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DODECANDRIN, A NEW RIBOSOME-INHIBITING PROTEIN FROM *PHYTOLACCA DODECANDRA*

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Dodecandrin, a newly discovered ribosome-inhibiting protein, has been isolated and purified from the leaves of the African endod plant, *Phytolacca dodecandra*. Dodecandrin has a molecular weight of approx. 29000. It cross-reacts with antiserum prepared against pokeweed antiviral protein from *Phytolacca americana* and exhibits similar requirements for antiribosomal activity. It is more basic than pokeweed antiviral protein, and comparison of the first 30 amino-terminal residues of the two proteins reveals 83% homology. This level of homology is greater than that between pokeweed antiviral protein and pokeweed antiviral protein S, another antiviral protein found in *P. americana*. Such conservatism in sequence, coupled with the high efficiency of the proteins in deactivating ribosomes and with their abundance in plant tissue, suggests that they serve an important function in the life of the plant, probably as a defense against infection.

Introduction

In recent years, it has become evident that many species of angiosperms contain proteins which act as powerful inhibitors of eukaryotic ribosomes. These ribosome-inhibiting proteins have been isolated from a number of phylogenetically diverse plants. *Phytolacca americana* (Phytolaccaceae) produces three known inhibitors, pokeweed antiviral protein, pokeweed antiviral protein II and pokeweed antiviral protein S [1–3]. Other inhibitors which have been isolated and partially characterized include luffin [4] from *Luffa cylindrica roem* and mormordin [5] from *Mormordica charantia* (Cucurbitaceae), gelonin [6] from *Gelonium multiflorum* (Euphorbiaceae) and tritin [7] from wheat germ (Gramineae). Pokeweed antiviral protein and gelonin have been well studied and shown to act by enzymatically modifying the stroying the EF-2-dependent GTPase activity of the subunit and preventing the elongation step of protein synthesis [8].

Ribosome-inhibiting proteins strongly resemble in their action the effective subunits (A-chains) of such dimeric protein toxins as ricin and abrin [9], modeccin [10], and viscumin [11]. These extremely potent toxins contain two disulfide-linked subunits, one of which (the B-chain) binds to the cell surface and mediates the entry of the other (the A-chain) into the cytoplasm. The A-chain, once within the cell, attacks the 60 S ribosomal subunit, causing protein synthesis to cease and killing the cell.

Both toxins [12–14] and ribosome-inhibiting proteins [14–17] have been used as components of cell-specific immunotoxins. These enzyme-antibody complexes have been shown to destroy targeted cells in vitro selectively [12–17] and have been used in vivo to prolong effectively the lives of tumor-bearing animals [15,17]. It has been pointed out [15] that ribosome-inhibiting proteins are of

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Abbreviation: Mes, 2-[*N*-morpholino]ethanesulfonic acid.

special value in immunotoxin production. Lacking a cell-binding B-chain, they do not require rigorous and often difficult procedures for removing B-subunit contamination in order to eliminate nonspecific cytotoxicity.

Our laboratory has been involved in investigation of the structure [18] and function [19] of pokeweed antiviral protein; as mentioned above, however, this species contains two other similar enzymes. Pokeweed leaves also contain a protein called pokeweed antiviral protein II [2]. Pokeweed antiviral protein is the primary inhibitor present in young leaves. As the plant matures, pokeweed antiviral protein II levels increase until, in late summer, pokeweed antiviral protein II becomes the predominate form. Pokeweed antiviral protein II does not cross-react with antibody to pokeweed antiviral protein, but has a similar molecular weight and mode of action [2]. A third protein, named pokeweed antiviral protein S, found in pokeweed seeds, has a molecular weight approx. 29000 and exhibits antiribosomal activity [3]. It appears to be more closely related to pokeweed antiviral protein than to pokeweed antiviral protein II based on a partial immunological cross-reactivity. This similarity is born out by comparison of the amino-terminal sequences of the two proteins [20]. This variety of antiribosomal enzymes within a single plant suggests an important role for ribosome-inhibiting proteins and led us to ask whether closely related species would contain inhibitors of their own.

