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Molecular mechanism of inhibition of mammalian protein synthesis by some four-chain agglutinins

Proposal of an extended classification of plant ribosome-inactivating proteins (rRNA *N*-glycosidases)

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The four chain agglutinins from *Abrus precatorius*, *Viscum album* and *Ricinus communis* promote depurination of the 28 S rRNA from rabbit reticulocyte ribosomes characteristic of the common ribosome-inactivating proteins (RIPs). These agglutinins inhibited mammalian protein synthesis at nanomolar concentrations but they do not affect plant protein synthesis under the same conditions. Therefore, they should also be considered as true RIPs but of a new class, the four-chain RIPs. An extended classification of RIPs is presented based on the former one from Stirpe et al. [Bio/technology 10 (1992) 405–412].

Ribosome-inactivating protein; Protein synthesis; Inhibition; Four-chain agglutinin; rRNA *N*-glycosidase

1. INTRODUCTION

Abrus precatorius, *Ricinus communis* and *Viscum album* contain four-chain agglutinins [1]. These proteins have also been reported to inhibit in vitro mammalian protein synthesis [1]. Typically, these agglutinins are composed of two types of polypeptide chains. The best known is *R. communis* agglutinin which is formed of two enzymic A chains of nearly 32 kDa each and two galactose-binding B chains of nearly 34 kDa each [1,2]. The *R. communis* agglutinin A chain has also been shown to be inhibitory to both in vitro rabbit reticulocyte lysates and plant protein synthesis at concentrations of 1.5 $ng \cdot ml^{-1}$ [3] and 580 $\mu g \cdot ml^{-1}$ [4], respectively. The A and B chains of *R. communis* four-chain agglutinin share good sequence homology with the corresponding A and B chains of the ribosome-inactivating protein (RIP) ricin, a highly poisonous dimeric toxin isolated from *Ricinus communis* [5]. This suggested the consideration of *R. communis* four-chain agglutinin as a RIP [6]. In fact, the recombinant *R. communis* agglutinin A chain has recently been proved to be a true RIP [3]. With the exception of this report on the recombinant protein, there are no data on the molecular mechanism of protein synthesis inhibition by the native four-chain agglutinins and consequently, in a recent general review of plant RIPs, they were not compiled as such [7]. In this report we present evidence that indicates that the three four-chain agglutinins above mentioned, in their native state, are rRNA *N*-glycosidases thus inhibiting mammalian protein synthesis at nanomolar concentrations and must therefore be classified as true RIPs. This, together with the recent discovery of two non-toxic two-chain RIPs, namely ebulin 1 [8] and nigrin b [9], prompted us to propose an extended classification of plant RIPs.

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2. MATERIALS AND METHODS

2.1. *Materials*

Highly purified preparations of *Abrus precatorius*, *Ricinus communis*, *Viscum album*, *Wisteria floribunda*, *Glicine max*, *Solanum tuberosum*, *Sophora japonica*, *Erythrina corallodendron*, *Arachis hypogaea*, *Caragana arborescens*, *Phaseolus coccineus*, *Robinia pseudoacacia* and *Momordica charantia* agglutinins were purchased from Sigma (St. Louis, MO, USA). All chemicals, biochemicals and radioactive materials were of the highest purity available and were obtained as in previous reports [10,11].

2.2. *Cell-free translation extracts and polypeptide synthesis*

Rabbit reticulocyte lysates were prepared as described elsewhere [12]. Rat liver and plant cell-free translation systems (wheat germ, *Vicia sativa* and *Cucumis melo*) were prepared as described previously [10,11,13]. The cell-free extracts were filtered through Sephadex G-25 to remove low M_r compounds and stored in small aliquots under liquid N_2 until their use. Translation was coded by endogenous messengers [10–13].

