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INHIBITION OF *ESCHERICHIA COLI* PROTEIN SYNTHESIS BY A LIMITED TRYPTIC DIGEST OF RICIN, THE TOXIN OF *RICINUS COMMUNIS* L. SEEDS

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SUMMARY

Protein synthesizing systems derived from crude extracts as well as from partially purified 70S ribosomes and elongation factors from Escherichia coli were treated with ricin, ricin A and B chains or ricin tryptic hydrolysate. Ricin and its isolated chains do not inhibit protein synthesis in these systems. However, a tryptic hydrolysate of ricin inhibits both Escherichia coli protein synthesizing systems. These results demonstrate that, although ricin and its two polypeptides A and B have no activity on Escherichia coli protein synthesis, some tryptic product(s) of ricin are able to inhibit protein synthesis in procaryotes. Thus it appears that fragments smaller than ricin or its two polypeptides can inactivate 70S ribosomes.

INTRODUCTION

Ricin, isolated from *Ricinus communis* L. seeds, is known for its severe toxicity in animals. Since Dirheimer *et al.* (1) demonstrated that ricin inhibits protein synthesis at the ribosomal level in rats, evidence has accumulated showing that the toxin inhibits protein synthesis in intact cells (2,3) as well as in cell-free systems (4,5), but only in eucaryotes (5,6). The target of ricin action is the 60S ribosomal subunit (7,8) where it inhibits EF-2 catalyzed translocation (9). However, EF-2 is not directly affected by the toxin (9).

Ricin is formed by two polypeptide chains joined together by a disulfide bond (10,11). Only the intact or reassociated molecule has a toxic effect on cells (10,11), the B chain being necessary only for the entry of the toxic A

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ABBREVIATIONS : TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone HCl ; EF , Elongation factor.

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chain into the cell (12). In a cell-free system ricin must be split into its two chains by the ribosomes during protein synthesis giving the A chain which inhibits protein synthesis without a lag period (13).

We have reported that ricin inhibits protein synthesis both in isolated mitochondria and in a submitochondrial system from *Saccharomyces cerevisiae* (6) as well as in isolated chloroplasts and in a subchloroplastic system prepared from spinach (*Spinacia oleracea*) (14,15). However, ricin has been described as being inactive on procaryotes (5,6). We suggested that native ricin cannot interact with procaryotic ribosomes due to steric hindrance. Although ricin is very resistant to proteolytic enzymes, after mild denaturation it can be hydrolyzed by trypsin into toxic peptides (16,17). We show that such a tryptic hydrolysate is able to inhibit procaryotic protein synthesis.

MATERIALS AND METHODS

Materials

Castor bean seeds were a generous gift of "Société Française du Ricin, Marseille, France" and came from Paraguay. Biochemical reagents were "analytical grade" and were purchased from Sigma. |^{14}C|-L-phenylalanine (300 Ci/mol) and |^{3}H|-L-phenylalanine (500 Ci/mol) came from "Commissariat à l'Energie Atomique, Saclay, France". Total *Escherichia coli* tRNA (containing 3.6% of the tRNA ^{Phe}) was purchased from "Boehringer, Mannheim, GFR".

Preparation of ricin, ricin A and B chains and ricin tryptic peptides

Ricin, ricin A and B chains, and ricin tryptic peptides were prepared as previously described (16-18).They were extensively dialysed against distilled water. Protein was measured according to Lowry *et al.* (19) using human serum albumin as a standard. To eliminate the interference due to trypsin, the hydrolysate was incubated with TLCK (10% w/w of trypsin) for 30 min at 25°C, and was then dialysed extensively against distilled water.

Escherichia coli protein synthesizing systems

Preparation of a crude cell-free system (System I) : Growth conditions for *E.coli* MRE 600 were as described by Parisi *et al.* (20). Crude cell-free system from *E.coli* was prepared as described by Nirenberg (21). The system was incubated for 45 min at 37°C in presence of 3 µg/ml DNAase, 3 mM phosphoenolpyruvate, 20 µg/ml pyruvate kinase, 1.57 mM ATP and a mixture of 0.08 mM of each of the 20 amino acids, then dialysed against three changes of the grinding buffer (21) and stored in liquid nitrogen.

The incubation mixture (120 µl) contained : 3.33 mM Tris-HCl, pH 7.4, 74 mM KCl, 5.55 mM $MgCl_2$, 0.32 mM GTP, 0.55 mM ATP, 5.55 mM phosphoenolyruvate, 40 µg/ml pyruvate kinase, 226 µg/ml Poly(U), 41.6 µCi |^{3}H|-L-phenylalanine and 83 µg of protein/ml crude *E.coli* extract.

