Enzymic removal of two oligosaccharide chains from ricin B-chain

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Peptide:*N*-glycosidase F removed both the asparagine-linked oligosaccharide chains of ricin B-chain in the absence of lactose. In the presence of lactose, which binds specifically to the B-chain, only one oligosaccharide chain was removed. Lactose also protected *Ricinus communis* agglutinin B-chain against the removal of one of the two susceptible oligosaccharides present in each B-chain subunit.

Ricin *Agglutinin* *Ricin B-chain* *Peptide:N-glycosidase F* *Deglycosylation*

1. INTRODUCTION

Ricin, the cytotoxic protein from the castor bean, *Ricinus communis*, is a heterodimer of two glycosylated subunits, the A- and B-chains, linked by a single disulphide bond. The A-chain (apparent molecular mass 32 kDa) catalytically inactivates the 60 S subunit of eukaryotic ribosomes. The B-chain (34 kDa) contains two saccharide-binding sites by which the toxin binds to galactose-terminating oligosaccharides present on the cell surface. The B-chain also appears to facilitate the transfer of the A-chain from the plasma membrane to its site of action in the cytosol [1,2]. The feasibility of targeting the A- and B-chains to specific cell types in animals by linkage to monoclonal antibody has been demonstrated in many model systems [3,4]. However, a problem with in vivo targeting has been that the immunotoxins are cleared rapidly from the bloodstream. This is because both the A- and B-chain contain oligosaccharides terminating in mannose residues which are recognised by receptors on the Kupffer cells of the reticuloendothelial system. This recognition can be abolished by chemically modifying the mannose residues [5,6] or by removing them with glycosidases [7,8].

Ricin B-chain (262 amino acid residues) contains two oligomannose-type oligosaccharide chains [9–11] linked to Asn^{95} and Asn^{135}. Foxwell et al. [7] demonstrated that one of the two oligosaccharide chains in B-chain could be removed by endo H or endo F, which cleave between the *N*-acetylglucosamine residues of the *N,N'*-diacetylchitobiose core structure adjacent to the Asn residue, whereas both were removable after denaturation of the protein with SDS. In contrast with ricin B-chain, *R. communis* agglutinin (RCA) B-chain has two oligosaccharide chains which are susceptible to endo H and also a third fucosylated chain which is not cleaved by the enzyme [12].

Commercial preparations of endo F contain a second endoglycosidase, PNGase F, which cleaves the asparaginyl-*N*-acetylglucosamine bond in *N*-linked oligosaccharides but which has a different pH optimum from endo F [13]. We have investigated the effect of digesting ricin B-chain with endo F/PNGase F in buffers of different pH in the

Abbreviations: endo H, endo-β-*N*-acetylglucosaminidase H; endo F, endo-β-*N*-acetylglucosaminidase F; PNGase F, peptide:*N*-glycosidase F; $PhMeSO_2F$, phenylmethylsulphonyl fluoride

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presence or absence of lactose. Both oligosaccharide chains were removed from the B-chain without prior denaturation by SDS when the digestion was performed in the absence of lactose. In the presence of lactose, only one of the oligosaccharide chains could be removed with the enzymes. Two oligosaccharide chains of RCA B-chain were likewise removed by endo F/PNGase F in an identical lactose-inhibitable manner.

2. MATERIALS AND METHODS

Ricin was purified from castor bean cake of Kenyan origin (Croda Premier Oils, Hull, England) as described [7]. Ricin B-chain was isolated by reducing the toxin on propionic acid-treated Sepharose 4B (Pharmacia, Milton Keynes, England) as described by Fulton et al. [14]. It was dialysed extensively against phosphate-buffered saline (PBS) and used without further purification.

Endo F (from *Flavobacterium meningosepticum*) was purchased from New England Research Products, Stevenage, England. This product is reported to contain 138 mU/ml endo F activity and 240 mU/ml PNGase F activity as assayed by the method of Tarentino et al. [15].