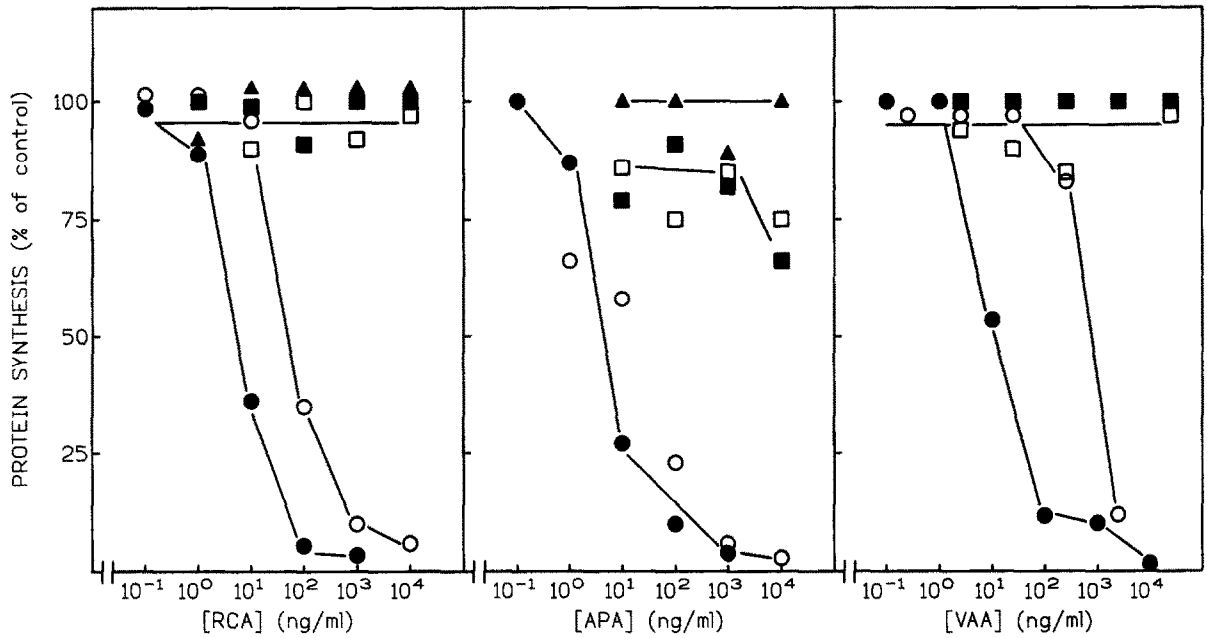

Fig. 1. Effects of four-chain agglutinins on protein synthesis carried out by mammalian and plant cell-free translation systems. Varying concentrations of RCA, APA or VAA were added to translation mixtures of each in vitro system as indicated in section 2. Protein synthesis is expressed as the percentage of label incorporated into proteins refered to controls run in the absence of agglutinin. Control mixtures of rabbit reticulocyte lysates, rat liver, wheat germ, *Vicia sativa* and *Cucumis melo* incorporated 16200, 59000, 67000, 94000, and 170000 dpm per mg of protein of cell-free extract, respectively. The agglutinins were incubated with 1% mercaptoethanol at 37°C for 30 min prior to their assay on protein synthesis. Symbols: ●, rabbit reticulocyte lysate; ○, rat liver; ■, *Vicia sativa* germ; □, *C. melo* germ; ▲, wheat germ.

2.3. *rRNA N-glycosidase activity of four-chain agglutinins*

100 μl of rabbit reticulocyte lysate were incubated with agglutinins or known RIPs for 15 min at 37°C under the same conditions as described elsewhere [14].

