INHIBITION OF PROTEIN SYNTHESIS BY SEED-EXTRACTS

A screening study

Anna GASPERI-CAMPANI, Luigi BARBIERI, Enzo LORENZONI and Fiorenzo STIRPE
Istituto di Patologia generale dell'Università di Bologna, 40126 Bologna, Italy

Received 16 December 1976
Revised version received 8 February 1977

1. Introduction

Powerful inhibitors of protein synthesis by 80 S ribosomes have been found in several plants. Of these, ricin (from the seeds of *Ricinus communis*) and abrin (from the seeds of *Abrus precatorius*) are highly toxic to animals or cells in vitro (see for review [1]) whereas PAP (from the leaves of *Phytolacca americana*) [2,3], crotin II (from the seeds of *Croton tiglium*) and curcin II (from the seeds of *Jatropha curcas*) [4] have low or no toxicity. All these inhibitors are proteins, and seem to act catalytically through the same mechanism, i.e., by irreversibly inactivating in a still unknown way the 60 S ribosomal subunit, which becomes unable to react with elongation factor 2 [2,5–7].

The present study was undertaken to ascertain whether similar inhibitors were present in seeds of other plants, initially selected by their taxonomic proximity or for their analogous toxic properties. It was observed that extracts from the seeds of several plants had an inhibitory effect on protein synthesis in a cell-free system (a lysate of rabbit reticulocytes). The same extracts had scarce or nil effect on protein synthesis by whole Ehrlich ascites cells, and were not toxic to mice at doses much higher than those sufficient to inhibit protein synthesis in vitro.

Address correspondence to: Professor F. Stirpe, Istituto di Patologia generale, Via S. Giacomo 14, 40126 Bologna, Italy

2. Experimental

2.1. *Materials*

Seeds were collected in the Botanical Garden of this University or in the fields, or were purchased from Mr F. G. Celo, Zweibrücken, FRG.

All chemicals were obtained from the same sources as in previous work [4].

2.2. *Preparation of the extracts*

Seeds were shelled, when feasible, and were ground 5–8-times with 4–5 vol. ethyl ether in a blender or with an Ultra-Turrax apparatus. The ether was removed by filtration through filter paper on a Buchner funnel. The resulting powder was dried in the air then stirred, with 10 vol. cold 0.2 M NaCl, containing 0.005 M sodium phosphate buffer, pH 7.2 (PBS), for 3 h on a magnetic stirrer at 4°C, and was left overnight at the same temperature. The mixture was centrifuged at 20 000 × *g* for 20 min and the supernatant, referred to as the crude extract, was collected and tested for inhibitory activity on protein synthesis. Active extracts were fractionated with ammonium sulphate. Usually three fractions were collected, at 0–40%, 40–60% and 60–100% saturation, respectively. When no precipitate was obtained at 40% saturation, additional salt was added to 60% saturation or, if no precipitate was formed, to 100% saturation. Precipitates were collected by centrifugation and were dissolved in the minimum volume of PBS and dialysed overnight against PBS.

In one case (seeds of *Trewia nudiflora*) no precipi-

tate was obtained even with saturated ammonium sulphate, and protein was precipitated from the crude extract with cold ethanol (50%, v/v). The crude extract of *Croton bonplandianum* seeds contained a very viscous material which was precipitated with ethanol (30%, v/v) and removed by centrifugation. The residual ethanol was removed by overnight dialysis against PBS, and the extract treated with ammonium sulphate. The seeds of *Ecballium elaterium* were washed extensively with water prior to grinding to remove a material present on the outside which became mucilaginous during the extraction with PBS.

All preparations could be stored at −25°C for months without appreciable loss of activity.

2.3. *Biochemical determinations*

Protein synthesis was measured as described previously [4] with a lysate of rabbit reticulocytes or with Ehrlich ascites cells. The lysate was diluted to obtain a fairly constant rate of protein synthesis (1000–1500 dpm in a 25 μl sample after 5 min of incubation).

RNAase activity was assayed by the method of Kunitz [8] and protein was determined by the method of Lowry et al. [9] with bovine serum albumin as a standard.

2.4. *Toxicity experiments*

Toxicity was evaluated in male Swiss mice weighing 25–28 g, supplied with food and water ad libitum. Samples of the extracts, diluted with 0.9% NaCl, were injected intravenously into groups of four animals/dose.

