Ricinus communis agglutinin B chain contains a fucosylated oligosaccharide side chain not present on ricin B chain

J. Michard Lord and Suzanne M. Harley

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, England

Received 27 June 1985

Ricinus communis agglutinin (RCA) B chain, in contrast to ricin B chain, contains fucose. Since both RCA and ricin B chain lose two oligosaccharide side chains when treated with β-endo *N*-acetylglucosaminidase H, it is proposed that fucose is present on a third oligosaccharide. This third oligosaccharide is not present on the ricin B chain and accounts for the larger relative molecular mass of the RCA B chain.

Ricinus communis *agglutinin* *Ricin* *B chain* *Fucose*

1. INTRODUCTION

Ricinus communis seeds contain 2 distinct but closely related lectins, ricin and RCA [1]. Ricin is a potent cytotoxin consisting of an enzymic subunit (A chain), which attacks 60 S ribosomal subunits, joined by a single disulphide bond to a galactose binding subunit (B chain) [2]. Both the A and B chains are *N*-glycosylated and have apparent relative molecular masses of 32 and 34 kDa, respectively [3]. RCA is a tetramer composed of 2 ricin-like heterodimers each consisting of an enzymic A chain (32 kDa) and a galactose binding B chain (36 kDa), both of which are also *N*-glycosylated [4].

Purified RCA B chain thus has an apparent relative molecular mass some 2000 Da larger than that of purified ricin B chain. When the B chains are treated with endo H, both of the oligosaccharide side chains known to be present on the ricin B chain are removed giving a deglycosylated polypeptide of M_r = 30000 [5,6]. Two oligosaccharide side chains are likewise removed from RCA B chain, reducing its apparent M_r to 32000 [6].

Abbreviations: RCA, *Ricinus communis* agglutinin; endo H, β-endo *N*-acetylglucosaminidase H; $PhMeSO_2F$, phenylmethylsulphonyl fluoride

We have recently been able to deduce the primary structure of both ricin and RCA B chains from the nucleotide sequence of cloned DNA complementary to the appropriate mRNA [7,8]. The 2 B chains share extensive homology differing in only 41 out of a total of 262 amino acid residues and have very similar relative molecular masses, the RCA B chain being just 163 Da larger than its ricin counterpart [8]. One significant feature of the B chain primary sequence differences is that the RCA chain contains a third potential *N*-glycosylation site in addition to the 2 sites common to both subunit sequences [8].

Here we present evidence that the apparent 2000 Da difference in relative molecular mass between RCA and ricin B chains [6] results from the presence of an extra oligosaccharide side chain on the RCA B chain. The apparent size difference is maintained after enzymic deglycosylation because the additional oligosaccharide on the RCA B chain, in contrast to the oligosaccharides common to both polypeptides, is resistant to endo H digestion. This resistance is apparently due to the presence of fucose.

2. MATERIALS AND METHODS

R. communis plants were grown from seed in a greenhouse. Endosperm tissue was excised from

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

the ripening seeds during testa formation; at this developmental stage the lectins are being rapidly synthesized [9].

Lectins were labelled in vivo by applying [^{3}H]fucose or [^{35}S]methionine to the upper surface of endosperm halves which had been placed on moist filter paper in a petri dish. The tissue was incubated overnight (16 h) at room temperature. When appropriate the tissue was pretreated with tunicamycin as described [10].

Ricin and RCA were purified by the method of Cawley et al. [3] modified as described [6]. The A and B subunits of ricin and RCA, reduced in the presence of 5% (v/v) β-mercaptoethanol and 0.5 M galactose, were separated according to published procedures [11,12].

Susceptible oligosaccharide side chains were removed from lectins or their subunits by endo H digestion. 20–50 μg of protein were precipitated with 10% (w/v) trichloroacetic acid. The pellets were washed in acetone and then resuspended by heating at 100°C for 2 min in 10 μl of 0.1 M sodium citrate, pH 5.6, 1% (w/v) SDS and 1 mM $PhMeSO_2F$. The SDS concentration was diluted to 0.1% by adding 90 μl of citrate buffer, pH 5.6. Each protein solution was divided into 2 equal aliquots and 2.5 mU of endo H was added to one aliquot. After incubation at room temperature overnight, samples were taken for electrophoretic analysis.

