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Recombinant proricin binds galactose but does not depurinate 28 S ribosomal RNA

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Preproricin transcripts microinjected into *Xenopus* oocytes were expressed and the product was segregated by the oocyte endoplasmic reticulum and core glycosylated. Recombinant proricin was soluble, stabilised by intramolecular disulfide bonds and biologically active in that it could bind to immobilized lactose (selectin 2) or immobilized asialofetuin. Affinity-purified proricin did not catalyse the depurination of 28 S ribosomal RNA unless it was reduced, when slight but significant activity was observed. Gel filtration of the reduced proricin fraction showed that this depurination activity was not associated with proricin. The activity was apparently due to ricin A chain released by reduction from mature ricin which was, in turn, generated from proricin, presumably via endogenous oocyte endoprotease activity.

Proricin; Galactose binding; Depurination

1. INTRODUCTION

Ricin is a potently cytotoxic, heterodimeric protein found in the seeds of the castor oil plant, *Ricinus communis*. One polypeptide subunit (the A chain) is an enzyme which catalytically inactivates 60 S subunits of eukaryotic ribosomes and thereby causes cell death [1]. The second polypeptide (the B chain), which is covalently joined to the A chain by a single disulfide bond, is a galactose-specific lectin [1]. Recently, it has been shown that ricin A chain is an *N*-glycosidase which removes a specific adenine residue located within a highly conserved region of 26 S and 28 S ribosomal RNAs [2,3].

During its biosynthesis in *Ricinus* seeds, mature ricin is derived from a precursor – preproricin – by a series of contranslational and post-translational modifications during intracellular transport from the site of synthesis in rough ER, via the Golgi complex, to the site of accumulation within organelles termed protein bodies [4,5]. The preproricin polypeptide consists of a 35-residue N-terminal leader peptide followed by the mature A chain sequence which, in turn, is joined to the B chain sequence by a 12 amino acid linking sequence [6]. This linking sequence is proteolytically removed within the protein bodies [7].

There are, at present, no data available on the biological activity of ricin precursor polypeptides. Here we demonstrate that preproricin can be expressed by microinjecting in vitro-generated transcripts into *Xenopus laevis* oocytes. The expressed product was segregated into the oocyte endomembrane system, core glycosylated and the N-terminal signal peptide was removed. This in vitro expression system has previously been shown to segregate, process and fold efficiently recombinant ricin B chain into a soluble, biologically active confirmation [8]. Recombinant proricin has been purified from oocyte homogenates and its biological activity has been determined.

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2. EXPERIMENTAL

2.1. *Bacterial strains and plasmid constructs*

Escherichia coli K12 strain DH1 was routinely used except during oligonucleotide site-directed mutagenesis when strains of 71.18 and 71.18 mut L were substituted [9]. Preproricin cDNA was created from a clone, pRCL617, which lacks the full signal sequence by just three bases [10]. The missing ATG was introduced into the 5′-poly(G) tail of the cDNA, together with a unique *Xho*I restriction site, by oligonucleotide site-directed mutagenesis. The oligonucleotide used had the following sequence:

5′G_9C*T*C*GA*GGA*T*GAAACC$G_3$3′

where the asterisks indicate mismatches.

Mutagenesis was carried out using the oligonucleotide and a recombinant M13 template following standard procedures [11]. The length of the oligonucleotide (27-mer) and its unusually high G content precluded conventional plaque screening in 6×SSC at the $T_d - 5°C$ and the T_d of the mutagenic oligonucleotide probe. Instead, sequential hybridizations were performed in 6×SSC and 5°C steps from room temperature to 60°C. Mutant sequences were excised with *Xho*I and *Sal*I and recloned into the *Sal*I site of the transcription vector pGEM1. Plasmid DNAs were prepared by the alkline lysis method [12] and purified by centrifugation in cesium chloride-ethidium bromide.

2.2. *Synthesis of preproricin mRNA and microinjection*

Preproricin mRNA was synthesized in vitro in the presence of the capping dinucleotide 7-Me(5′)GpppG(5′)OH and T_7 RNA polymerase as described [8]. Purified RNA was dissolved in distilled water at approx. 100 μg/ml. Microinjection into batches of 100 oocytes from *X. laevis*, pulse labelling with [^{35}S]methionine and oocyte homogenization were performed exactly as in [8].

2.3. *Assay for galactose binding activity*

Recombinant proricin was assayed for sugar binding activity by measuring its ability to bind to immobilized asialofetuin. Bound proricin was measured using rabbit anti-ricin-B chain antibodies followed by ^{125}I-labelled protein A, as described elsewhere [13].