Native ricin B-chain was digested at 37°C by adding 1 μl endo F/PNGase F to 50 μl of a 0.5 mg/ml solution of B-chain in 50 mM sodium citrate (pH 5.5), 50 mM sodium phosphate (pH 7.0) and 50 mM Tris-HCl or 50 mM sodium borate (pH 9.0) in the presence or absence of 0.2 M lactose. Denatured B-chain was prepared by heating the protein solution at 95°C for 3 min in the presence of 0.1% SDS and 0.1% 2-mercaptoethanol. Digestion was performed at 37°C in buffer containing 1% Nonidet P-40.

SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli [16] and protein bands were visualised by Coomassie blue staining.

3. RESULTS

When ricin B-chain was digested with endo F/PNGase F at pH 5.5, SDS-polyacrylamide gel electrophoresis revealed the presence of three protein bands (fig.1, lane 2). One of the bands corresponded to undigested B-chain (34 kDa). The other two bands had apparent molecular masses of

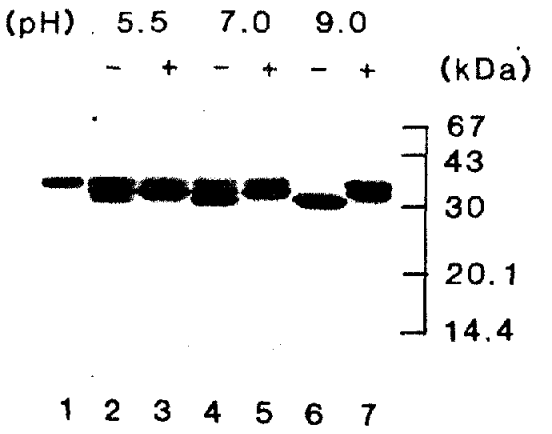

Fig.1. Digestion (48 h) of ricin B-chain (lane 1) with endo F/PNGase F at pH 5.5, 7.0 and 9.0 in the absence (lanes 2,4,6 respectively) or presence (lanes 3,5,7) of 0.2 M lactose.

approx. 32.5 and 31 kDa corresponding to B-chain from which one and two oligosaccharide chains respectively had been removed. Only a single band (31 kDa) was present after the digestion of ricin B-chain at pH 9.0 (fig.1, lane 6). This modified ricin B-chain was unable to bind to Sepharose-immobilised concanavalin A, which recognises mannose-containing glycoproteins even after denaturation by SDS confirming that both high-mannose oligosaccharide chains had been removed. When the digestion was performed in the presence of 0.2 M lactose, only one oligosaccharide chain was efficiently removed from ricin B-chain (fig.1, lanes 3,5,7) and the modified protein bound to concanavalin A-Sepharose. In this case, the digestion was apparently most efficient at pH 5.5. Lactose also protected RCA B-chain against removal of one of the two susceptible oligosaccharide chains per B-chain subunit (fig.2).

Once ricin B-chain had been denatured by SDS, both oligosaccharide chains were susceptible to endo F/PNGase F at pH 7.0 regardless of the presence or absence of lactose (fig.3, lanes 1,2). However, extensive proteolysis occurred at pH 9.0 since no protein bands >18 kDa were visible after digestion (fig.3, lanes 3,4). This proteolysis was not inhibited if digestion was performed in the presence of 1 mM $PhMeSO_2F$. The excision of oligosaccharides from ricin B-chain cannot be due

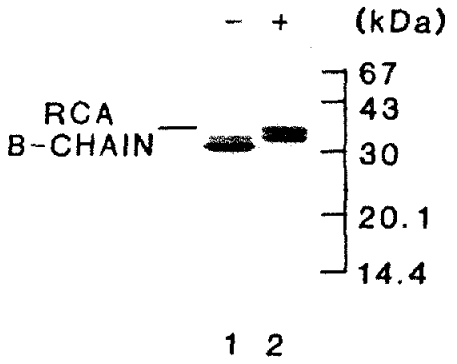

Fig.2. Digestion (24 h) of RCA B-chain with PNGase F at pH 9.0 in the absence (lane 1) or presence (lane 2) of 0.2 M lactose.

