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Biological activity of recombinant *Ricinus communis* agglutinin A chain produced in *Escherichia coli*

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DNA encoding *Ricinus communis* agglutinin A chain was ligated into the *E. coli* expression vector pDS 5/3. Induced *E. coli* 71.18 cells which had been transformed with this plasmid express *Ricinus communis* agglutinin A chain in a soluble and biologically active form. Recombinant *Ricinus communis* agglutinin A chain had ribosomal RNA *N*-glycosidase activity and was approximately 10-fold less active than ricin A chain in a cell-free protein synthesis inhibition assay.

Ricinus communis agglutinin A chain; Ricin A chain; Expression; *E. coli*

1. INTRODUCTION

Castor beans (*Ricinus communis* seeds) contain two closely related types of lectins, ricin and *Ricinus communis* agglutinin (RCA) [1,2]. Ricin is a heterodimeric protein consisting of a catalytically active polypeptide (the A chain) covalently linked via a single disulphide bond to a galactose-specific lectin (the B chain) [3]. RCA is a tetramer in which two ricin-like heterodimers are held together by non-covalent forces. The corresponding polypeptides of ricin and RCA share extensive sequence homology. The A chains, which contain 267 amino acid residues, differ in only 18 residues, and the 262 residue-long B chains differ at 41 position [4].

In spite of this extensive sequence homology, ricin and RCA differ markedly in their biological properties. RCA is 60–75 times more potent than ricin in the agglutination of erythrocytes [2]. However, RCA is 2000-fold less toxic than ricin when injected intraperitoneally into mice [5]. After entering the cytosol of target cells, ricin A chain specifically depurinates a conserved surface loop present in 28 S ribosomal RNA [6,7]. Ribosomes containing depurinated 28 S RNA can no longer carry out protein synthesis, which leads to cell death.

The apparent difference in cytotoxicity between ricin and RCA does not appear to simply reflect a difference in protein synthesis inhibition by the corresponding A chains [2]. It appears that differences in quaternary structure also influence the ease with which the A chain enters target cells in vivo. In an attempt to confirm this, we have produced soluble, biologically active RCA A chain in *E. coli*. The ability of recombinant RCA A chain to inhibit protein sythesis by mammalian ribosomes in vitro and its cytotoxicity to cultured cells after reassociation with purified ricin B chain has been determined and compared to recombinant ricin A chain.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction endonucleases, T4 ligase, [^{35}S] methionine, [^{14}C] leucine and biotinylated protein A/streptavidin-horseradish peroxidase were obtained from Amersham (Bucks, England). Isopropyl β-D-thiogalactopyranoside (IPTG), ampicillin, plenylmethylsulphonyl fluoride (PMSF) and lysozyme (grade 1) were obtained from Sigma (Dorset, England). Polybuffer exchanger PBE94 and polybuffer 74 were from Pharmacia (Uppsala, Sweden). Vector pDS5/3 was kindly provided by Dr. B. Dobberstein (EMBL, Heidelberg, Germany).

2.2. *General methods*

Plasmid DNAs were prepared by the alkaline lysis method [8] and purified by centrifugation in cesuim chloride–ethidium bromide. Restriction enzyme digests and ligations were carried out as recommended by the suppliers.

2.3. *Construction of pRCA/A*

An RCA-encoding plasmid, pRCL52, has been described earlier [4]. The 3′ end of a ricin A chain-encoding cDNA was substituted into this clone by taking a 260 bp *Bgl*II–*Eco*RI fragment from a plasmid containing ricin A chain DNA followed by a stop codon, and ligating with the large *Bgl*II–*Eco*RI fragment pRCL52. A *Hin*dIII–*Eco*RI fragment containing the complete RCA A chain coding sequence with the stop codon derived from pUC8RA, was subcloned into M13 and an *Xho*I site created by site-directed mutagenesis at −15 in the RCA presequence region. An *Xho*I–*Sal*I fragment was subsequently retrieved, passaged through pUC18 and a *Bam*HI fragment cloned into pDS5/3 exactly as described for the construction of a ricin A chain-encoding expression plasmid [9].