2.4. *Purification of proricin*

Homogenate from approx. 25 oocytes in 1 ml oocyte homogenization buffer (20 mM Tris-HCl, pH 7.6, 100 mM PMSF) was passed through 1 ml immobilized lactose (selectin 2, Pierce, Rockford, IL). The homogenate was passed through the column a total of three times. The column was washed with homogenization buffer (minus PMSF) until no further protein emerged. Bound material was eluted in the same buffer containing 100 mM galactose. Samples from the collected fractions were then prepared for immunoprecipitation [14] and SDS-polyacrylamide gel electrophoresis [15] or for reduction and further fractionation by gel filtration. Usually the first 1 ml of the 100 mM galactose eluate contained virtually all of the bound material. This was reduced at room temperature for 30 min in 50 mM dithiothreitol. The sample was then passed through a 1×30 cm Sephadex G-75 column equilibrated in homogenization buffer containing 50 mM dithiothreitol. Typically, 48×1-ml fractions were collected and analysed by SDS-polyacrylamide gel electrophoresis or for depurination activity.

2.5. *Ricin A chain catalysed depurination of 28 S ribosomal RNA*

Samples of the crude oocyte homogenate or 30 μl aliquots of the fractions from the selectin 2 affinity chromatography or gel filtration steps were incubated with an equal volume of rabbit reticulocyte lysate for 30 min at 30°C. An equal volume of water was added and the solution was made 1% in SDS. RNA was prepared from the lysates by standard phenol/chloroform extraction followed by ethanol precipitation. The RNA was redissolved in water and a volume equivalent to 8 μg RNA was incubated with 40 μl of 1 M aniline, pH 4.5, for 2 min at 60°C in the dark. Ethanol-precipitated RNA samples were then dissolved in 20 μl of 60% formamide in 0.1×E buffer (3.6 mM Tris, 3 mM NaH_2PO_4, 0.2 mM EDTA) [16] and incubated at 65°C for 5 min before cooling. RNA was electrophoresed in 1.2% agarose, 0.1×E buffer and 50% formamide.

2.6. *Other methods*

mRNAs were translated in vitro in wheat germ lysates as described [17]. Immunoprecipitated or affinity-purified protein samples were treated with endo H (Miles, Elkhart, IN) as in [5].

3. RESULTS AND DISCUSSION

The preproricin signal sequence is part of a 35-residue N-terminal leader peptide and is responsible for directing proricin into the lumen of the rough endoplasmic reticulum [5,6]. A preproricin cDNA clone initially isolated encoded 34 of these 35 leader residues [10] but lacked the 5′ ATG codon subsequently identified as the translational start site [18]. A 1.94 kb *Pst*I fragment encoding preproricin but missing only the extreme 5′ coding region ATG was ligated into M13mp19. Oligonucleotide site-directed mutagenesis using a synthetic 27-mer containing 6 mismatches simultaneously created an XhoI site and introduced the missing initiation codon at the extreme 5′-end of the coding region (fig.1). After confirming the mutation by sequencing, an *Xho*I-*Sal*I fragment containing the entire preproricin coding sequence was subcloned into *Sal*I-linearized pGEM1 and the orientation of the preproricin coding sequence with respect to the T_7 promoter was confirmed by restriction mapping (fig.1). This construct was used to generate preproricin mRNA in vitro using the T_7 promoter and T_7 RNA polymerase.

Fig. 2 shows the expressed products of preproricin mRNA transcripts after translation in a wheat germ cell-free lysate or after microinjection

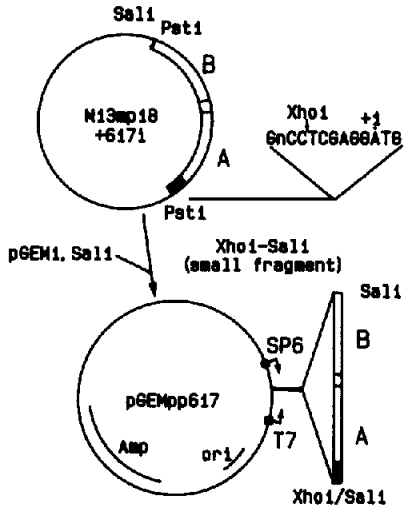

Fig.1. Construction of preproricin in a transcription vector. Site-directed mutagenesis was used to create an ATG and *Xho*I site within the *Pst*I insert of pRCL617 [10] which had been subcloned into M13mp18. The resulting *Xho*I-*Sal*I fragment containing preproricin cDNA was recloned into the *Sal*I site of pGEM1 to generate pGEMpp617.