The species tested, *Phytolacca dodecandra* (endod), is separated from *P. americana* at the subgeneric level (*Pircunia* vs. *Euphytolacca*) [21], a relationship sufficiently distant for significant evolutionary divergence to have occurred. The two species are split geographically. *P. dodecandra* is found in tropical and southern Africa. *P. americana* is originally New World, although it has been introduced worldwide by man.

In this paper we report the isolation of ribosome-inhibiting proteins from *P. dodecandra*, the purification and partial characterization of one of them, and a comparison of its N-terminal amino acid sequence to those previously published for two inhibitors from *P. americana*. To our knowledge, this represents the closest phylogenetic comparison that has been made for ribosome inhibitors from different organisms.

Materials and Methods

Pokeweed antiviral protein. Pokeweed antiviral protein was prepared according to the method of Irvin [1], and was stored frozen in 50-μl aliquots at $-70°C$.

Antibody. Immunological experiments were performed using rabbit anti-pokeweed antiviral protein prepared as previously described [19].

Immunological methods. Ouchterlony immunodiffusion was performed in petri dishes containing 1.5% agar in phosphate-buffered saline plus 0.005% sodium azide. The plates were incubated for 48 h in a moist chamber, then washed for 24 h in deionized water to diffuse out unprecipitated protein. Gels were stained in 0.2% Coomassie brilliant blue G-250 in 25% isopropanol/10% acetic acid, destained and pressed dry into filter paper.

Immunoelectrophoresis was performed according the method of Graber and Williams [22]. 1.5% agarose gels buffered in 50 mM Tris-barbital (pH 8.6) were electrophoresed for 4 h at 100 V. Troughs were then cut and antibody added to fill the wells. Gels were incubated in a moist chamber for 48 h, then pressed dry. Unprecipitated protein was removed by washing the gel overnight in phosphate-buffered saline before staining.

Polyphenylalanine synthesis assays. The polyuridylic acid-directed polyphenylalanine synthesis system used to determine ribosome inhibition was as previously described [19]. KCl-washed wheat-germ ribosomes and wheat-germ postribosomal supernatant (S150) were prepared by the method of Walthall et al. [23]. In determining salt, ATP and S150 requirements, ribosomes were preincubated with inhibitory proteins under the various conditions described under Results. Unless otherwise stated, the preincubation was terminated by addition of 40 μg rabbit antibody and the reaction mix was brought to standard pseudophysiological conditions as described in Ref. 19 prior to initiation of protein synthesis.

Amino-terminal sequence determination. Sequence analysis was performed at the University of Texas Protein Sequencing Center. 10-nmol samples of protein were lyophilized from water. Samples were sequentially degraded on a Beckman 890C Sequencer equipped with a Sequemat P-6 autoconverter. An Altex 345C HPLC and a

Hewlett-Packard 3390A integrator were used to analyze the products, according to the method of Tarr [24].

Results and Discussion

The purification protocol for one inhibitor from *P. dodecandra* is shown in Table I. Leaves, typically 500 g, were ground in a Waring blender with 1000 ml of buffer A (10 mM Tris-HCl (pH 7.5)/1 mM β-mercaptoethanol/0.2 mM EDTA). This extract was stirred overnight at 4°C, then filtered and centrifuged to remove solids.

The 65–100% saturated ammonium sulfate cut of the raw extract, dialyzed back into buffer A, was applied to a 5 × 60 cm column of DEAE-Sephadex equilibrated in the same buffer. Most contaminants bound to the column, while the inhibitory activity eluted in a broad peak.