Electrophoresis in the presence of SDS and dithiothreitol was performed as described [13]. Polypeptides were visualized by staining with Coomassie blue and radioactive polypeptides by fluorography [14].

Immunoprecipitation was performed using antibodies raised in rabbits against RCA as described [13].

3. RESULTS AND DISCUSSION

Rabbit antibodies raised against RCA cross react with both RCA and ricin. When both lectins are labelled in vivo by incubating developing castor bean endosperm with [^{35}S]methionine, polypeptides immunoprecipitated by RCA antibodies run as 3 major bands on reduced, denaturing gels (fig.1, lane 2). The fastest migrating band is a mixture of ricin and RCA A chains, the intermediate band contains ricin B chain and the slowest band

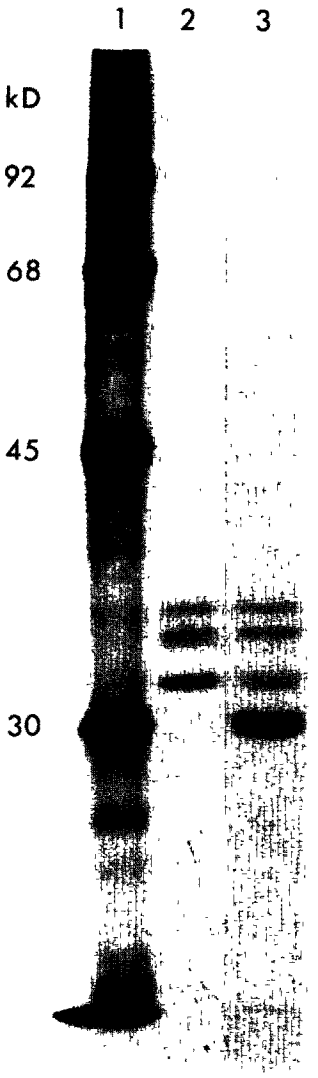

Fig.1. Polypeptides immunoprecipitated by anti-RCA serum from extracts of developing castor bean endosperm which had been incubated with [^{35}S]methionine in the absence (lane 2) or presence (lane 3) of tunicamycin. Lane 1, molecular mass markers. Bands visualized by fluorography.

is RCA B chain. The intermediate band also contains a mixture of both ricin and RCA 'heavy' A chains. In the case of ricin it has been shown that while both heavy and normal A chains are identical in terms of their amino acid composition, heavy A chain had approximately twice the carbohydrate content of normal A chain [5]. This suggests that

in the case of normal A chain only one of 2 potential *N*-glycosylation sites [7] is occupied by an oligosaccharide while in the case of heavy A chain both are occupied. Further, all ricin A chain oligosaccharide side chains apparently contain fucose and as such both normal and heavy A chain oligosaccharides are resistant to endo H digestion. In contrast, neither of the 2 ricin B chain oligosaccharides contain fucose and both can be removed by endo H [5].

RCA B chain has a higher apparent relative molecular mass than ricin B chain (fig.1, lane 2). When purified RCA B chain is treated with endo H 2 oligosaccharide side chains are also removed, but the apparent size difference between the RCA and ricin B chains is still observed for their enzymically deglycosylated forms [6]. Although this suggests that the RCA B chain polypeptide is approx. 2000 Da larger than its ricin counterpart, recent work in our laboratory has shown that this is not the case [8]. The relative molecular masses of the 2 B chains have been deduced from the nucleotide sequences of their cDNAs and differ by only 163 Da. The deduced ricin and RCA A chains are also almost identical in size and are slightly larger than the B chains. This can also be seen when *N*-glycosylation in vivo is prevented by tunicamycin (fig.1, lane 3). Although inhibition of glycosylation was not complete, the non-glycosylated lectin chains run as a tight doublet of apparent relative molecular masses around 30 kDa (fig.1, lane 3). The uppermost band of the doublet is a mixture of ricin and RCA A chains, the lower of B chains. The deduced RCA B chain amino acid sequence showed one particularly significant difference from that of ricin. Residue number 79 in the ricin B chain is Asp but is Asn in the RCA B chain. This Asn residue (in the sequence Asn-Cys-Ser) becomes a third possible *N*-glycosylation site unique to the RCA B chain. If this site were occupied by an oligosaccharide resistant to endo H digestion, the experimentally observed size differences between RCA and ricin B chains could be accounted for.

