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Ricin inhibition of *in vitro* protein synthesis by plant ribosomes

(*Ricinus communis* agglutinin/castor bean/wheat germ)

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ABSTRACT ***In vitro*** **translation systems were prepared with supernatant factors from wheat germ and 80S ribosomes from wheat germ, barley embryos, watermelon cotyledons, pea cotyledons, and castor bean endosperm. Ricin A-chain, which strongly inhibits protein synthesis by mammalian ribosomes, inhibited all of the plant ribosomal systems by 50% when present at 25–45 μg/ml—≈23,000 times the concentration needed to inhibit mammalian systems.** ***Ricinus communis*** **agglutinin A-chain, a protein similar to ricin A-chain, inhibited translation by the plant systems 50% at concentrations 5–10 times those of the ricin A-chain. Ribosomes from castor bean endosperm, the source of ricin and the agglutinin, were just as susceptible to the inhibitors as were ribosomes from the other four plants. Compartmentation of the inhibitors within vacuoles derived from protein bodies of the endosperm appears to be responsible for protecting cytoplasmic protein synthesis during germination of castor beans.**

Ricin and *Ricinus communis* agglutinin (RCA) are two glycoproteins present in the protein bodies of the endosperm tissue of seeds of castor bean (*Ricinus communis*) (1). Ricin, a potent toxin for animal cells, is composed of two subunits. The B-chain interacts with cell surfaces, allowing the toxin to enter cells, whereas the A-chain acts on ribosomes, thus inhibiting protein synthesis (2). In cell-free translation systems, the A-chain alone has a greater effect on translation than does the complete ricin molecule (2). RCA is structurally similar to ricin, as determined by immunologic relatedness (3) and amino-terminal sequences (4). However, RCA is composed of two A-chains and two B-chains and is much less toxic than ricin (5). The RCA A-chain can inhibit cell-free protein synthesis (4, 5), but Cawley *et al.* (4) have noted that ricin A-chain is 2 to 5 times more effective than RCA A-chain.

Although there is general agreement in the literature on the ability of ricin A-chain to inhibit *in vitro* synthesis by mammalian 80S ribosomes, the information available for plant systems is confined to conflicting results on wheat germ. Olsnes *et al.* (2) cited unpublished work that demonstrated that a cell-free system from wheat germ "was strongly inhibited." Later, Cawley *et al.* (6) found no effect when ricin A-chain was added at 50 μg/ml to a poly(U)-directed wheat germ system, whereas ricin A-chain at 10 ng/ml inhibited a similarly prepared rat liver system by 50%. Recently, Lugnier and Rether (7) have demonstrated that ricin at 24 μg/ml inhibited a poly(U)-directed wheat germ system by 65%.

The purpose of the present investigation was to study the effects of ricin, RCA, and their A-chains on cell-free translation systems composed of soluble factors from wheat germ and 80S ribosomes from wheat germ, barley embryos, watermelon cotyledons, pea cotyledons, and castor bean endosperm—the source of ricin and RCA. The results suggest some general conclusions about the response of plant translation systems to the two lectins.

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MATERIALS AND METHODS

Plant Materials. Castor bean seeds (*Ricinus communis* L. cv. Hale) and watermelon seeds (*Citrullus vulgaris*, Schrad.) were soaked for 24 hr in running water, sown in moist vermiculite, and germinated in the dark at 30°C in a humidified chamber. Pea seeds (*Pisum sativum* L. Little Marvel) were surface-sterilized by stirring for 10 min in a 10% (vol/vol) solution of commercial bleach, followed by two 10-min rinses with water. The seeds were imbibed for 6 hr in aerated water, sown in moist vermiculite, and germinated in the dark at 25°C. Embryos excised from barley seeds (*Hordeum vulgare* var. Himalaya) were germinated on moist filter paper in a Petri dish in the dark at 25°C for 24 hr. Wheat germ was obtained from the College Market at Loma Linda University (Riverside, CA).