The DEAE eluate, concentrated to about 40 ml, was dialyzed against buffer B (10 mM Mes (pH 5.2)/0.2 mM EDTA/0.1 mM β-mercaptoethanol) before being applied to a 1 × 30 cm column of Whatman P-11 phosphocellulose equilibrated in the same buffer. The column was washed with several volumes of buffer and developed with a linear 1.2 l 0–500 mM NaCl gradient in buffer B. Two major peaks eluted from the column, both exhibiting extremely potent antiribosomal activity (Fig. 1).

SDS-polyacrylamide gel electrophoresis showed that the broad first peak contained two poorly resolved bands of apparent molecular weights 31 000 and 32 000.Both bands cross-react with anti-pokeweed antiviral protein, as determined by Ouchterloney immunodiffusion. To date, however, these two protiens have proven difficult to resolve and will not be discussed further in this paper.

The second phosphocellulose peak is sharp and well-resolved. SDS-polyacrylamide gel electrophoresis reveals a single protein which comigrates with pokeweed antiviral protein, suggesting it has a molecular weight of about 29 000 (Fig. 2a). We have named this protein dodecandrin. Non-denaturing gels at pH 4.3 indicate that doedecandrin is slightly more basic than pokeweed antiviral protein (Fig. 2b).

Ouchterloney immunodiffusion experiments show that dodecandrin cross-reacts completely with antibody to pokeweed antiviral protein, indicating that all antigenic sites are held in common. When subjected to immunoelectrophoresis by the method of Graber and Williams, dodecandrin forms a single arc slightly farther toward the cathode than pokeweed antiviral protein, again indicating a slightly greater basicity. We also found that addition of 8 μg antibody completely inhibited the action of 10 ng dodecandrin on polyphenylalanine synthesis by wheat-germ ribosomes.

We have previously determined that the pokeweed antiviral protein reaction is strongly dependent on salt concentration, being most active at low salt concentrations (20 mM KCl/2 mM $MgAc_2$) and virtually inactive at higher pseudophysiological salt concentrations (90 mM KCl/4 mM $MgAc_2$). However, the presence of ATP and a heat-labile factor present in wheat-germ postribosomal supernatant (S150) allow the reaction to

TABLE I

PURIFICATION OF DODECANDRIN FROM 500 g *P. DODECANDRA* LEAVES

One unit is defined as the amount of protein required to inhibit 50% of the polyphenylalanine synthesis activity of 35 pmol wheat-germ ribosomes in 250 μl total volume during a 15 min incubation.

Purification step	mg protein	Units/mg	Units × 10^{-7}	Yield (%)
Raw extract	79001 [a]	1083	8.56	100
65–100% satd. $(NH_4)_2SO_4$ cut	1364 [a]	63291	8.63	101
DEAE filtrate	110.8 [a]	238095	2.64	31
Phosphocellulose eluate	18.1 [a]	714286	1.29	15
H_2O dialysate	16.9 [b]	769231	1.30	15

[a] Based on A_{280}/A_{260}.

[b] Based on modified Folin-Lowry protein determination using pokeweed antiviral protein as a standard.

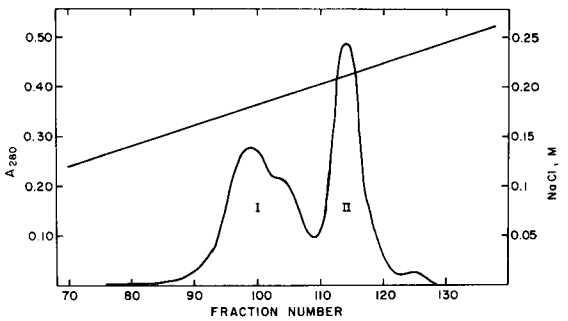

Fig. 1. Elution of dodecandrin from phosphocellulose. The diagonal line respresents NaCl concentration. Fraction size was 5 ml. Only that portion of the profile with A_{280} greater than background is illustrated. Peak I contains two major proteins and exhibits substantial antiribosomal activity. Peak II contains dodecandrin.

proceed unhindered at high salt levels [19].

