ACTION OF RICIN FROM *RICINUS COMMUNIS* L. SEEDS ON EUKARYOTE RIBOSOMAL PROTEINS

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1. Introduction

Ricin, isolated from *Ricinus communis* L. seeds, is known for its very high toxicity on animals; particularly after parenteral administration. Since the discovery by Dirheimer et al. [1] that ricin inhibits protein synthesis at the ribosome level in rats, evidence has accumulated showing that this toxin exerts its protein synthesis inhibition in intact cells [2–5] as well as in cell-free systems [6–8], but only on eukaryotes [9,10]. Montanaro et al. [11] and Sperti et al. [12] have shown that the target of action of ricin in rat liver protein synthesis is on the 60-S ribosomal subunits. Further, ricin inhibits both the ribosome-linked GTP hydrolysis catalyzed by elongation factor 2 (EF-2) and the binding of EF-2 to ribosomes [11,13–17]. Other evidence suggests an additional effect on elongation factor 1-linked reactions [14–16].

An enzymatic activity has been suggested to explain the activity of ricin [18] and was further investigated [17] after the conclusion that protein synthesis inhibition occurred at a ricin/ribosome molar ratio very much lower than 1 [9,11,12,15]. It was suggested that ricin may act as a specific nuclease on rRNAs or as a specific protease on ribosomal proteins [10]. However, no change was detected in the electrophoretic mobility of baker's yeast rRNAs after they were treated with ricin [10], and no small fragments of labelled RNA were detected after treating ^{32}P-labelled L cell polyribosomes with ricin [19]. These results suggest that ricin does not have nucleolytic activity, unless it is both very selective and very limited.

We report here our investigation of the ability of ricin to act as an enzyme which alters ribosomal proteins, using ribosomes from rat liver and rabbit reticulocytes. We utilized a two-dimensional polyacrylamide gel electrophoresis system to study the ribosomal proteins. No differences in the electrophoretic pattern of the ribosomal proteins from ricin-treated and control ribosomes were observed.

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2. Materials and methods

2.1. *Materials*

Pure ricin was obtained as described earlier [20]. Ricin prepared in this way is different [21] from castor bean lectins isolated by affinity chromatography [22–24]. Concentrations of ricin were determined according to Lowry et al. [25] using human serum albumin as a standard.

Rabbit reticulocyte lysate was prepared from male New Zealand White rabbits 2–3 kg according to the method of Lockard and Lingrel [26], as modified by Rhoads et al. [27]. Crude reticulocyte ribosomes as well as purified subunits were prepared from the lysate according to Howard et al. [28].

Rat liver ribosomes were prepared from male Wistar rats (200–250 g) basically as described by Moldave and Skogerson [29], and purified subunits from them essentially with the procedure of Blobel and Sabatini [30], with minor modifications [31].

Surviving activities after ricin treatment of ribosomes were assayed in a poly(U)-dependent phenylalanine incorporation system as described elsewhere [32]. Activity assays for ricin-treated reticulocyte lysate were done in a cell-free protein synthesizing system [33].

Antibodies which specifically cross-reacted with eukaryote ribosomal proteins L40 and L41 were prepared as described elsewhere [34].

All chemicals used were the purest available from Fluka (Switzerland), Merck (Darmstadt) or Boehringer (Mannheim).

2.2. *Ricin treatment of ribosomes*

Ricin (500 ng) was incubated with NH_4Cl-washed rat liver 80S ribosomes (125 μg) for 15 min at 37°C. The reaction mixture was promptly layered on a sucrose gradient and the 40S and 60S subunits isolated for protein analysis. With reticulocyte lysate the procedure was the same except that ricin was incubated with the total lysate in the presence of an energy source. The crude reticulocyte ribosomes were then centrifuged (4°C; 3 h; 50 000 rev/min) through a 0.5 ml cushion of 20% sucrose containing 0.05 M Tris-HCl (pH 7.4), 0.025 M KCl, 0.005 M $MgCl_2$, 0.002 M β-mercaptoethanol, in a Beckman SW 50.1 rotor with 0.8 ml adapters. Control experiments without ricin were also done in each case.

2.3. *Two-dimensional gel electrophoresis*

Ribosomal proteins were analyzed by the two-dimensional polyacrylamide gel electrophoresis system described elsewhere [35] after extraction of the proteins from isolated 80S, 60S and 40S particles using 66% acetic acid in the presence of 0.1 M $MgCl_2$ [36]. Two gels were used for electrophoresis in the first dimension – one from cathode to anode and the other in the opposite direction. They were layered simultaneously on the second dimension gel.

3. Results and discussion

Under the conditions described in Materials and methods for the ricin treatment of the rabbit reticulocyte lysate system, protein synthesis was inhibited by greater than 95%. Crude ribosomes were isolated by centrifugation from such reaction mixtures, and their proteins analysed by two-dimensional polyacrylamide gel electrophoresis. Fig.1A shows the pattern of stained spots from control ribosomes, and fig.1B from ricin-treated ribosomes. The patterns are identical in all respects; even to the extent that various extraneous non-ribosomal proteins are indistinguishable (present here because the ribosomes were never treated with high concentrations of KCl). We considered the analysis of the crude ribosomes to be the most favorable, as this minimizes the possibility of missing some effect, since some proteins have been claimed to be more distinct with ribosomes than with subunits [37]. Moreover, we thought that the use of crude ribosomes would involve less manipulation of the ribosomes, and hence would yield more precise results as to the effect (if any) of ricin on the electrophoresis pattern of the proteins. However, when such analyses were also performed on the 40S or 60S subunits isolated from such crude ribosomes, or from ricin-treated rat liver ribosome subunits, no significant differences were observed either (data not shown).