3. RESULTS AND DISCUSSION

Early reports on plant protein inhibitors of protein synthesis described that the four-chain agglutinins found in *Ricinus communis*, *Abrus precatorius* and *Viscum album* (RCA, APA and VAA respectively) inhibit in vitro translation by mammalian systems [1]. Since neither the mechanism nor the biological significance of such effects on translation have been addressed, we renewed the investigation of this issue. As shown in Fig. 1, native APA, RCA and VAA four-chain agglutinins inhibit translation by rabbit reticulocyte lysates and rat liver cell-free extracts, in agreement with earlier reports [1]. In addition, it shows that the agglutinins were rather inactive under the same conditions on translation by wheat germ, *Vicia sativa* germ and *Cucumis melo* germ cell-free systems. The IC_{50} (concentration giving 50% of inhibition) values for inhibition of translation by rabbit reticulocyte lysates were 5.5, 6 and 12 $ng \cdot ml^{-1}$ for *Ricinus communis*, *Abrus precatorius* and *Viscum album* agglutinins, respectively; these values are close to those described for common RIPs [7].

Nine other lectins (*Wisteria floribunda*, *Glicine max*, *Solanum tuberosum*, *Sophora japonica*, *Erythrina coralodendron*, *Arachis hypogaea*, *Caragana arborescens*, *Phaseolus coccineus* and *Robinia pseudoacacia*) assayed for protein synthesis inhibition in the rabbit reticulocyte lysate, were shown to be inactive. Other non-toxic agglutinins such as the agglutinins from *Momordica charantia*, *Crotalaria juncea*, *Rutilus rutilus*, *Phytolacca americana* and *Vicia cracca* at a concentration of 100 $\mu g \cdot ml^{-1}$ also inhibit protein synthesis by the rabbit reticulocyte lysates but the mechanism of inhibition has not been studied to date [15]

Abrus precatorius, *Ricinus communis* and *Viscum album* also contain the highly poisonous toxins ricin, abrin and viscumin, respectively, which are classified as typical type 2 RIPs [1,7,16]. Therefore, despite the fact that the native agglutinins were highly purified, we decided to ascertain whether small amounts of them might be contaminating the native four-chain agglutinin preparations, this being the reason of the inhibition. Ricin and RCA may be efficiently separated by chromatography on Sephacryl S 200 [17]. As shown in Fig. 2, *R. communis* agglutinin and ricin eluted from a Sephacryl S 200 column in very different positions. The only inhibitory activity of the agglutinin preparation was detected in the sharp peak of agglutinin and not in the position corresponding to ricin. This agglutinin preparation was therefore indeed free of ricin. The same results were obtained with repurified APA and VAA (data not shown).

The fact that both toxic RIPs, ricin and abrin, and the

recombinant RCA A-chain are *N*-glycosidases acting on the eukaryotic rRNA [3,7], raised the question of whether the native four-chain agglutinins might trigger ribosomal inactivation by the same mechanism, i.e. depurination of the largest rRNA. As shown in Fig. 3, even at very high dilutions both agglutinins displayed a strong *N*-glycosidase activity that enabled the release of the RIP diagnostic RNA fragment upon treatment of the rRNA isolated from agglutinin-inactivated ribosomes with acid aniline. Also *Momordica charantia* agglutinin displayed *N*-glycosidase activity but at 17 $\mu g \cdot ml^{-1}$ (data not shown).

As illustrated in Fig. 4, neither repurified APA nor RCA were toxic to mice at concentrations below 33.3 μg per kg of body weight. In contrast, VAA displayed a mild toxicity compared with ricin (aproximately ten-fold less). Overall, native APA, RCA, and VAA four-chain agglutinins must be considered as true RIPs of

