22], a signal peptide is cleaved between residues 23 and 24. The resulting polypeptide, which matches perfectly the N-terminal sequence of IrisRIP.A1, has a molecular mass of 30.9 kDa and an isoelectric point of 9.70. RIPIriHol.A2 and RIPIriHol.A3 show the same overall structure as RIPIriHol.A1 and exhibit 84–95% sequence identity at the deduced amino acid level. The calculated molecular masses of the isoforms 2 and 3 (after removal of the signal peptide) are 31.0 and 30.9 kDa respectively, and their isoelectric points 9.88 and 9.97 respectively. The isoelectric-point values of the three proteins are in agreement with the sequence of their elution from the cation-exchange column.

Northern blot analysis

Northern blot analysis was performed to determine the total length of the RIP mRNAs. Hybridization of the blot using the synthetic oligonucleotide as a probe yielded one band of approx.
Type 1 ribosome-inactivating proteins from Iris

Figure 5  Stereoviews of the three-dimensional models of ricin A-chain (A) and IrisRIP.A3 (B)

Helices (thick lines) and left-handed twisted 6-strand β-sheet (heavy lines) are indicated. The deep cleft shown on the right side of the chains corresponds to the active site which binds adenine of 28S RNA.

1300 nucleotides (results not shown). Identical results were obtained when hybridization was performed with the random-primer-labelled cDNA clones encoding the Iris RIP. The size of the RNA is in good agreement with the length of the cDNA clones that were analysed.

Molecular modelling

The amino acid sequences of the type 1 RIP from Iris exhibit a high degree of identity and similarity with other type 1 RIPs and with the A-chains of type 2 RIPs. RIPIriHol.A3, for instance, has 35% identity and 62% similarity with the A-chain of the classical type 2 RIP ricin. Since the high degree of similarity between this type 1 RIP and the A-chain of ricin indicates that both polypeptides are closely related structurally, the sequence RIPIriHol.A3 encoding IrisRIP.A3 was modelled using the co-ordinates of ricin, the three-dimensional structure of which has been resolved by X-ray crystallography [20]. Although it must be emphasized that the results of these modelling studies have to be interpreted with care, they can give interesting information about structural similarities between related proteins.

The catalytically active A-chain of ricin is built up of three distinct domains and contains regular secondary structures such as eight α-helices, and six strands of β-sheet, which exhibit a left-handed twist of approx. 110° when observed along the hydrogen bonds [23]. A comparison of the HCA plots of the A-chain of ricin and IrisRIP.A3 shows that α-helices and strands of β-sheet are readily conserved in both proteins, although some discrepancies exist between both sequences due to gaps or insertions of a few amino acid residues occurring between the secondary-structure features of IrisRIP.A3 (Figure 4). Once the structurally conserved regions common to both proteins were identified, the coordinates of ricin could be used to build a three-dimensional model of IrisRIP.A3.
Modelling of IrisRIP.A3 yielded a three-dimensional model very similar to that of the ricin A-chain (Figure 5). Despite a few discrepancies due to the deletions and insertions between the secondary structures, the overall folding of the ricin A-chain is fully conserved in IrisRIP.A3. IrisRIP.A3 contains eight α-helices and a six-stranded β-sheet with a left-handed twist similar to those found in the ricin A-chain.

All amino acid residues that build up the active site of the A-chain of ricin (Tyr<sub>49</sub>, Tyr<sub>123</sub>, Glu<sub>177</sub>, Arg<sub>180</sub> and Trp<sub>211</sub>) [23–25] are fully conserved in IrisRIP.A3. IrisRIP.A3 contains Tyr<sub>119</sub>, Tyr<sub>122</sub>, Glu<sub>161</sub>, Arg<sub>164</sub> and Trp<sub>199</sub>). However, Ala<sub>178</sub>, another invariant residue, which is conserved in all A-chains and is probably responsible for the stable state of the active site of the ricin A-chain [23], is replaced by Thr<sup>162</sup> in IrisRIP.A3, as well as in all other type 1 RIPs from Iris. With the exception of two changes, all residues that are located in the vicinity of the active site of the ricin A-chain, and are probably necessary to maintain its catalytic conformation (Asn<sub>78</sub>, Arg<sub>134</sub>, Gln<sub>173</sub>, Glu<sub>208</sub> and Asn<sub>220</sub>), are conserved in IrisRIP.A3 (Asn<sub>78</sub>, Arg<sub>119</sub>, Gln<sub>157</sub>, Gln<sub>199</sub> and Thr<sub>199</sub>) (Figure 6). This high degree of conservation is in good agreement with the RIP activity of IrisRIP.A3.