Ricin and RCA were purified and separated from extracts of developing castor bean seeds which had been labelled in vivo with [^{3}H]fucose. Both lectins were labelled by [^{3}H]fucose (fig.2). Electrophoretic analysis confirmed that ricin and RCA had been clearly separated (fig.3a, lanes 1 and 2). Treatment with endo H increased the mobilities of the lectin B chains, that of ricin ran slightly faster than the A chain (fig.3a, lanes 3) while the deglycosylated RCA B chain had the same mobility as the A chain (fig.3a, lane 4). Fluorography demonstrated the presence of [^{3}H]fucose in the RCA B chain (fig.3b, lane 2).

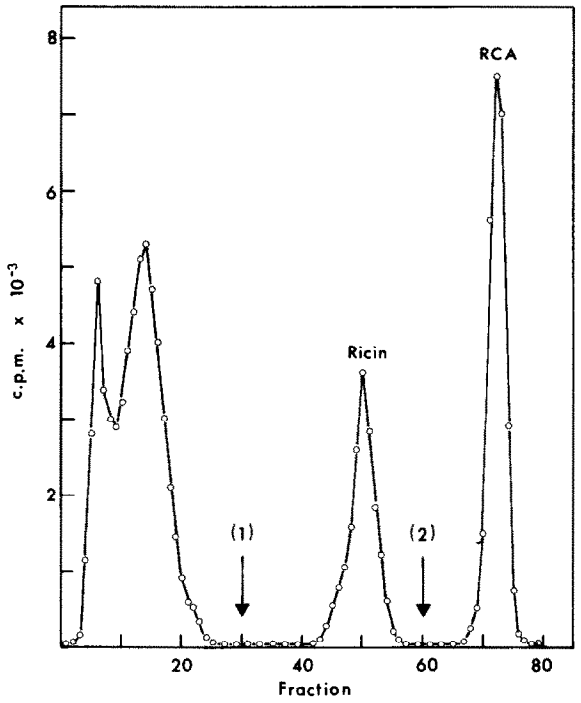

Fig.2. Separation of [^{3}H]fucose labelled *Ricinus* lectins on Sepharose 6B. After washing through the unbound protein, ricin was eluted with 5 mM galactosamine (applied at arrow 1) before RCA was eluted with 100 mM galactose (applied at arrow 2). 10 ml fractions were collected and 100 μl was removed from each for [^{3}H] determination.

A clearer indication of [^{3}H]fucose distribution was obtained by fractionating the 2 lectins into their constituent subunits. In the case of ricin [^{3}H]fucose was exclusively confined to the A chain peak (fig.4a), but in the case of RCA both the A and the B chain peaks contained [^{3}H]fucose (fig.4b). Individual subunits, before or after endo H treatment, were separated electrophoretically. Comparison of the Coomassie stained gel with the fluorograph confirmed the distribution of [^{3}H]fucose containing oligosaccharides. In agreement with an earlier report [5], [^{3}H]fucose was