Preparation of Ricin, RCA, and Their Subunits. Ricin and RCA were purified from dry castor beans by elution from Sepharose 4B-200 (Sigma) with *N*-acetylgalactosamine and galactose, respectively, as described by Nicolson *et al.* (3). The A- and B-chains of ricin were separated on DEAE-cellulose (Whatman DE-52) as described by Olsnes and Pihl (8), except that 0.5% 2-mercaptoethanol was included in all of the buffers and the B-chain was eluted stepwise with 0.1 M NaCl. The RCA subunits were separated on DEAE-cellulose by the method of Saltvedt (5) except that 0.5% 2-mercaptoethanol was included in all of the buffers. The preparations were analyzed by electrophoresis in 10% polyacrylamide/$NaDodSO_4$ gels (9). Protein concentration was determined by the method of Lowry *et al.* (10) with ovalbumin (grade V, Sigma) as standard. For the ricin A-chain, the protein concentration determined by the Lowry method agrees with that obtained by using the A_{280} extinction coefficient (11). For ricin and RCA, the Lowry values are approximately half those calculated from the extinction coefficients (11).

Preparation of Supernatant Factors. The method was based on those of Gwóźdź and Bewley (12) and Marcus *et al.* (13). All operations were carried out at 4–6°C. Each gram of wheat germ was ground in a mortar and pestle with 5 ml of buffer (250 mM sucrose/40 mM KCl/4 mM Mg acetate/50 mM Tris·acetate, pH 8.1) and broken glass capillary pipets. The extract was centrifuged at 23,500 × *g* for 15 min. After removal of the floating fat pad, 6.5 ml of the supernatant solution was layered over a 1.5-ml sucrose cushion (36% sucrose/40 mM KCl/4 mM Mg acetate/50 mM Tris·acetate, pH 8.1) in a heavy walled polycarbonate centrifuge tube. The preparation was centrifuged at 47,000 rpm in a Beckman type 65 rotor (200,000 × *g*) for 3 hr. The clear, upper portion of the solution was removed and stored in aliquots at −20°C for a period not exceeding 1 wk. The so-

Abbreviation: RCA, *Ricinus communis* agglutinin.

lution was thawed and dialyzed against 150 vol of dialysis buffer (40 mM KCl/4 mM Mg acetate/50 mM Tris·acetate, pH 8.1) for 2 hr immediately before use. For protein determinations, 1 vol of dialyzed solution was mixed with 2 vol of cold 12% (wt/vol) trichloroacetic acid. The protein that precipitated was dissolved in 1 M NaOH and assayed (10).

Preparation of Ribosomes. The ribosomes from wheat germ were those pelleted during the preparation of the wheat germ supernatant factors (above). Four pellets were suspended in 4 ml of translation buffer (80 mM KCl/10 mM Mg acetate/50 mM Tris·acetate, pH 8.1). Ribosomes from barley embryos were prepared in a similar manner.

Ribosomes from other plant sources were prepared as follows. Endosperm tissue from 25 3-day-old castor bean seedlings, cotyledons from 30 4-day-old pea seedlings, or cotyledons from 45 4-day-old watermelon seedlings were ground in a mortar with 20 ml of Fourcroy buffer B (14). For the castor bean endosperm, Na heparin (Grade II, Sigma) was added to the buffer at 1,000 units/ml (15). An additional 12 ml of buffer was added, and the extraction was continued. The extract was centrifuged at 23,500 × g for 15 min. The supernatant solution was carefully removed to avoid disturbing any floating fat, and 6.5 ml of the supernatant solution was layered onto a 1.5-ml sucrose cushion (36% sucrose/400 mM KCl/20 mM Mg acetate/50 mM Tris·acetate, pH 8.1). The ribosomes were pelleted by centrifuging at 200,000 × g for 2 hr. Four such pellets were resuspended in 3 ml of translation buffer. The RNA concentration was determined by the modified orcinol assay (16) with RNA type XI (Sigma) as standard. Ribosomes were precipitated with 1 vol of 20% perchloric acid and redissolved in 3 M NaOH prior to the assay.