2.4. *Expression of RCA A chain in* E. coli

For expression, recombinant plasmids were introduced into *E. coli*

strain 71.18 by calcium chloride-mediated transformation. 500 ml cultures were grown at 30°C in the presence of ampicillin (50 μg/ml) and IPTG (2 mM) to an A_{550} of 0.8. *E. coli* cells were harvested by centrifugation and resuspended in 2 ml of 0.15 M NaCl, 10 mM Na phosphate, pH 7.4, containing 1 mM PMSF. Lysozyme was added to 2 mg/ml and, after standing for 10 min on ice, the cells were lysed on ice by sonication using three 20 s bursts from an MSE ultrasonic disintegrator (amplitide 24). Lysates were cleared by centrifugation at 12,000 × *g* for 30 min at 4°C.

2.5. *Isolation of recombinant RCA A chain*

Cell-free lysate supernatants were dialysed against 25 mM imidazole/HCl, pH 7.4, and passed over a chromatofocusing column of polybuffer exchanger PBE94 (1.6 × 30 cm) equilibrated in the same buffer. A pH gradient (pH 7.4–4.0) was developed by passing through the column 600 ml of polybuffer 74 (diluted 1:10 in H_2O). Fractions having a pH of 5.9 (the pI of RCA A chain) were analysed by SDS-polyacrylamide gel electrophoresis [10] and Western blotting [11]. Blots were probed with rabbit antibodies raised against ricin A chain purified from *Ricinus* seeds and developed using the biotinylated protein A/streptavidin-peroxidase system (according to the manufacturer's instructions).

2.6. *Activity of recombinant RCA A chain in reticulocyte lysates*

The biological activity of recombinant RCA A chain was determined by its ability to inhibit [^{14}C]leucine incorporation into protein in a rabbit reticulocyte lysate [12]. Various concentrations of recombinant RCA A chain or recombinant ricin A chain [9] (0.5–50 ng/ml) were assayed for inhibition with respect to controls over a 5 min translation period at 28°C. Each concentration was assayed in triplicate.

2.7. *Depurination assay*

Reaction mixtures containing rabbit reticulocyte ribosomes (30 μg) were incubated with recombinant toxins in 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.6, at 30°C for 1 h. Control reactions lacking toxin were similarly incubated. RNA was extracted [13] and dissolved in sterile distilled water at 3 μg/ml. Aniline treatment of the rRNA and electrophoresis in agarose/formamide gels were carried out as described previously [14].

2.8. *Inhibition of protein synthesis in cultured cells*

Vero cells (2×10^4) in 0.1 ml Dulbecco's modified Eagles medium (DMEM) containing 5% foetal calf serum supplemented with 0.1% glutamine were added to the wells of 96-well microtitre plates and incubated at 37°C in 5% CO_2. Toxin or control solutions (25 μl) were added to the cells which had been washed in phosphate-buffered saline (PBS). After 20 h, each well was pulsed for 2 h with 1 μCi [^{35}S]methionine in 50 μl PBS at 37°C. After washing the cells in PBS followed by 5% TCA they were lysed in 0.5 M NaOH and the precipitate was collected and counted.

3. RESULTS AND DISCUSSION

The RCA A chain construct used here was generated by fusing DNA encoding the bulk of the RCA A chain (except for a small region encoding the carboxyl terminus), with DNA encoding the missing carboxyterminal region but which had been taken from ricin A chain cDNA. This strategy was used to incorporate a stop codon which had previously been engineered into the ricin A chain DNA. The resulting gene encoded a mutant form of RCA A chain which differed from the wild type at a single amino acid residue; the ricin A chain-derived DNA encodes serine at residue 241 rather than the asparagine normally present in RCA A chain.