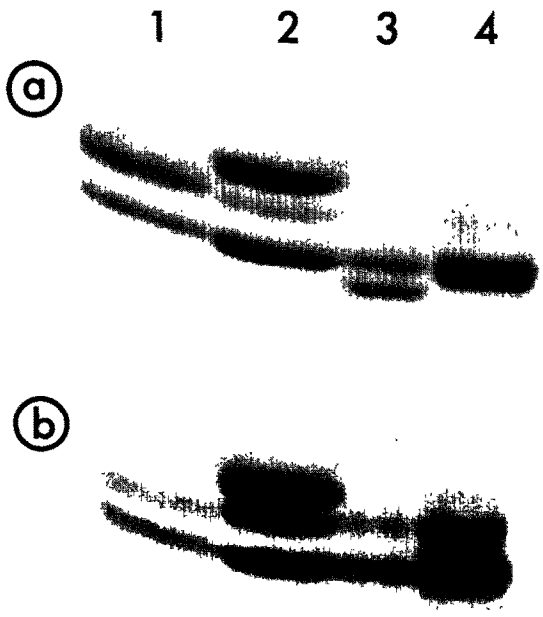

Fig.3. Aliquots of the ricin (lanes 1 and 3) and RCA (lanes 2 and 4) peaks from fig.2 were analysed electrophoretically without (lanes 1 and 2) or after (lanes 3 and 4) endo H digestion. (a) Coomassie blue stained gel; (b) fluorograph of (a).

found in ricin A and heavy A chains (fig.5b, lane 2) but not in ricin B chain (fig.5b, lane 3). The ricin A chains were insensitive to endo H digestion (fig.5a and b, lanes 4) in contrast to ricin B chain (fig.5a and b, lanes 5). The separation of RCA subunits was only partially effective (fig.5a, lanes 6 and 7). In spite of this it can be clearly seen that RCA A and heavy A chains (fig.5b, lane 6) and RCA B chain (fig.5b, lane 7) all contain [^{3}H]fucose. Once again the RCA A chains were insensitive to endo H digestion (fig.5b, lane 8), but RCA B chain was reduced to a size approximately equal to that of the A chain (fig.5b, lane 9).

In conclusion, therefore, we propose that the RCA B chain has 3 oligosaccharide side chains. In addition to 2 shared with the ricin B chain, both of which lack fucose and are removed by endo H, the third oligosaccharide contains fucose and is resistant to endo H. The presence of this extra oligosaccharide accounts for the apparent size difference between ricin and RCA B chains.

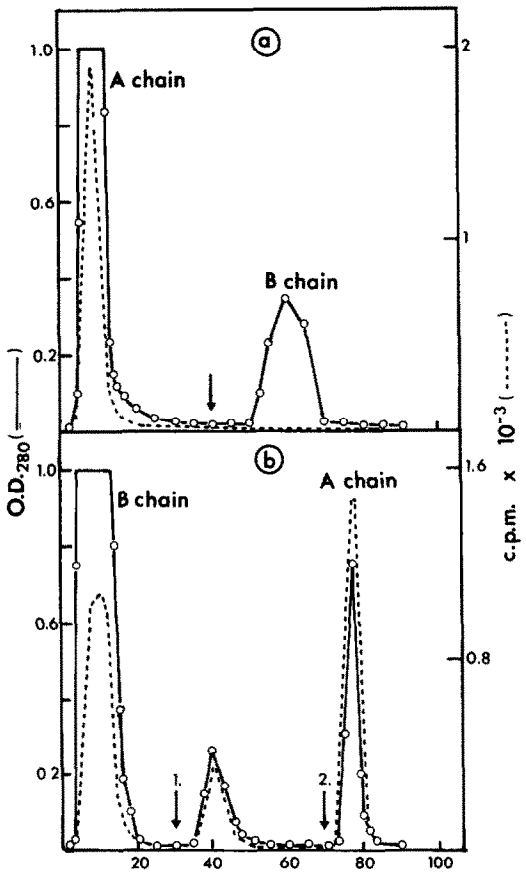

Fig.4. Separation of lectin subunits. Ricin and RCA were incubated with 5% β-mercaptoethanol and 0.5 M galactose. (a) Ricin subunits were applied to a DE 52 column equilibrated in 50 mM Tris-HCl, pH 8.5. The A chain did not bind to the column and the B chain was eluted with buffer containing 100 mM NaCl added where indicated by the arrow. (b) RCA subunits were applied to a DE 52 column equilibrated in 10 mM Tris-HCl, pH 8.0. The B chain did not bind and the column was washed with 150 mM NaCl/buffer (arrow 1) before eluting the A chain in 500 mM NaCl/buffer added at arrow 2. 5 ml fractions were collected and samples were removed for A_{280} and radioactivity determination. β-Mercaptoethanol contributed significantly to the A_{280} of material which did not bind to the columns.