Poly(U)-Directed *in Vitro* Translation. The translation system was that of Gwóźdź and Bewley (12), with a few modifications. A total assay volume of 0.4 ml contained 80 mM KCl, 10 mM Mg acetate, 50 mM Tris·acetate (pH 8.1), 3.2 mM dithiothreitol, 0.5 mM GTP (trilithium salt, Calbiochem), 1 mM ATP, 6.2 mM phosphocreatine, 2.1 units of creatine phosphokinase (EC 2.7.3.2; rabbit muscle type I, Sigma), 0.25 units of tRNA (Baker's yeast type X, Sigma), 200 μg of poly(U) (Calbiochem), 30 μl of ribosomes, 100 μl of supernatant factors, and 0.1 μCi (3.7 × 10^3 Bq) of L-[U-^{14}C]phenylalanine (495 mCi/mmol, Amersham). The assays were run at 30°C and stopped by the successive addition of 0.2 ml of cold 20% trichloroacetic acid/50 mM unlabeled phenylalanine and 1 ml of 5% trichloroacetic acid/25 mM phenylalanine. The samples were left on ice for 10 min, placed in a boiling water bath for 15 min, and then cooled on ice for 10 min. An additional 2 ml of 5% trichloroacetic acid/25 mM phenylalanine was added. The insoluble material was collected on Whatman GF/A glass fiber filters, and washed twice with 3 ml of cold 5% trichloroacetic acid/25 mM phenylalanine and twice with 1.5 ml of cold 95% ethanol. The filters were placed in scintillation vials, allowed to dry for 1–2 hr, and assayed for ^{14}C in 10 ml of scintillation fluid {0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene}. The data presented have been corrected for background and zero-time values.

RESULTS

Characteristics of the Translation Systems. Preliminary studies showed that incorporation of [^{14}C]phenylalanine was linear for 30 min. In the experiments in which many different treatments were compared, reactions (in triplicate) were terminated at 20 min. The results in Table 1 establish, for each of the five systems with different tissues as the source of ribosomes, that incorporation was completely dependent on added ribosomes and was strongly inhibited by cycloheximide but not by chloramphenicol. Thus, the incorporation observed is strictly due to 80S ribosomes and, because added serum albumin had no effect, the responses to the inhibitors (below) are not due to nonspecific protein interactions. Although roughly equivalent amounts of ribosomes and supernatant factors were used, the actual rates of incorporation in the five systems were widely different (Table 2).

Table 1. Response of the translation systems to different treatments

Treatment		Incorporation of [^{14}C]Phe into protein, % of control				
Addition	Amount, μg/ml	Wheat germ	Barley	Pea	Watermelon	Castor bean
Control	—	100	100	100	100	100
Without ribosomes	—	0	0	0	1	0
Albumin*	200	99	92	103	94	99
Chloramphenicol	100	103	100	97	93	100
Cycloheximide	50	11	18	24	19	20
Ricin	25	90	85	90	99	93
	50	82	75	82	92	90
	100	79	64	80	86	85
	200	72	61	—	79	78
RCA	50	97	99	100	—	97
	100	94	97	91	90	88
	200	93	90	83	86	80

Assays were conducted at 30°C and terminated at 20 min. Control assays contained only the components described in *Methods*.
* Bovine serum albumin.

Response to Ricin, RCA, and Subunits. The responses of all five systems to the castor bean lectins were remarkably similar (Table 1; Fig. 1). RCA had essentially no effect in concentrations as high as 200 μg/ml, whereas ricin showed significant inhibition in all systems at >50 μg/ml. The A-chain of RCA induced some inhibition and the A-chain of ricin was clearly the most effective inhibitor.

When percentage inhibition was plotted against log A-chain concentration, straight lines were obtained over most of the concentration range (Fig. 1); from these data, the amounts of ricin and RCA A-chains required for 50% inhibition of protein synthesis in the five systems could be deduced. The values for ricin A-chain in the five systems were all clustered within a twofold range of concentration around 37 μg/ml (Table 3), and those from the RCA chain were some 5- to 10-fold higher than this. To compare the susceptibility of plant systems to that of ribosomes derived from mammalian tissues, published values of Cawley *et al.* (4) are included in Table 3. Clearly, concentrations of ricin A-chain some 20,000-fold greater are required to produce equivalent inhibition in the systems derived from plants. Nevertheless in both systems, the relative responses to RCA A-chain and ricin A-chain are similar in the two systems, with the ricin A-chain some 5- to 10-fold more effective (4).

Table 2. Characteristics of the translation systems

System	Additions, μg		[^{14}C]Phe,‡ cpm
	RNA*	Protein†	
Wheat germ	81.6	820	39,844
Barley	66.0	770	2,831
Pea	39.6	690	3,812
Watermelon	36.7	740	1,057
Castor bean	47.4	725	703

* Added as ribosomes.
† Added as supernatant factors.
‡ Incorporated in 20 min.