The system was incubated with different amounts of ricin, ricin A and B chains and ricin tryptic hydrolysate at 37°C. At various times samples of 50 µl were transferred to Whatman 3MM paper discs which were treated and counted as previously described (16).

Preparation of System II : Ribosomes were prepared and washed 8 times using the method of Lucas-Lenard and Lipmann (22). The 105,000 x *g* supernatant enzymes were partially purified (20). Preparation of |^{14}C|-L-phenylalanyl-tRNA^{Phe} was done as reported by Kaji *et al.* (23) so that 1,000 cpm/µl/pmol of phenylalanyl-tRNA^{Phe} was obtained (one $A_{260\ nm}$ Unit corresponds to 43 µg tRNA/ml).

The incubation mixture (250 µl) contained : 50 mM ammonium maleate, pH 6.6, 12 mM $MgCl_2$, 2.5 mM β-mercaptoethanol, 0.5 mM GTP, 5 mM creatine phosphate, 1 mM reduced glutathione, 25 µg creatine phosphokinase/ml, 40 µg spermine/ml,

TABLE I

ACTION OF RICIN, A AND B CHAINS, AND OF A PARTIAL TRYPTIC HYDROLYSATE ON *E.COLI* PROTEIN SYNTHESIS

System used	Concentration of the toxic fraction (in µg)	CPM per ml	Percent of inhibition of protein synthesis
SYSTEM I			
Without Poly(U)	-	840	-
Complete	-	9,460	-
Trypsin (minus phosphate + ricin)	-	1,515	
Trypsin + TLCK	-	9,305	
Complete + ricin	450	9,380	-
Complete + ricin A chain	450	9,305	-
Complete + ricin B chain	450	9,524	-
Complete + partial ricin hydrolysate	450	1,314	94.5
Complete + boiled partial ricin hydrolysate	450	9,523	-
SYSTEM II			
Without Poly(U)	-	1,702	-
Complete	-	17,764	-
Trypsin (minus phosphate + ricin)	-	3,142	
Trypsin + TLCK	-	17,621	
Complete + ricin	450	17,794	-
Complete + ricin A chain	450	17,825	-
Complete + ricin B chain	450	17,704	-
Complete + partial ricin hydrolysate	450	4,557	72.7
Complete + boiled partial ricin hydrolysate	450	17,744	-
SYSTEM III			
Complete	-	336,791	-
Trypsin (minus phosphate + ricin)	-	22,692	
Trypsin + TLCK	-	334,678	
Complete + ricin	450	336,802	-
Complete + ricin A chain	450	336,891	-
Complete + ricin B chain	450	336,598	-
Complete + partial ricin hydrolysate	450	34,689	89.7
Complete + boiled partial ricin hydrolysate	450	336,839	-

Protein synthesis was estimated after 30 min of incubation at 37°C.
Trypsin = solution of 2% (w/v) of trypsin in water.
TLCK was at a concentration of 10% (w/w) compared to trypsin. Trypsin + TLCK was incubated 3 h at +25°C before experiments.

80 µg Poly(U)/ml, 40,000 cpm (40 pmol) $|^{14}C|$-L-phenylalanyl-$tRNA^{Phe}$/ml, 100 µg of ribosomes/ml and 84 µg of the purified enzyme fraction/ml.

Preparation of System III : It contained ribosomes washed only once with a buffer containing 10 mM Tris-HCl, pH 7.4, 0.5 M NH_4Cl, 0.1 mM $MgCl_2$. All other components were identical to those described for System II.

Radioactivity was estimated in Systems II and III as with the crude system (System I).

RESULTS AND DISCUSSION

As reported in Table I, a total tryptic hydrolysate of ricin inhibits *E.coli* protein synthesis in whatever system was used. On the other hand, intact ricin and ricin A and B chains are without effect.

Kinetics of *E.coli* protein synthesis inhibition in System I (crude system) indicate that inhibition increases with increasing amounts of partial tryptic ricin hydrolysate (Fig. 1).

In this study we have confirmed that native ricin, as well as its two chains, do not inhibit procaryotic (*E.coli*) protein synthesis. This was not due to an excess of EF-G adsorbed on the 70S ribosomes that could compete with ricin, as reported for EF-2 in eucaryotic systems (3,9), since no inhibition was observed in assays using either ribosomes washed once (System III) that still contained EF-G nor ribosomes washed 8 times (System II) and deprived of detectable EF-G.