Table 1
The effect of seeds extracts on protein synthesis by a reticulocyte lysate

Source of extract	Most active preparation	ID_{50} (extract protein μg/ml)	Remarks
PHYTOLACCACEAE			
Phytolacca americana	60–100%	0.004	
EUPHORBIACEAE			
Croton bonplandianum	0–100%	3.16	
Euphorbia lathyris	60–100%	1.53	
Euphorbia pilulifera	60–100%	64.0	
Mallotus philippinensis	60–100%	50.9	
Putranjiba roxburghii	60–100%	64.5	Heat-resistant
Trewia nudiflora	50% ethanol	2.9	Heat-resistant
RHAMNACEAE			
Rhamnus cathartica	60–100%	11.5	
CUCURBITACEAE			
Bryonia dioica	60–100%	0.17	
Citrullus colocynthis			
Seeds	60–100%	0.22	
Fruit-pulp (dried powder)	40– 60%	12.8	
Cucurbita pepo	40– 60%	67.4	Partially heat-resistant Contains RNAase activity
Ecballium elaterium	60–100%	23.6	

Extracts were prepared from seeds, unless stated otherwise. The most active preparation is identified by the saturation of ammonium sulphate or by the concentration of ethanol giving the most active precipitate. ID_{50} is the concentration of seeds protein which inhibits protein synthesis by 50%, and was calculated by the linear regression method. Protein synthesis was assayed as described previously [4]. Incubation was at 27°C for 5 min.

3. Results

The following seed-extracts had no effect on protein synthesis by the reticulocyte lysate, at extract protein concentration 100 μg/ml, and were not examined further:

LEGUMINOSAE
Canavalia ensiformis, Cytisus laburnum, Phaseolus vulgaris, Robinia pseudo-acacia, Vicia sativa
EUPHORBIACEAE
Aleurites moluccana, Chrozophora plicata, Chrozophora tinctoria, Euphorbia heterophilla, Hura crepitans, Sarcococca pruniformis
THYMELEACEAE
Daphne laureola
CAPRIFOLIACEAE:
Viburnum rhytidophyllum
DIOSCOREACEAE
Tamus communis

The activity of the extracts inhibiting protein synthesis was recovered almost entirely in the ammonium sulphate fractions. The activity of the most active fraction of each extract is given in table 1. The inhibitory effect was abolished by boiling the extracts for 30 min, except in the case of *Putranjiba roxburghii* and of *Trewia nudiflora,* whose extracts retained approximately 80% of their inhibitory activity, and of *Cucurbita pepo,* which retained 40% of the activity after boiling. All heat-stable extracts were not examined further.

A time-course study was performed with the extracts showing an $ID_{50} < 10$ μg/ml. The results were consistent with the representative experiment shown in fig.1. The incorporation of [^{14}C]leucine was arrested immediately upon addition of the extracts, and there was no decrease of the radioactivity incorporated when the incubation was continued after the addition of the inhibitors.

The effect of active extracts on the incorporation of [^{14}C]leucine into protein by Ehrlich ascites cells in vitro was tested at extract protein concentrations up to 100 μg/ml. Only the extracts from *Croton bonplandianum, Bryonia dioica* and *Citrullus colocynthis* seeds hindered incorporation to a significant extent, although at concentrations much higher than those inhibiting protein synthesis in the reticulocyte lysate system (table 2).

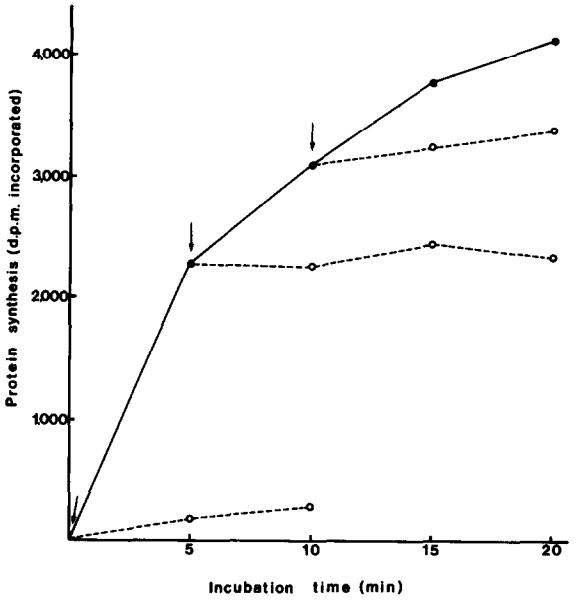

Fig.1. The effect of the extract from *Citrullus colocynthis* seeds on protein synthesis by a reticulocyte lysate. Experimental conditions were as described in table 1. The extract (protein 1 μg/ml) was added at the times indicated by the arrows. (●——●) No additions (○- - -○) Extract added.