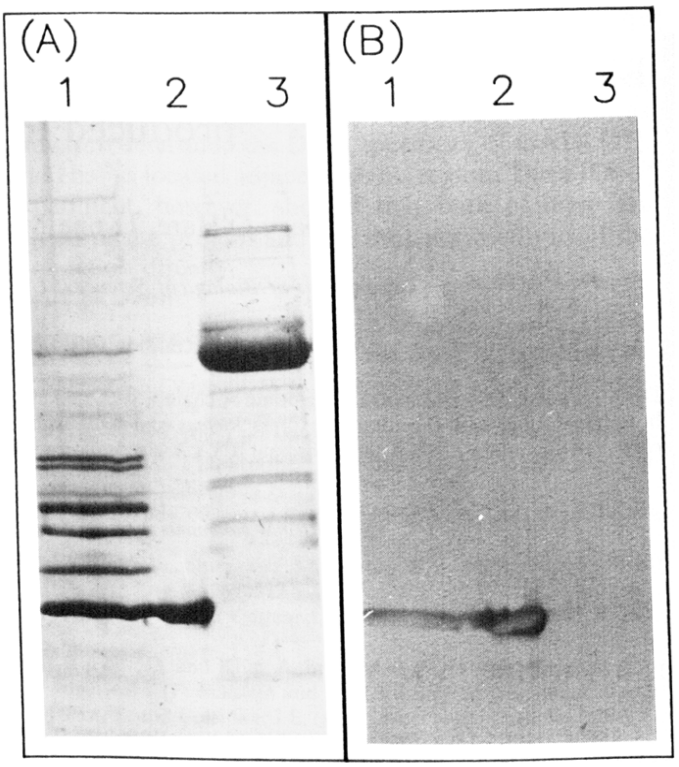

Fig. 1. Expression of RCA A chain. Cytoplasmic supernatants from *E. coli* cells transformed with the expression plasmid were subjected to chromatofocusing and the pH 5.9 fraction (lane 1) and pH 7.0 fraction (lane 3) were analysed by SDS-PAGE and silver stained (A) or blotted onto nitrocellulose and probed with anti-ricin A chain antibodies (B). Lane 2 contained purified recombinant ricin A chain.

The strategy used to modify the 5' end of the RCA A chain coding sequence was exactly the same as that described previously in the case of ricin A chain [9]. A *Bam*HI fragment containing the RCA A chain sequence was cloned into the expression site of the vector pDS5/3 [15]. This vector contain the strong coliphage T5 promoter P_{N25} fused to the *E. coli lac* operator, with a ribosome binding site and initiation codon immediately upstream from the unique *Bam*HI expression site. Downstream are sequences for efficient transcription termination. The expression plasmid generated directs the synthesis of a fusion protein in which the recombinant RCA A chain sequence (267 amino acid residues) is preceded by a 10 residue N-terminal extension, of which the initiator methionine is derived from the vector, 5 residues are from the pUC18 polylinker and 4 residues are from the RCA A chain N-terminal leader [9]. The recombinant fusion protein was calculated to have a molecular mass of about 30 kDa.

E. coli 71.18 cells were transformed with vector alone or with a recombinant plasmid containing the RCA A chain insert either in the correct orientation for expression or in the reverse orientation. Transformants were grown at 30°C in L broth supplemented with ampicillin and the inducer IPTG. After homogenization, dialysed