Dodecandrin exhibits similar requirements when tested against wheat-germ ribosomes. In the absence of added cofactors, dodecandrin functions well at low levels of K^+ and Mg^{2+} and is inhibited by increasing the concentration of either cationic species. In the presence of ATP and S150, dodecandrin action becomes relatively independent of salt concentration with a slight peak in activity occurring at physiological salt levels.

In vitro examination of the antiribosomal activity of dodecandrin under pseudophysiological conditions showed it to be at least as potent as pokeweed antiviral protein in inhibiting the polyphenylalanine synthesizing ability of wheat-germ ribosomes (Fig. 3). This high level of inhibitory activity should make dodecandrin an excellent candidate for use in cell-specific immunotoxins.

We examined the N-terminal amino acid sequence of dodecandrin for comparison with sequences previously published for pokeweed antiviral protein and pokeweed antiviral protein S [20]. The first 32 residues were considered to be definitely determined; these are shown in Fig. 4 along with the corresponding residues of pokeweed antiviral protein and pokeweed antiviral protein S. Dodecandrin shows a marked similarity to pokeweed antiviral protein, the differences being the substitution of alanine for phenylalanine at position 15, of Met-Asp-Asn for Leu-Asn-Asp beginning at position 20, and of arginine for glutamic

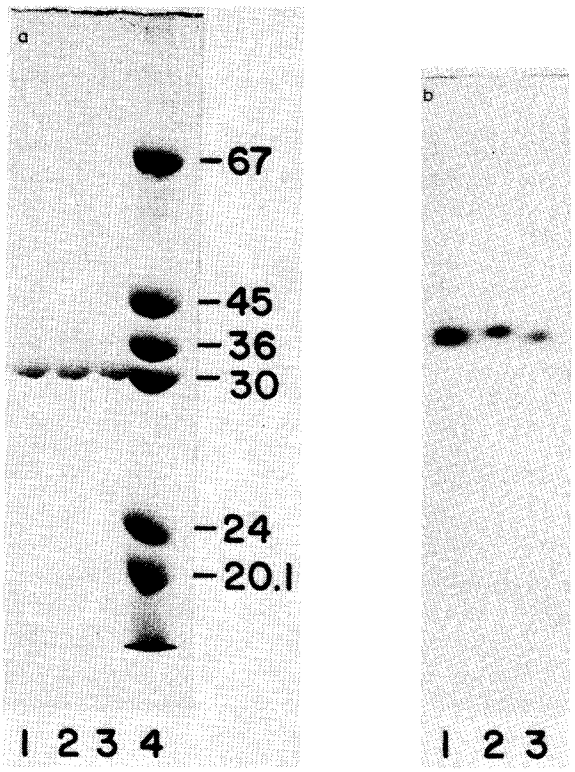

Fig. 2. (a) 10% SDS-polyacrylamide gel electrophoresis of dodecandrin and pokeweed antiviral protein. Lane 1: 2.5 μg dodecandrin + 2.5 μg pokeweed antiviral protein. Lane 2: 5 μg pokeweed antiviral protein. Lane 3: 5 μg dodecandrin. Lane 4: molecular weight markers: bovine serum albumin (67 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa). (b) 10% disc-polyacrylamide gel electrophoresis of dodecandrin and pokeweed antiviral protein at pH 4.3. Lane 1: 1.5 μg dodecandrin + 1.5 μg pokeweed antiviral protein. Lane 2: 1.5 μg dodecandrin. Lane 3: 1.5 μg pokeweed antiviral protein.

acid at position 24. It should be noted that the methionine at position 20 corresponds to an identical residue in pokeweed antiviral protein S.