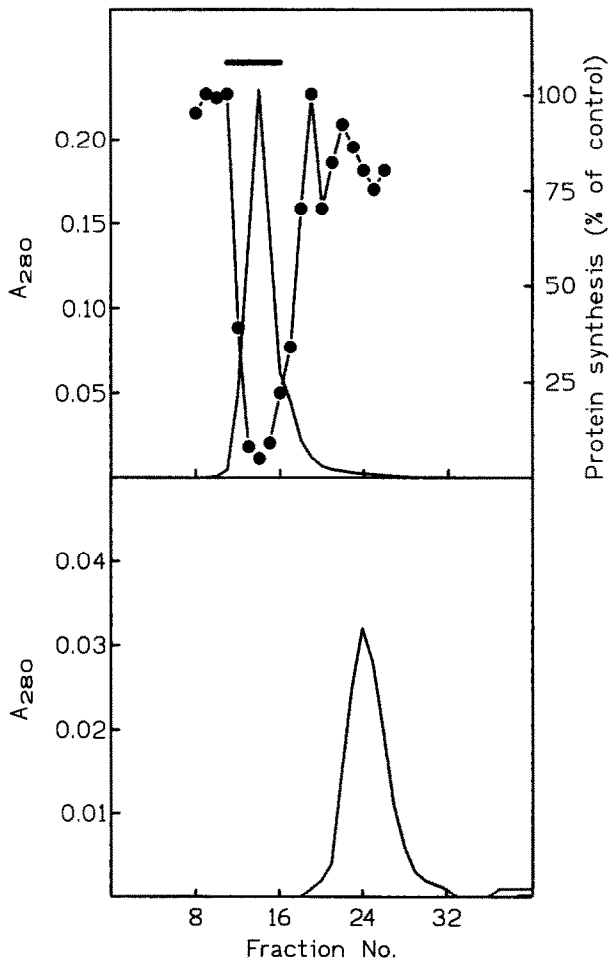

Fig. 2. Sephacryl S 200 chromatography of *Ricinus communis* agglutinin. Either 525 μg of *R. communis* agglutinin (upper panel) or 125 μg ricin (lower panel) were chromatographed through a column of Sephacryl S 200 (0.7 × 30 cm). In the upper panel the fractions were assayed for inhibition of protein synthesis in the rabbit reticulocyte lysate (●). The horizontal bar indicates those fractions that were used in subsequent studies.

Table I

Extended classification of plant RIPs (rRNA *N*-glycosidases) according to their structure

Type 1 (one chain)	non-toxic	i.e. gelonin [18], trichosanthin [19], petroglaucin [10], etc.
Type 2 (two chains)	non-toxic	ebulin 1 [8], nigrin b [9]
	toxic	ricin, abrin, modeccin, volkensin and viscumin (reviewed in [7])
Type 4 (four chains)	non-toxic	RCA [3,7], APA [7], *M. charantia* agglutinin [15]
	toxic	VAA [7]

two new classes: the four-chain or type 4 non-toxic and toxic RIPs.

In the light of the present and very recent data [8,9], plant RIPs should be classified in at least three categories as outlined in Table I. Type 1 RIPs, non-lectin usually single-chain RIPs such as gelonin [18], trichosanthin [19], etc. and with an exception of two short polypeptides RIP in maize [20]. Type 2 RIPs, lectins, which can be divided in two groups: non-toxic RIPs such as ebulin l [8] and nigrin b [9], and toxic RIPs such ricin, abrin, modeccin, viscumin and volkensin [7]. Type 4 RIPs, lectins formed of four chains which can also be divided into non-toxic such as *Momordica charantia*, APA and RCA four-chain agglutinins [1,15], and toxic such as VAA.

Non-toxic type 2 and type 4 RIPs, as suggested recently [8], may be either defective in the membrane translocation protein domain, either in the B or the A chain, or alternatively they may have some sort of defect in cell binding or perhaps an increased degradation [8]. As a consequence, the RIP catalytic A chain cannot reach the cytosol and therefore such RIPs do not affect protein synthesis in intact cells [21]. The non-toxic type 2 and type 4 RIPs cannot be detected by the current test of toxicity for intact animals, usually mice. Instead, they would be revealed by the inhibitory ability of agglutinins on translation and thereafter by looking at the rRNA *N*-glycosidase activity in inhibitory agglutinins.

