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The mechanism of action of the cytotoxic lectin from *Phoradendron californicum*: the RNA *N*-glycosidase activity of the protein

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A toxic lectin from *Phoradendron californicum* (PCL) was found to inactivate catalytically 60 S ribosomal subunits of rabbit reticulocytes, resulting in the inhibition of protein synthesis. To study the mechanism of action of PCL, rat liver ribosomes were treated with the toxin and the extracted rRNA was treated with aniline. A fragment containing about 450 nucleotides was released from the 28 S rRNA. Analysis of the nucleotide sequence of the fragment revealed that the aniline-sensitive phosphodiester bond was between A4324 and G4325 of the 28 S rRNA. These results indicate that PCL inactivates the ribosomes by cleaving an *N*-glycosidic bond at A4324 of 28 S rRNA in the ribosomes as does ricin A-chain.

rRNA; Ribosome inactivation; Lectin; Toxin action

1. INTRODUCTION

Cytotoxic proteins that catalytically inactivate eukaryotic ribosomes have been isolated from a variety of plants [1–3]. These proteins, the best studied examples of which are ricin and abrin, consist of two non-identical subunits (A- and B-chain) that are joined by a disulfide bond [1–5]. The B-chain binds to receptors on the surface of cells and promotes the uptake of the A-chain into cells [1–5]. The entry of the A-chain into the cytoplasm of a cell then results in the death of the cell by inactivation of its ribosomes [1–4]. We have already shown that ricin A-chain (and the A-chain of many other cytotoxins including bacterial toxins) is a specific RNA *N*-glycosidase that inactivates ribosomes by hydrolyzing a single *N*-glycosidic bond between an adenine and ribose at a specific nucleotide (A4324) of the 28 S rRNA in the ribosomes [3,6–10].

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Recently, we have purified and characterized another cytotoxic Viscaceae lectin (PCL) from *Phoradendron californicum* [11]. PCL (69 kDa) consists of one A chain (31 kDa) and one B chain (38 kDa). The carbohydrate content is 14.1%. PCL is D-galactose-specific and has no blood group specificity. The lethal dose (i.p.) for mice (25 g) is 100 μg. The toxicity of mistletoe lectin 1 (ML 1) under the same conditions is 0.7 μg. However, the mechanism of cytotoxic action of PCL has not been elucidated as yet. Here, we demonstrate that PLC inhibits protein syntehsis in rabbit reticulocytes by inactivating the 60 S ribosomal subunit. This inactivation is a result of the hydrolysis by the A chain of PCL of the *N*-glycosidic bond of the residue A4324 of rat liver 28 S rRNA, a mechanism identical to that of ricin A-chain.

2. MATERIALS AND METHODS

2.1. *General*

The following procedures were either described or cited previously [6,7,12,13]: isolation and purification of PCL from

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the extract of *P. californicum*, preparation of rabbit reticulocyte and the incubation conditions for globin synthesis, preparation of rat liver ribosomes, incubation of the ribosomes with PCL, extraction of rRNA with phenol and dodecyl sulfate, aniline-induced chain scission at the modified site in 28 S rRNA, analysis of the nucleic acids by polyacrylamide-agarose composite gel electrophoresis, preparation of 5′-^{32}P-labeled fragment and the method for sequencing of RNA. We thank Dr F. Hawskworth (US Department of Agriculture, Forest Service Station, Fort Collins, USA) for collecting the plant material and Dr P. Muller (Staatliches Institut für Immunpräparate und Nährmedien, Berlin, GDR) for preparing PCL.

2.2. *Polyphenylalanine synthesis*

Ribosomal subunits were prepared from rabbit reticulocytes by the method of Schreier and Staehelin [14]. The concentration of ribosomes was calculated from the extinction at 260 nm based on a value $E^{1\%}_{260}$ of 100 and an M_r of 4.3×10^6 [15]. The reaction mixture (0.1 ml) contained the following: 25 mM Hepes-KOH buffer (pH 7.4), 75 mM potassium acetate, 8 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 0.25 mM GTP, 10 mM creatine phosphate, 10 μg creatine kinase (Boehringer, Mannheim), 25 μg rat liver tRNA, 25 μg poly(U) (Pharmacia P-L Biochemicals), 1.2 A_{260} units purified ribosome components (0.4 A_{260} unit of 40 S, and 0.8 A_{260} unit of 60 S subunits), 500 μg protein of tRNA-free reticulocyte S-100 fraction, and 1 μCi [^{3}H]phenylalanine (spec. act. 430 μCi/mmol, Amersham). After incubation at 30°C for 30 min, 20-μl aliquots (duplicate for each reaction) were spotted on 3MM Whatman paper filters. Radioactivity insoluble in hot trichloroacetic acid was counted as described [16].