Active extracts were tested for the presence of RNAase, and a measurable activity was found only in the extract from *Cucurbita pepo*.

The acute toxicity of the three most active extracts was evaluated. The extracts from *Bryonia dioica* and from *Citrullus colocynthis* seeds did not cause any apparent harm when injected intravenously to mice

Table 2
The effect of seed-extracts on protein synthesis by Ehrlich ascites cells

Source of extract	Preparation tested	ID_{50} (extract protein μg/ml)
Croton bonplandianum	0–100%	46.6
Bryonia dioica	60–100%	62.7
Citrullus colocynthis	60–100%	127.5

Extracts were prepared from seeds. Cells (5 × 10^6/ml) were incubated for 1 h at 37°C in 1 ml medium E 2a [10] containing 5% calf serum and 0.5 μCi L-[^{14}C]leucine. Radioactivity was determined from 25 μl samples. Other details are as in table 1.

at extract protein dose 5 mg/animal. The extract from *Phytolacca americana* killed all animals at dose 5 mg/mouse, but had no effect at dose 1 mg/mouse.

4. Discussion

About one-half of the seed-extracts examined with present experiments inhibit protein synthesis in a cell-free mammalian system but not, or scarcely, in Ehrlich ascites cells. In cases considered in this discussion, the inhibitory activity is thermolabile and can be recovered in a non dialysable fraction precipitated with ammonium sulphate. This suggests that the active principles may be protein, probably similar to those found in other plant materials (see Introduction) and particularly to PAP, crotin II and curcin II, all of which have no effect on cells. Thus the inhibitors of protein synthesis of plant origin can be divided into two categories; one, including so far ricin and abrin, which are highly toxic to animals, and another, including all other inhibitors with low toxicity, the difference being probably due to different capacity of penetration inside cells, or to a different rate of destruction of the inhibitor molecule by the cells.

The most active extract is that from *Phytolacca americana* seeds. Presumably these seeds contain the same inhibitor (PAP) found in the leaves of the same plant [2,3,11]. This inhibitor is different from the pokeweed mitogen (PWM) since it has no mitogenic activity [12]. We have checked that the extract from *Phytolacca americana* seeds has no mitogenic activity, but observed also that PWM (from Grand Island Biological Co., Grand Island, NY, USA) inhibits protein synthesis in the reticulocyte lysate system, although at higher concentrations than PAP (40% inhibition was obtained with PWM 1 μg/ml). It is possible that this effect is due to traces of the more powerful PAP which may be present in the PWM preparation.

The very high potency of some of these crude preparations suggests that the active component(s) might act in a catalytical manner, i.e., enzymatically, as it has been proposed for those already described [1,4,11]. RNAase activity was found only in the extract from *Cucurbita pepo*. The presence of a protease seems also unlikely, since the radioactivity incorporated into lysate protein does not decrease when the incubation is continued after the addition of the inhibitor (fig.1). However, the possibility cannot be excluded that these extracts act by damaging specifically ribosomal RNA or protein, in a similar way as does colicin E3 on RNA of bacterial ribosomes [13]. Protein synthesis was arrested immediately by the inhibitors, and the extent of inhibition was the same when the extracts were added before or well after starting the reaction. This would suggest an inhibition of peptide chain-elongation rather than of initiation [14] and it is possible that all these principles inhibit protein synthesis through the same mechanism as do ricin and those already known.

Acknowledgements

We thank Professor E. Bonetti for his interest and Professors A. Pirola and F. Corbetta of the Istituto di Botanica for advice and for gifts of seeds. The work was aided by grants from the Consiglio Nazionale delle Ricerche, Rome, and by the Pallotti's legacy for cancer research.

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