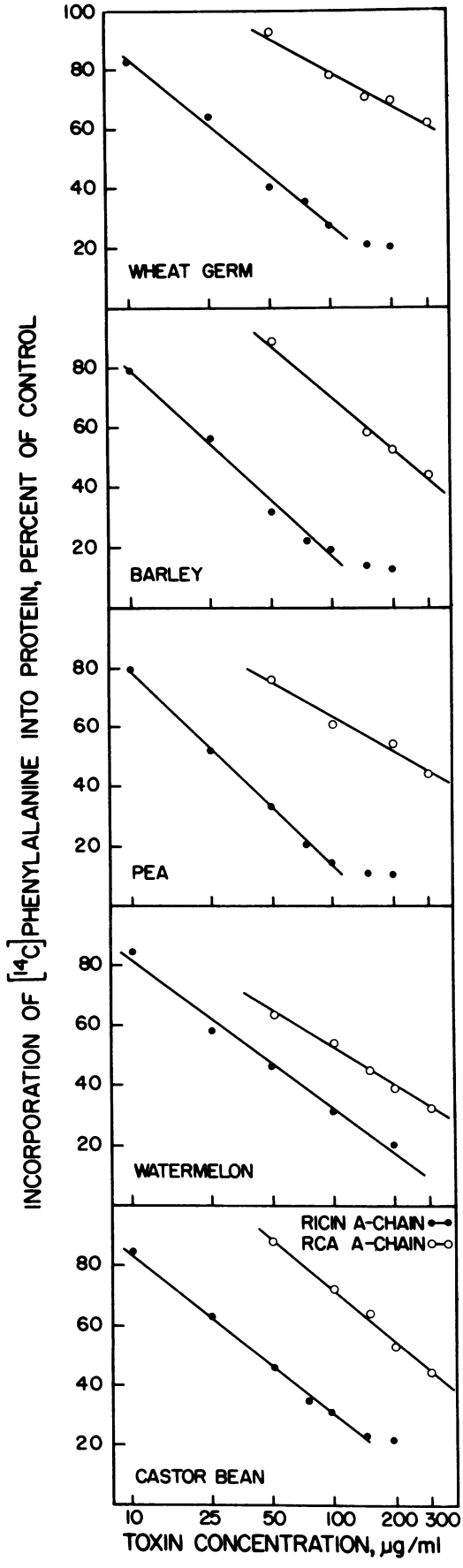

FIG. 1. The effects of increasing A-chain concentration on [^{14}C]-phenylalanine incorporation by the different translation systems.

Table 3. Concentration of A-chains (μg/ml) required for 50% inhibition of [^{14}C]phenylalanine incorporation

System	A-chain source	
	Ricin	RCA
Wheat germ	39.4	580
Barley	29.4	227
Pea	27.7	225
Watermelon	44.2	113
Castor bean	44.1	245
Rat liver*	0.0016	0.0079

* From ref. 4.

DISCUSSION

In all five translation systems from plants, including wheat germ, 25–45 μg of ricin A-chain per ml is sufficient to inhibit poly(U)-directed synthesis by 50%. This is in contrast to the findings of Houston and colleagues (6), who recorded no inhibition in their wheat germ system by ricin A-chain at 50 μg/ml. We see significant inhibition by ricin itself only when the concentration exceeded 50 μg/ml, whereas Lugnier and Rether (7) observed 65% inhibition at 24 μg/ml. The explanations offered by Lugnier and Rether for the differences between their results (7) and those of Houston's group (6) probably hold here also. What is now clear is that 80S ribosomes from plants are uniformly susceptible to inhibition by ricin. Although the plant translation systems do not show the extreme sensitivity of the mammalian ones, they are nevertheless strongly inhibited by ricin A-chain concentrations in the micromolar range. (The molecular weight of ricin A-chain is 30,000.)

The ribosomes from the endosperm of castor bean are clearly no less responsive to ricin than are the ribosomes from other plant species, yet the castor bean tissue used here, in which active protein synthesis is occurring, contains high levels of endogenous ricin. The concentration of ricin expressed on the basis of the water content is 3–5 mg/ml (unpublished data), some 100-fold higher than the concentration of A-chain required for inhibition of protein synthesis by the extracted ribosomes.

Clearly there must be some mechanism by which the susceptible ribosomes in the endosperm are protected from the endogenous ricin. In the dry seed, the ricin is present as a water-soluble component of the protein body matrix (1). During germination, the protein bodies give rise to vacuoles in which hydrolysis of the components of the protein body occur. Thus, it appears that it is the strict compartmentation of ricin within the vacuole that protects the cytoplasmic protein synthesis from inactivation during germination and early growth. How protection is imposed during the development of the seed on the parent plant, when ricin is being synthesized, remains to be established.

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