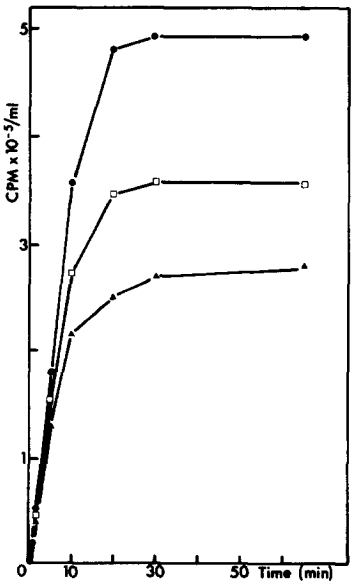

Fig. 1. Kinetics of inhibition of protein synthesis by ricin partial tryptic hydrolysate in an *E.coli* cell-free system.
●——●: control ; □——□: 23.6 µg/ml of hydrolysate ; ▲——▲: 96.6 µg/ml of hydrolysate.

However, a limited tryptic hydrolysate inhibits *E.coli* protein synthesis (Table I and Fig. 1). This inhibition was not due to residual trypsin because the tryptic hydrolysate was treated with TLCK and controls showed that no residual tryptic activity was detectable. Thus, degraded ricin acts effectively on 70S ribosomes even if EF-G is present. The concentration of ricin hydrolysate that inhibits 50% of *E.coli* protein synthesis in the crude system (System I) was 125 µg/ml (not shown). This dose is much higher than that which inhibit 50% of eucaryotic protein synthesis (10 ng/ml) (16). Thus the *E.coli* ribosome even if it can be inhibited by a ricin hydrolysate is less sensitive than the eucaryotic one.

Since the mechanisms for protein synthesis is better known in procaryotes than in eucaryotes, these results may provide a new tool for determining the mode of action of ricin.

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REFERENCES

1. Dirheimer, G., Haas, F. and Métais, P. (1968) In : Hépatonéphrites Toxiques. Ed. Roche L., Collection de Médecine Légale et de Toxicologie Médicale, Série B : Toxicologie Médicale, p. 45-50, Masson et Cie, Paris.
2. Lin, J.-Y., Liu, K., Chen, C.-C. and Tung, T.-C. (1971) Cancer Res. 31, 921-924.
3. Refsnes , S., Olsnes, S. and Pihl, A. (1974) J. Biol. Chem. 249, 3557-3562.
4. Olsnes, S. and Pihl, A. (1972) FEBS Lett. 20, 327-329.
5. Olsnes, S. Heiberg. R. and Pihl, A. (1973) Mol. Biol. Rep. 1, 15-20.
6. Lugnier, A.A.J., Küntzel, H. and Dirheimer, G. (1976) FEBS Lett. 66, 202-205.
7. Montanaro, L., Sperti, S. and Stirpe, F. (1973) Biochem. J. 136, 677-683.
8. Sperti, S., Montanaro, L., Mattioli, A. and Stirpe, F. (1973) Biochem. J. 136, 813-815.
9. Gessner, S.L. and Irvin, J.D. (1980) J. Biol. Chem. 255, 3251-3253.
10. Funatsu, M. and Funatsu, G. (1968) Tanpakushitsu Kakusan Kôso (Protein, Nucleic Acid, Enzyme) 13, 905-928 (Tokyo, in japanese).
11. Olsnes, S. and Pihl, A. (1972) FEBS Lett. 28, 48-50.
12. Sandvig, K., Olsnes, S. and Pihl, A. (1978) Eur. J. Biochem. 82, 13-23.
13. Lugnier, A.A.J., Creppy, E.-E., Le Meur, M.-A., Gerlinger, P. and Dirheimer, G. (1976) FEBS Lett. 76, 166-172.
14. Kohn, L., Lugnier, A.A.J., Creppy, E.-E., Tiboni, O., Ciferri, O. and Dirheimer, G. (1980) In : Natural Toxins. Ed. Eaker, D., Pergamon Press, Oxford, New York.
15. Kohn, L. and Lugnier, A.A.J. (1979) Toxicon 17 S1, 107.
16. Lugnier, A.A.J., Le Meur, M.-A., Gerlinger, P. and Dirheimer, G. (1976) Eur. J. Toxicol. 9, 323-333.
17. Lugnier, A.A.J., Le Meur, M.-A., Gerlinger, P. and Dirheimer, G. (1974) Biochimie 56, 1287-1289.
18. Lugnier, A.A.J. and Dirheimer, G. (1971) C.R. Acad. Sc. Paris 273D, 704-707.

19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
20. Parisi, B., Milanesi, G., Van Etten, J.L., Perani, A. and Ciferri, O. (1967) J. Mol. Biol. 28, 295-309.
21. Nirenberg, M.W. (1963) In : Methods in Enzymology. Eds Colowick, S.P. and Kaplan, N.O., 6, 17-23, Academic Press, New York and London.
22. Lucas-Lenard, J. and Lipmann, F. (1966) Proc. Natl. Acad. Sci. USA 55, 1562-1566.
23. Kaji, A., Kaji, H. and Novelli, G.D. (1965) J. Biol. Chem. 240, 1185-1191.