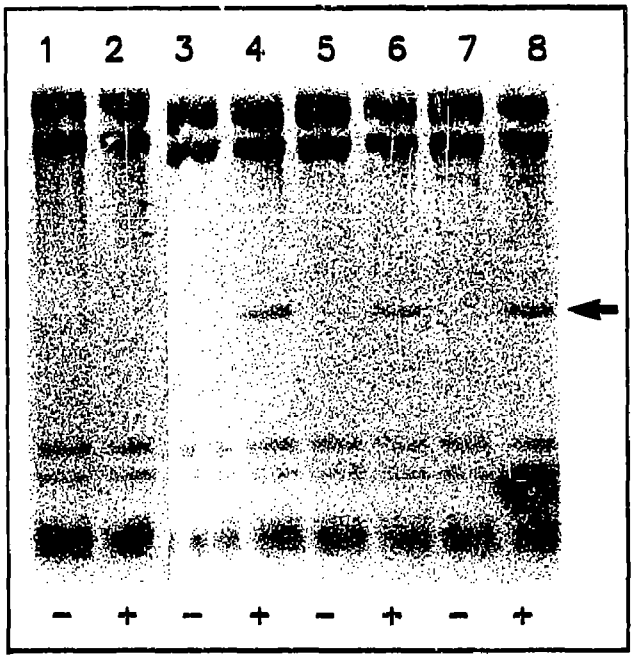

Fig. 2. Depurination of rabbit reticulocyte ribosomes. Ribosomes were incubated with test protein, rRNA was extracted and 3 μg aliquots were treated with aniline. These samples together with non-aniline treated controls were fractionated on a 2.2% agarose-formamide gel and the bands were visualized by ethidium bromide staining. Lanes 1 and 2, untreated control ribosomes; lanes 3 and 4, ribosome incubated with 100 ng native ricin A chain; lanes 5 and 6, ribosomes incubated with 100 ng of recombinant ricin A chain; lanes 7 and 8, ribosomes incubated with 100 ng recombinant RCA A chain. +, indicates aniline treatment; −, indicates no aniline treatment.

cell-free supernatants were passed down a chromatofocusing column which was then treated with a pH gradient (pH 7.4–4.0). Collected fractions having a pH of ~5.9 (the pI of RCA A chain) were analysed by SDS/polyacrylamide gel electrophoresis (Fig. 1A, lane 1). This fraction contained a major band of molecular mass ~30 kDa whose mobility on the gel was identical to that of purified recombinant ricin A (Fig. 1A, lane 2) and which was not present in a fraction of higher pH (Fig. 1A, lane 3). The 30 kDa band was identified as RCA A chain since, in addition to having the correct pI, it (i) reacted strongly with rabbit antibodies raised against ricin A chain (Fig. 1B, lane 1), and (ii) it was not present in supernatants from cells transformed with vector alone or with vector containing the insert in the reverse orientation (data not shown).

Recombinant RCA A chain produced in *E. coli* was catalytically active and capable of specifically depurinating 28 S rRNA. Depurinated 28 S rRNA is susceptible to aminecatalysed hydrolysis of the sugar–phosphate backbone at the depurination site, releasing a small fragment of around 390 nucleotides from the 3' end of the rRNA. This fragment is diagnostic for ribosome-inactivating N-glycosidases and is readily observed following agarose/formamide gel electrophoresis. Hydrolysis of recombinant RCA A chain-treated rabbit reticulocyte ribosomes produced the diagnostic fragment (Fig. 2, lane 4), which was identical in size to that released from ricin A chain-treated ribosomes (Fig. 2, lanes 6 and 8).

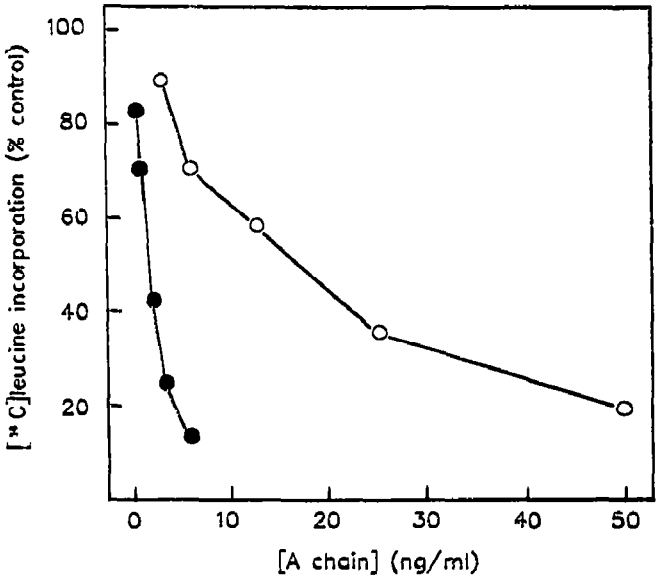

Fig. 3. Biological activity of recombinant A chains. Various concentrations of ricin A chain (●) or RCA A chain (○) were tested for their ability to inhibit cell-free protein synthesis in the rabbit reticulocyte assay.