3. RESULTS AND DISCUSSION

When small amounts of PCL were added to a cell-free protein synthesizing system (S-30) from rabbit reticulocytes, incorporation of [^{3}H]leucine into acid-insoluble material was strongly inhibited (fig.1). A concentration of PCL of 4.0×10^{-8} M (protein/ribosome ratio of 0.1) partially inhibited leucine incorporation, while a higher concentration (2.0×10^{-7} M) led to almost complete inhibition of protein synthesis. However, the inhibitory activity of PCL was much lower than that of ricin A-chain. This finding seems to be consistent with our previous observation, in which PCL is less toxic in mice than other typical toxic lectins such as MLT [11]. Table 1 clearly shows that ribosomes derived from the lectin-treated lysate have reduced polymerizing activity, indicating that the target of the action of the toxic lectin is on ribosomes. When the 60 S ribosomal subunits were derived from the PCL-treated lysate, significant reduction in polyphenylalanine synthesis was observed. The PCL-treated 40 S subunits, however, were less active than those of control when assayed with the

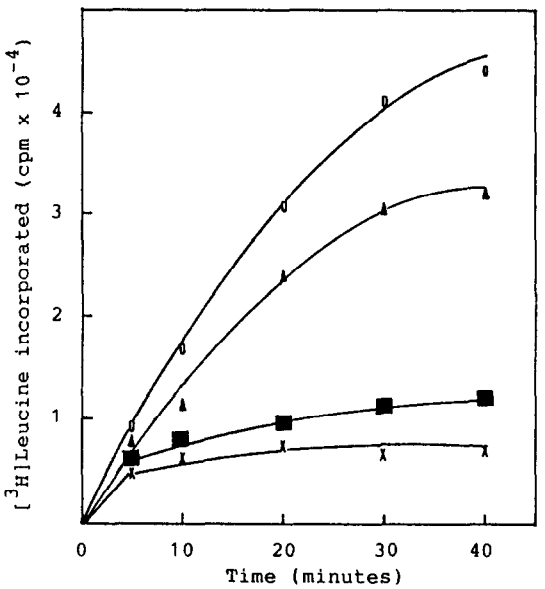

Fig.1. Effect of A-chain of PCL on protein synthesis in rabbit reticulocyte lysate system. Reaction mixtures (50 μl) containing 4.16×10^{-7} M ribosomes were incubated at 30°C and at the times indicated, aliquots were removed and acid-insoluble radioactivities were counted. (○—○) Control; (△—△) 4.0×10^{-8} M; (■—■) 2.0×10^{-7} M or 1.0×10^{-6} M of the A-chain of PCL; (×—×) 1.0×10^{-9} M ricin A-chain.

combination of control 60 S. This may result from the action of PCL adsorbed onto the ribosomal subunits even after extensive washing during the purification procedures. Therefore we conclude that the A-chain of PCL inhibits protein synthesis in the lysate system by inactivating 60 S ribosomal subunits catalytically.

Table 1

Activity of the ribosomal subunits derived from the toxin-treated rabbit reticulocyte lysate in poly(U)-dependent polyphenylalanine synthesis

Subunit		[^{3}H]Phe incorporated (cpm)	% activity
40 S	60 S		
Control	control	21591	100
Treated	treated	4347	20.1
Control	treated	3870	17.9
Treated	control	16368	75.8

Ribosomal subunits were prepared from control or toxin-treated (1.0×10^{-6} M A-chain) reticulocyte lysate as described in section 2. Incubation was at 30°C for 30 min. Aliquots were removed and acid-insoluble radioactivities were counted