Because of the high degree of both sequence identity (> 85%) and similarity (> 95%) between the three type 1 RIP isoforms from Iris, all these proteins exhibit a three-dimensional structure that is very similar to that of the ricin A-chain, and their HCA plots are almost identical (results not shown). Like in IrisRIP.A3, the residues responsible for the N-glycosidase activity of the ricin A-chain are also conserved in the other two isoforms, except for Ala<sub>178</sub>, which is replaced by Thr<sup>162</sup>.

The three-dimensional models of the Iris sequences, built from the co-ordinates of ricin, can nicely be superimposed on the structures of the type 1 RIP trichosanthin (PDB code 1mrj) and momordin I (α-momorcharin, PDB code 1mri) [26], and on that of the A-chain of abrin (PDB code 1abr) [27] (Figure 7). This holds especially true (r.m.s. deviation < 0.5 Å) for the α-helices and strands of β-sheet, which constitute the overall folding pattern of the proteins. The only discrepancies occur in the loop conformations. As a result, the network of amino acid residues forming the adenine-binding site of all these protein models is well superimposable.

**Phylogenetic tree**

A search in the database indicated that the deduced amino acid sequences of the type 1 RIP from Iris exhibit a high degree of sequence similarity to the previously cloned type 1 RIPs, as well as to the A-chains of type 2 RIPs. As shown in Figure 8, the Iris RIPs form a separate cluster of the phylogenetic tree built from the amino acid sequences of various type 1 RIPs and the A-chains of type 2 RIPs. It is also noteworthy that the type 1 RIPs
The present paper describes the isolation and molecular cloning of three isoforms of a protein from the bulbs of *Iris* that could be identified as type 1 RIPs on the basis of their inhibitory effect on cell-free protein synthesis and the release of adenine from ribosomes. Like other RIPs, they act not only on whole ribosomes, but also on purified rRNA, poly(A) and DNA, confirming that at least the effect on DNA is a common property of all RIPs tested so far [6]. Sequence comparisons and molecular modelling indicated that the *Iris* RIPs are structurally and evolutionarily related to type 1 RIPs from other species and to the A-chain of type 2 RIPs. The discovery of the *Iris* RIPs is important since it demonstrates for the first time that the occurrence of these proteins within the monocotyledoneae is not restricted to the Gramineae (like wheat, barley, rye, rice and maize) and Asparagaceae (asparagus). In addition, the identification of the most abundant bulb protein as a type 1 RIP shows that not only seeds but also typical vegetative storage organs can accumulate large quantities of a presumably defence-related protein. The latter observation has two important consequences. First, *Iris* bulbs are a readily available and rich source of a type 1 RIP that can easily be isolated by simple techniques. Secondly, the high concentration of type 1 RIPs in the bulbs addresses the question of their physiological role. By analogy with the double role of some abundant plant lectins [28], the hypothesis can be put forward that the *Iris* type 1 RIPs are storage proteins, which due to their particular enzymic activity can be used as defence proteins whenever the plant is challenged by a pathogen or a predator. It is evident, however, that such a defence-related role does not preclude another more specific role in the metabolism of the plant.

### REFERENCES


**Figure 7** Superimposition of the three-dimensional models of IrisRIP.A3 (thick line) with those of the A-chains of ricin and abrin, as well as the type 1 RIPs  z-momorcharin and trichosanthin (thin lines).

The secondary-structure features (a-helices and b-sheets) are well superimposed. Most of the discrepancies occur in the loop regions.

**Figure 8** Phylogenetic tree built up from the amino acid sequences of different RIPs.

The phylogenetic tree is built from the sequences of *Iris* hollandica (RIPIriHol.A1, RIPIriHol.A2 and RIPIriHol.A3), *Luffa cylindrica* (luffin-a and luffin-b), Trichosanthes kirilowii (trichosanthin, karasurin), Momordica charantia (momorcharin), Momordica balsamina (momordin), Mirabilis jalapa (mirja), Saponaria officinalis S6 (sapof) and the A-chains from the type 2 RIPs of *Ricinus communis* (Ricin-A and RCA-A), *Abras precatorius* (Abrin-A) and *Sambucus nigra* (SNAI-A and SNAV-A). The scale indicates the number of amino acid changes.

from *Iris* are apparently more closely related to the type 2 RIPs from *Abras precatorius* and *Ricinus communis* than to most other type 1 RIPs.

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