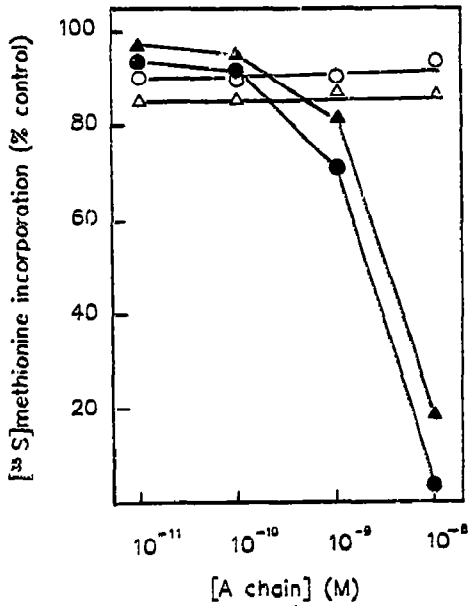

Fig. 4. Cytotoxicity of recombinant A chains to Vero cells. Cells were plated out at 2×10^5 cells/ml in 96-well plates and treated with ricin A chain (○), RCA A chain (△), ricin A chain which has been preincubated with 10^{-8} M ricin B chain (●) or RCA A chain which had been preincubated with 10^{-8} M ricin B chain (▲). Cells were incubated for 20 h at 37°C. Each well was then pulsed with 1 μCi [^{35}S]methionine for 2 h before determining the radioactivity incorporated into protein. The incorporation observed was expressed as percentages related to untreated control cells.

By scanning gels containing recombinant RCA A chain and comparing the intensity of the 30 kDa band with scans of gels containing known amounts of recombinant ricin A chain, the quantity of RCA A chain in various preparations was determined. Inhibition of protein synthesis in rabbit reticulocyte lysates was measured in the presence of either recombinant RCA A chain or recombinant ricin A chain (Fig. 3). The IC_{50} value (the concentration required for 50% inhibition of protein synthesis) for RCA A chain was estimated to be 16.5 ng/ml, 11-fold higher than that estimated for ricin A chain (1.5 ng/ml).

Both recombinant RCA A chain and ricin A chain were capable of reassociating with purified ricin B chain to form cytotoxic heterodimers. Figure 4 indicates that preincubation of Vero cells with either RCA A chain or ricin A chain over the concentration range indicated had no effect on protein biosynthetic capacity when compared to untreated cells. When the A chains, at the concentrations indicated, were preincubated with 10^{-8} M ricin B chain prior to incubation with Vero cells, holotoxin which was cytotoxic at the higher A chain concentrations was generated. While holotoxin containing ricin A chain appeared to be slightly more cytotoxic than that containing RCA A chain, both recombinant A chains were clearly translocationally competent and potently toxic in this whole cell assay.

In conclusion, RCA A chain has been produced cytoplasmically in *E. coli* in a soluble, stable and biologically active form. RCA A chains have the ribosomal RNA N-glycosidase activity characteristic of all plant ribosome inactivating proteins, although it was roughly one order to magnitude less active than ricin A chain under the assay conditions employed here. RCA A chain was capable of associating with purified ricin B chain to produce a molecule, presumably heterodimeric, that was cytotoxic to Vero cells. While RCA A–ricin B conjugates appeared to be slightly less cytotoxic than native ricin, the difference in cytotoxicity was markedly less than the 2,000-fold difference reported between tetrameric RCA and dimeric ricin [5]. This difference in cytotoxicity observed with the purified lectins clearly does not simply reflect the difference in biological activity of the respective A chains. Rather, it appears to be due to B chain-mediated difference in quaternary structure which in turn probably influences the case with which the A chains are delivered into the cytosol of target cells.

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