It should be noted that the electrophoretic mobility of the protein labelled L5 [28] in fig.2 remained unaltered by the ricin treatment. This protein migrates identically to the rat liver ribosomal protein L3, which Terao et al. [38] have shown is associated with the 5S rRNA in a 7S complex; the

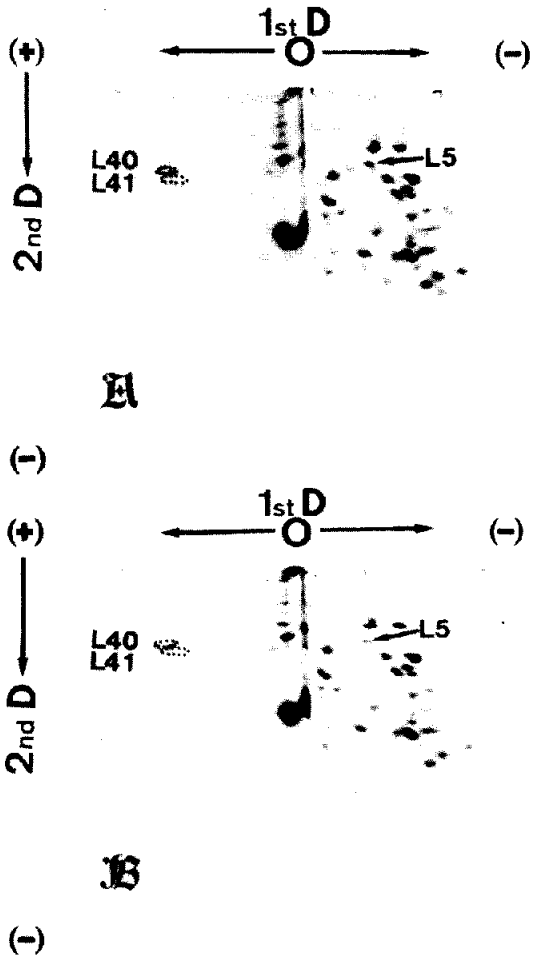

Fig.1. Two-dimensional gel electrophoresis patterns of rabbit reticulocyte 80S ribosomal proteins. The electrophoresis conditions were as described in Materials and methods. The dotted circles were placed around the stained area of proteins L40 and L41, as they stain quite faintly [35,39]. The large stain spot in the center of the pattern is due to hemoglobin contamination of the crude ribosomes. (A) Control; (B) ricin-treated.

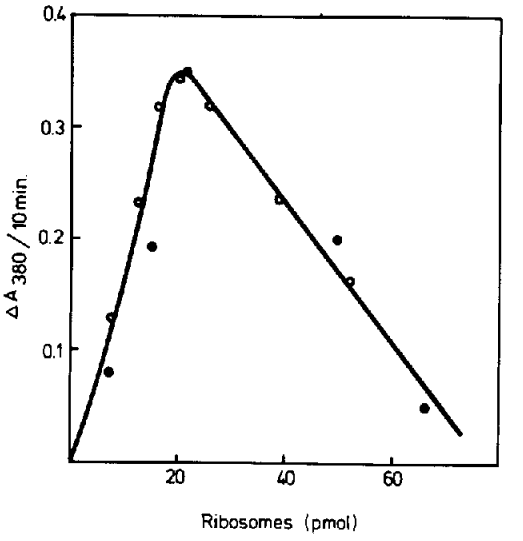

Fig.2. Turbidity formation by anti-ribosomal protein serum and rabbit reticulocyte 80S ribosomes. Turbidity measurement was done as previously described [43]. Each assay contained 50 μl of antiserum and reticulocyte 80S control (●——●) or ricin-treated (○——○) ribosomes as indicated on the abscissa. Buffer conditions: 0.02 M Tris-HCl (pH 7.4), 0.02 M NH_4Cl, 0.01 M $MgCl_2$, 0.005 M β-mercaptoethanol; final vol. 300 μl. The antiserum was added at zero time, and the increase in absorbance at 380 nm was recorded. The change in 10 min is indicated on the ordinate.

ATPase and GTPase activities of which are inhibited by ricin [16].

These results indicate that ricin has no apparent proteolytic action on ribosomal proteins. Of course, modifications which resulted in no overall charge difference and which did not markedly change the molecular weight of the proteins would not be visible. Only a detailed analysis of all the isolated proteins would give a definitive answer on whether or not ricin could have this slight and very specific proteolytic activity.

Since ricin specifically inhibits the elongation step of protein synthesis [17,39], it must be emphasized that the acidic proteins L40 and L41, which are intimately involved in ribosome-elongation factor interactions [34,40–42], are neither electrophoretically affected by the action of ricin (fig.1), nor removed from the ribosomes by ricin-treatment. The latter result is shown in fig.2. In this experiment antibodies which are immunologically specific for proteins L40 and L41 [34] show equal amounts of these proteins on control and ricin-treated ribosomes as determined by the turbidimetric assay. In addition, the post-ribosomal supernatant solution contained no antibody cross-reacting material (data not shown). These results allow us to conclude that at least the antigenic sites of these proteins are not modified since they give exactly the same equivalence point in treated and control; this further

supports the lack of modification indicated by the electrophoresis data above.

As ricin does not cause any modification in the electrophoretic mobility of yeast and L cell rRNAs [10,19], and is apparently without such an activity on the eukaryote ribosomal proteins examined here, the molecular mechanism of ricin's action remains obscure.

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