ACKNOWLEDGEMENTS

We thank Croda Premier Oils, Hull, England, for kindly providing the castor bean seeds. This work was supported by the Science and Engineering Research Council via grant GR/D 66442 (to

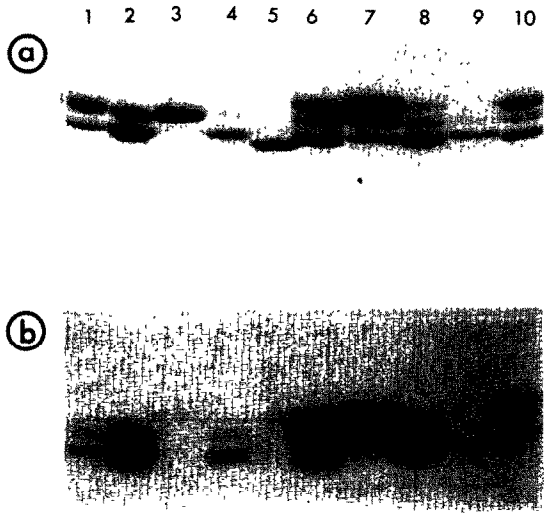

Fig.5. Ricin (lane 1) and its purified A and B subunits without (lanes 2 and 3) or after (lanes 4 and 5) endo H treatment and RCA (lane 10) and its purified A and B subunits without (lanes 6 and 7) or after (lanes 8 and 9) endo H treatment were analysed electrophoretically. (a) Coomassie blue stained gel; (b) fluorograph of (a).

J.M.L.) and by the National Science Foundation through a NATO Postdoctoral Fellowship (to S.M.H.).

REFERENCES

[1] Nicolson, G.L., Blaustein, J. and Etzler, M.E. (1974) Biochemistry 13, 196–204.
[2] Olsnes, S. and Pihl, A. (1982) in: Molecular Action of Toxins and Viruses (Cohen, P. and Van Heyningen, S. eds) pp.51–105, Elsevier, Amsterdam, New York.
[3] Cawley, D.B., Hedblom, M.L. and Houston, L.L. (1978) Arch. Biochem. Biophys. 190, 744–755.
[4] Olsnes, S., Saltvedt, E. and Pihl, A. (1974) J. Biol. Chem. 249, 803–810.
[5] Foxwell, B.M.J., Donovan, T.A., Thorpe, P.E. and Wilson, G. (1985) Biochim. Biophys. Acta, in press.
[6] Butterworth, A.G. and Lord, J.M. (1983) Eur. J. Biochem. 137, 57–65.
[7] Lamb, F.I., Roberts, L.M. and Lord, J.M. (1985) Eur. J. Biochem. 148, 265–270.
[8] Roberts, L.M., Lamb, F.I., Pappin, D.J.C. and Lord, J.M. (1985) J. Biol. Chem., submitted.
[9] Roberts, L.M. and Lord, J.M. (1981) Planta 152, 420–427.
[10] Lord, J.M. (1985) Eur. J. Biochem. 146, 411–416.
[11] Saltvedt, E. (1976) Biochim. Biophys. Acta 451, 536–548.
[12] Olsnes, S. and Pihl, A. (1973) Biochemistry 12, 3121–3126.
[13] Roberts, L.M. and Lord, J.M. (1981) Eur. J. Biochem. 119, 31–41.
[14] Bonner, W.M. and Lasky, R.A. (1974) Eur. J. Biochem. 46, 83–88.