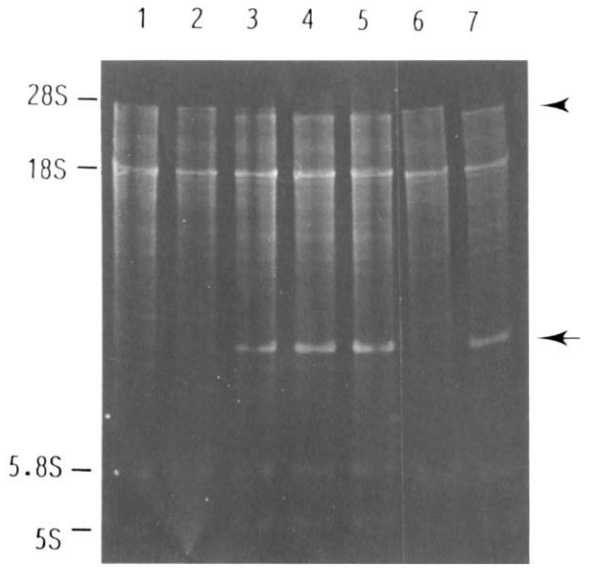

Fig.2. Analysis by gel electrophoresis of rRNA from toxin-treated rat liver ribosomes. Rat liver ribosomes (3.47×10^{-7} M) were incubated with the A-chain at 37°C for 30 min. RNA was extracted and separated on the composite gel electrophoresis after treatment with aniline. Lanes: 1, control; 2–5, 4.0×10^{-8}, 8.0×10^{-8}, 2.0×10^{-7}, 1.0×10^{-6} M A-chain, respectively; 6, same as lane 5, except RNA was not treated with aniline; 7, ricin A-chain/aniline-treated as a positive control. The arrow and arrowhead denote 28 S rRNA-derived fragments containing 3′- and 5′-ends, respectively. RNA bands were visualized with ethidium bromide.

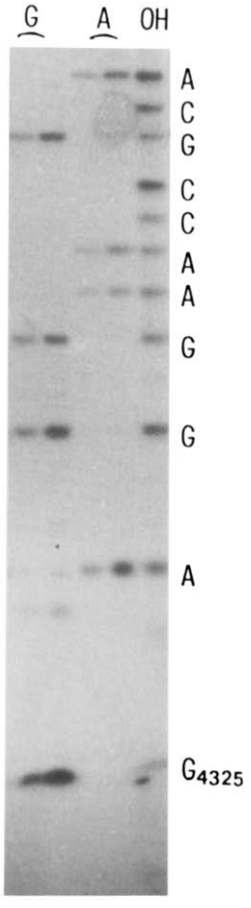

Fig.3. Autoradiograph of a sequencing gel of a fragment labeled at the 5′-end and digested with ribonucleases. The ribosomes were treated with A-chain (1.0×10^{-6} M) as described in fig.2. The smaller fragment (arrow in fig.2) produced by aniline treatment containing the 3′-end of 28 S rRNA was purified by sucrose density gradient centrifugation. The RNA was treated with bovine alkaline phosphatase and made radioactive at the 5′-terminal with [γ-^{32}P]ATP and T_4 kinase. The repurified fragment was partially digested with either alkali (OH), ribonuclease T_1 (G), or ribonuclease U_2 (A).

As can be seen in lanes 2–5 in fig.2, a new RNA band of about 450 nucleotides which is similar in size to that produced by ricin A-chain/aniline treatment (lane 7) was produced from PCL-treated rRNA at the cost of the disappearance of 28 S rRNA. The amounts of the fragments produced were dependent on the dose of PCL in the reaction mixture (cf. lanes 1–5). The results suggest that PCL A-chain acts by the same mechanisms as ricin A-chain. This point was confirmed by direct nucleotide sequencing of the fragment. For this purpose, the 450-nucleotide fragment (arrow in fig.2) was isolated and its 5′-end was labeled with [γ-^{32}P]ATP and the 5′-terminal nucleotide sequences were determined by the enzymatic method. The 5′-end of the fragment was identified as G4325 of the 28 S rRNA (fig.3). The result indicates that the A-chain of PCL cleaves the *N*-glycosidic bond at A4324 in 28 S rRNA, because the β-elimination reaction catalyzed by aniline at acidic pH on the 28 S rRNA isolated from the protein-treated ribosomes resulted in chain scission at the 3′-side of A4324 giving pG4325 at the 5′-end of the 450 nucleotide fragment.

From the above results, we conclude that the inhibitory activity of the lectin from *P. californicum* is due to its enzymatic activity. This RNA *N*-glycosidase has the same activity as that displayed by the A-chain of other lectins such as ricin and ML1 [3,6,7–10].

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