Although pokeweed antiviral protein and dodecandrin are clearly not identical proteins, differing in five of the first 30 N-terminal amino acid residues, they are obviously extremely similar. Given that the differences between pokeweed antiviral protein and pokeweed antiviral protein S – two proteins occurring within the same individual plant – are greater than the differences between it

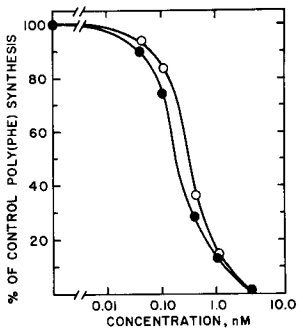

Fig. 3. Dose-response of wheat-germ ribosomes to dodecandrin (●) and pokeweed antiviral protein (○). Dilutions of inhibitor were added to a polyphenylalanine synthesizing mix containing 37 pmol wheat-germ ribosomes. The mix was then incubated for 15 min at 25°C. Uninhibited phenylalanine incorporation was 155 pmol.

and dodecandrin, a great deal of evolutionary conservatism seems to be implied. This is especially true since no function has definitely been assigned to the ribosome inhibitors of *Phytolacca*.

These proteins exhibit antiviral properties when applied to the leaves of other plants [25] or to animal cells in culture [25–27], due to their ability to enter the cell along with the virion and kill the cell before viral replication occurs. In order to be an effective antiviral agent, however, an enzyme would need to inhibit synthesis on its own ribosomes, at least in the presence of virus. Owens et al. [28] could demonstrate to inhibitory effect by pokeweed antiviral protein on isolated pokeweed ribosomes. Their pokeweed ribosomes, however, exhibited low levels of activity comparable to the activities they obtained for wheat germ and cowpea ribosomes after inhibition. Battelli et al. [29] obtained similar results, but their 'uninhibited' pokeweed ribosomes had less than 1% of the activity of their wheat-germ ribosomes. Isolation attempts in our laboratory have so far not produced high-activity pokeweed ribosomes. It seems at least possible that pokeweed antiviral protein and other similar enzymes are compartmentalized; breakage of the cells during preparation would then release their activity, inhibiting pokeweed ribosomes. We feel that the question of whether these enzymes are capable of inhibiting conspecific ribosomes is still an open one. Further studies using highly purified, active ribosomes will be needed before the issue can be clearly decided.

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                                    10                  20
DODECANDRIN          : V N T I I Y N V G S   T T I S N Y A T F M
POKE. ANTIVIRAL PROT.: V N T I I Y N V G S   T T I S F Y A T F L
PAP - S              : I N T I T F D A G H   A T I N K Y A T F M
                                    30
DODECANDRIN          : D N L R N E A K D P   S L
POKE. ANTIVIRAL PROT.: N D L E N E A K D P
PAP - S              : E S L ? N E A K D
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Fig. 4. Comparison of the amino-terminal sequences of dodecandrin, pokeweed antiviral protein and pokeweed antiviral protein S (PAP-S). Dodecandrin differs from pokeweed antiviral protein at five residues and from pokeweed antiviral protein S at 11 residues.

In assessing the role of ribosome-inhibiting proteins several points should be borne in mind. (1) Proteins like pokeweed antiviral protein and dodecandrin are highly conserved over the course of evolution. (2) Several separate such proteins may be produced during the life of an individual plant. (3) These proteins represent a significant resource investment by the plant (up to 0.5% of the total soluble protein). (4) The proteins are very efficient at dispatching ribosomes. The turnover number for pokeweed antiviral protein is 400 min^{-1} and the K_m for ribosomes is 0.2 μM [19]. It seems reasonable that these enzymes exist to inhibit protein synthesis, and they they probably inhibit synthesis on their own ribosomes, at least under some conditions. Pokeweed is susceptible to infection by some viruses [30] and ribosome-inhibiting proteins, unlike the heterodimeric cytotoxins, cannot afford protection against large eukaryotic herbivores. As a result, their physiological role is still a mystery, although it is likely to involve plant defense.

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