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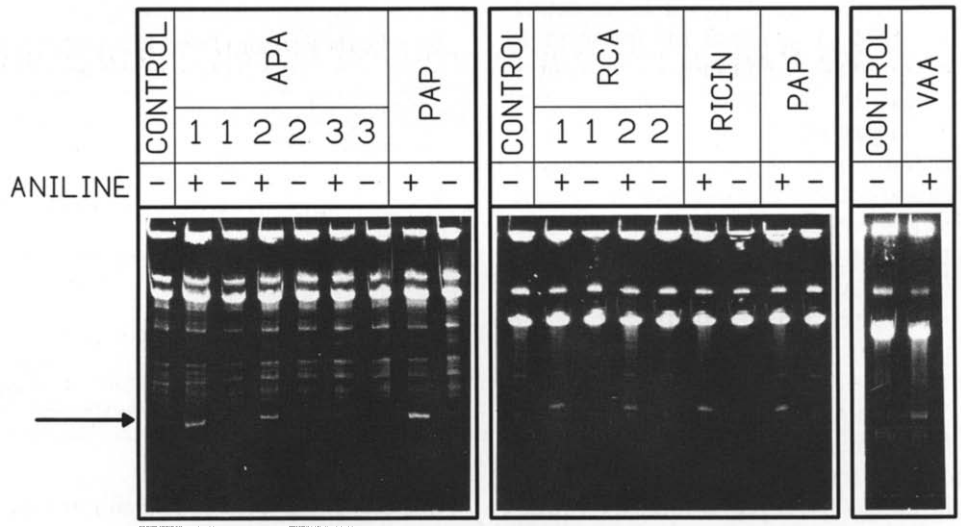

Fig. 3. rRNA *N*-glycosidase activity of four-chain agglutinins. Left: samples of 3 μg of rRNA either from control, APA-treated (lanes 1–3) ribosomes or PAP-treated ribosomes were subjected to RNA fragment release analysis and then electrophoresed in 5% polyacrylamide gel; the amounts of inhibitory protein were 2 μg (APA, lane 1), 23 ng (APA, lane 2), 2 ng (APA lane 3) and 7 μg (PAP). Center: samples of 3 μg of rRNA either from control, RCA-treated ribosomes (lanes 1–2), ricin-treated ribosomes or PAP-treated ribosomes were processed as in the left panel; the amounts of inhibitory protein were 2 μg (RCA, lane 1), 15 ng (RCA, lane 2), 2 μg (ricin) and 7 μg (PAP). Right: 3 μg of rRNA either from control or VAA-treated ribosomes were processed as in the left panel; the amount of inhibitory protein was 1 μg. The arrow indicates the RIP diagnostic RNA fragment.

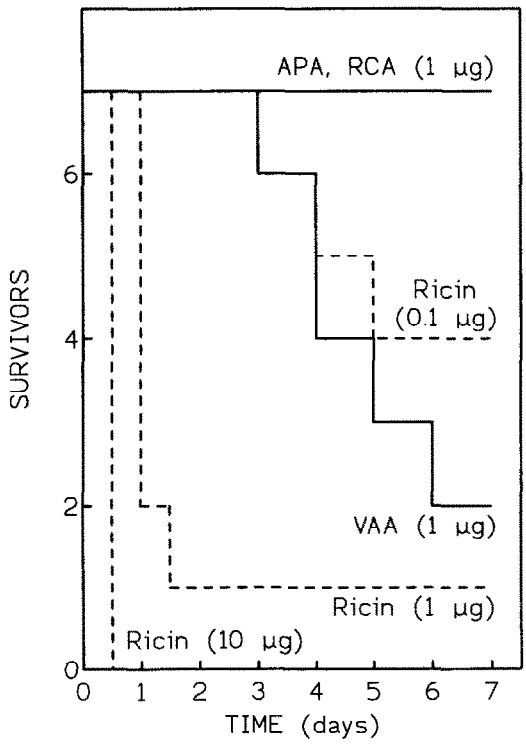

Fig. 4. Poisoning ability of four-chain agglutinins and ricin. Variable amounts of inhibitory proteins were injected intraperitoneally to seven mice. Poisoining was evaluated by death.

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