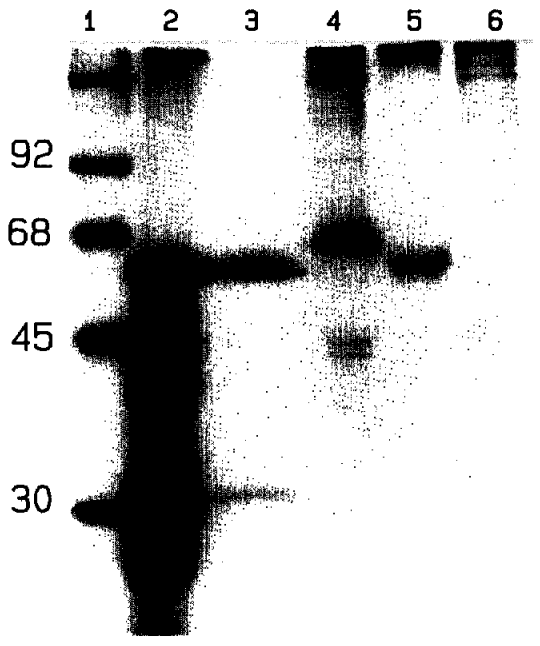

Fig.2. Synthesis of recombinant proricin. The in vitro transcript encoding preproricin was translated in a wheat germ cell-free system or microinjected into *Xenopus* oocytes in the presence of [^{35}S]methionine. Proricin was recovered from oocyte homogenates by immunoprecipitation using anti-ricin B chain antibodies, analysed by SDS-polyacrylamide gel elecrophoresis and visualized by fluorography. Lanes: (1) molecular mass markers (values indicated on the left, in kDa), (2) wheat germ product, (3) oocyte product after treatment with endoH, (4) oocyte product prepared under normal (reducing) conditions, (5) unreduced oocyte product, (6) control oocytes.

into *X. laevis* oocytes. Proricin was recovered from oocyte homogenates by immunoprecipitation with anti-ricin B chain antibodies. The wheat germ system gave a major product which indicates the apparent molecular size of unprocessed preproricin (fig.2, lane 2). The product immunoprecipitated by anti-B chain antibodies from a total homogenate of oocytes after injection of preproricin mRNA was clearly larger than the wheat germ product (fig.2, lane 4), suggesting that the former had been glycosylated. This was confirmed by the decrease in apparent size upon treatment of the oocyte product with endo-*N*-acetylglucosaminidase H (fig.2, lane 3). Fractionation of the oocytes prior to immunoprecipitation showed that the expressed proricin was present in the particulate vesicle fraction rather than the soluble cytosolic fraction (not shown), confirming that it had been segregated into the lumen of the endoplasmic reticulum to permit *N*-glycosylation. Proricin presumably contains 5 intrachain disulfide bonds (the bond which ultimately joins the A and B chains plus 4 bonds within the B chain [1]). The mobility of the proricin was significantly increased in the absence of dithiothreitol (fig.2, lane 5) compared to that in its presence (fig.2, lane 4), indicating that intramolecular disulfide bonds maintain the product in a compact structure of lower apparent molecular mass than its reduced counterpart. No immunoprecipitated product was found in homogenates from control oocytes that had been mock-injected with water (fig.2, lane 6). Homogenates from oocytes injected with preproricin mRNA and incubated with [^{35}S]methionine were applied to an immobilized lactose column. While the bulk of the proteins passed straight through, the column effectively bound the recombinant proricin, which was readily released from the column by 100 mM galactose (fig.3a). The identity of affinity-purified proricin was confirmed by immunoprecipitation (not shown). The amount of proricin capable of binding galactose was determined by measuring its ability to bind to asialofetuin, a glycoprotein with ter-

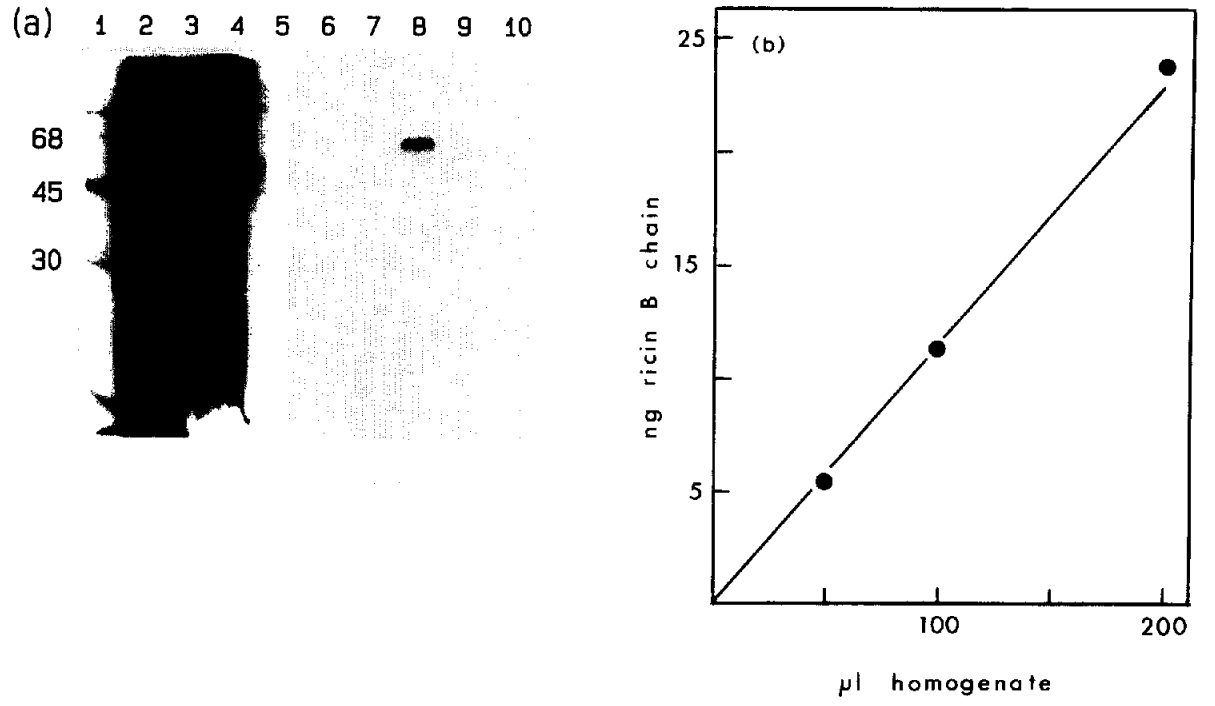

Fig.3. Affinity purification of recombinant proricin and confirmation of its lectin activity. (a) Total homogenates from [^{35}S]methionine-labelled oocytes expressing proricin were passed through an immobilized lactose (selectin 2) column. After washing the column to remove all unbound material, bound product was eluted with 100 mM galactose. Lanes: (1) molecular mass (in kDa) markers, (2) total homogenate, (3–7) unbound material passing through the column, (8–10) material eluted with galactose. Polypeptides were analysed by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. (b) Aliquots of homogenates from oocytes injected with preproricin mRNA were added to microtiter plate wells coated with asialofetuin. Bound proricin was measured by subsequent addition of anti-ricin B chain antibodies followed by ^{125}I-labelled protein A. The amount of bound proricin was estimated in terms of its B chain component using a calibration curve prepared with oocyte homogenates spiked with known amounts (0–50 ng) of biochemically purified ricin B chain. 50 µl homogenate is equivalent to one oocyte.

minal galactose residues. Results obtained with homogenates prepared from oocytes expressing proricin indicated that approx. 5 ng B chain binding equivalents were produced per oocyte (fig.3b). High-speed centrifugation of freshly prepared oocyte homogenates showed that the recombinant proricin was initially completely soluble.

The ability of proricin to depurinate 28 S ribosomal RNA was assayed. The specific, ricin A chain-mediated depurination of 28 S ribosomal RNA renders the RNA susceptible to hydrolytic cleavage of the sugar-phosphate backbone at the depurination site. Because this site is close to the 3′-end of 28 S ribosomal RNA, hydrolysis of ricin-treated RNA generates a small (390-nucleotide) RNA fragment which is diagnostic for the depurination reaction [2].

Aniline hydrolysis of rabbit reticulocyte ribosomal RNA treated with homogenates from oocytes expressing proricin to which dithiothreitol had been added generated a faint band visible on RNA gels which was apparently identical in size to the small fragment generated by treatment with purified ricin A chain (not shown). This band was not observed after treating the ribosomes with non-reduced homogenates of proricin-expressing oocytes or with reduced homogenates from control oocytes.

When the affinity-purified, [^{35}S]methionine-labelled proricin fraction was reduced with dithiothreitol, subsequent electrophoresis demonstrated not only the presence of proricin but two additional faint bands whose mobility was typically that of ricin A and B chains (not shown).

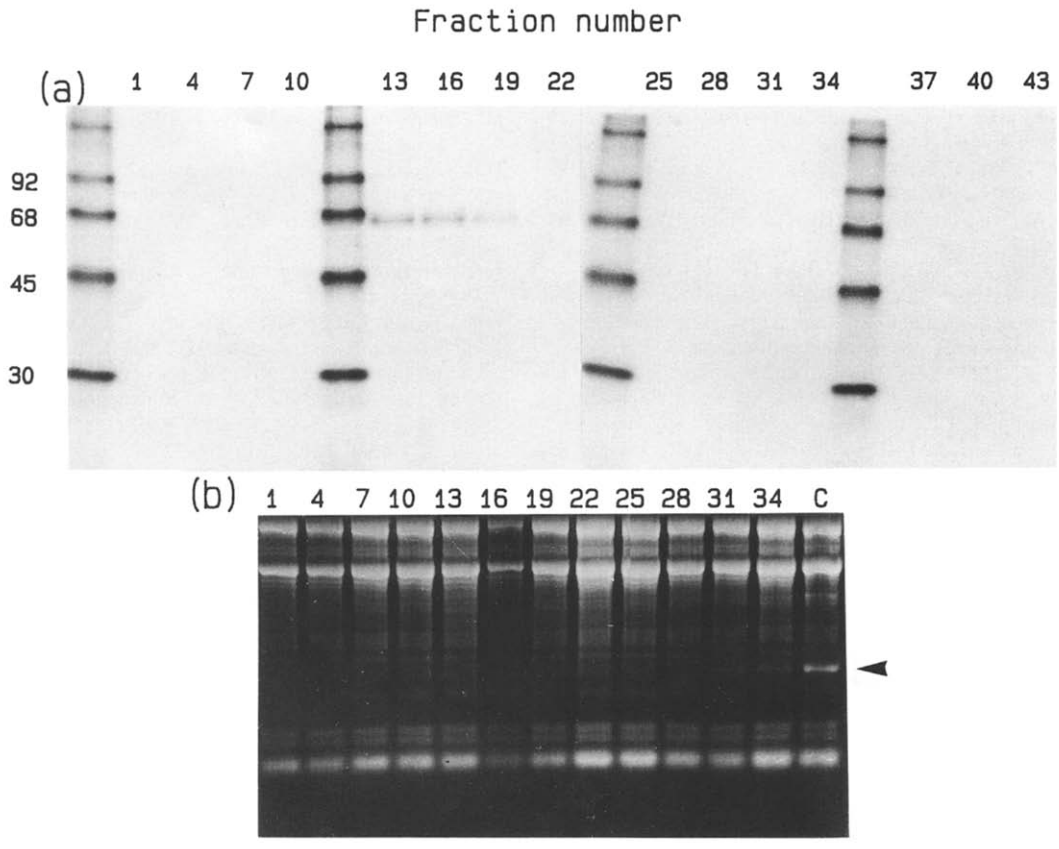

Fig.4. Depurination activity present in purified, reduced proricin is not associated with the precursor. Reduced proricin was fractionated on a Sephadex G-75 column and collected fractions were analysed by immunoprecipitation to locate proricin, and for their ability to depurinate 28 S ribosomal RNA. (a) Proricin immunoprecipitated from collected fractions was present in fractions 13, 16 and 19; (b) the same fractions were tested for depurination activity which was present in fractions 31 and 34.

Further, both of these smaller bands were immunoprecipitated, together with proricin, under non-reducing conditions using anti-ricin B chain-specific antibodies (not shown). Evidence that the depurination activity was associated with the smaller fragments, rather than proricin, was obtained by size fractionating the reduced mixture on a Sephadex G-75 column. Fig. 4b shows that depurination activity was present in fractions 31 and 34, whereas immunoprecipitation showed that proricin was confined to fractions 13, 16 and 19 (fig.4a). When reduced native ricin was fractionated on the G-75 column, the individual A and B subunits were recovered in fractions 30–34 (not shown).

Collectively, the present data show that *Xenopus* oocytes translate injected preproricin mRNA to produce soluble, recombinant proricin which is segregated, glycosylated and folded into a biologically active confirmation stabilized by intramolecular disulfide bonds. In this form proricin is capable of binding to galactose via its B chain component but is unable to depurinate 28 S ribosomal RNA. The A chain component is potentially active, however, because adventitious processing of a small proportion of the proricin, presumably by endogenous oocyte endoprotease(s), produces disulfide-linked holotoxin which is capable of depurinating 28 S ribosomal RNA after reductive release of the A chain.

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