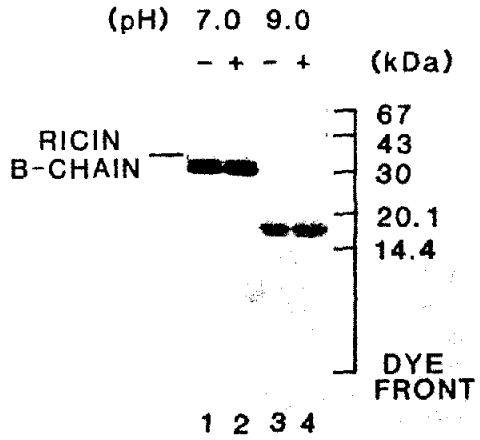

Fig.3. Digestion (24 h) of SDS-denatured ricin B-chain with endo F/PNGase F at pH 7.0 and pH 9.0 in the absence (lanes 1,3 respectively) or presence (lanes 2,4) of 0.2 M lactose. Samples were run on an 18% polyacrylamide gel.

to limited proteolysis since the oligosaccharide chains are attached to Asn residues located near to the middle of the B-chain primary structure and not near the N- or C-terminus.

A time course of digestion of ricin B-chain at pH 9.0 showed the stepwise removal of oligosaccharide chains with time (fig.4, lanes 2–4). In the presence of 0.2 M lactose, one oligosaccharide chain was completely protected against removal and the other chain was cleaved more slowly (fig.4, lanes 5–7). Ricin B-chain isolated from ricin which

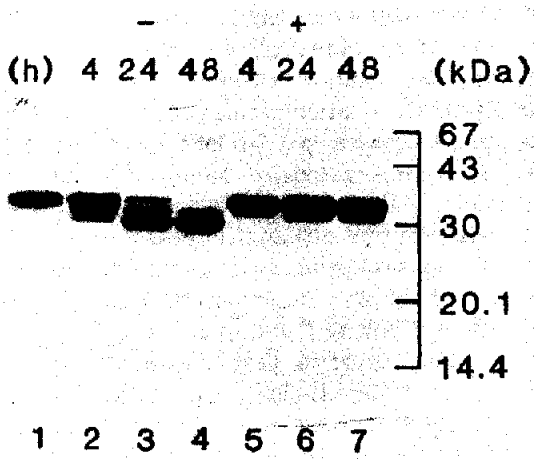

Fig.4. Incubation of ricin B-chain at 37°C without enzyme (48 h, lane 1) or with PNGase F in 50 mM sodium borate, pH 9.0 in the absence (lanes 2–4) or presence (lanes 5–7) of 0.2 M lactose. Samples were removed after 4, 24 or 48 h of digestion.

had been treated with a mixture of sodium metaperiodate and sodium cyanoborohydride to modify mannose residues in the oligosaccharide chains [5] was deglycosylated in an identical lactose-inhibitable fashion (not shown). At the end of 48 h digestion at pH 9.0 and 37°C, the fully deglycosylated ricin B-chain was soluble, but the protein aggregated irreversibly during dialysis into PBS. The presence of 0.2 M lactose during digestion prevented aggregation of the B-chain in PBS.

4. DISCUSSION

The two Asn-linked oligosaccharide chains of ricin B-chain can both be removed by digestion with an enzyme preparation containing endo F and PNGase F. The pH optima of these two enzymes are widely separated so that endo F is predominantly active at low pH whereas PNGase F predominates at high pH [13]. Both oligosaccharide chains were susceptible to digestion at pH values between 5.5 and 9.0 suggesting that each chain may be removed by both enzymes. Lactose completely protected one oligosaccharide against removal at pH between 5.5 and 9.0 and significantly reduced the cleavage of the other chain. In agreement with the results of Foxwell et al. [7], SDS denaturation of the B-chain, which inac-

tivates lactose binding, abolished the protective effect of lactose. However, in contrast with native B-chain, the denatured protein was susceptible to the action of a contaminating peptidase which was apparently active at pH 9.0 but not pH 7.0. RCA B-chain shares extensive amino acid sequence homology with ricin B-chain including two Asn glycosylation sites at identical positions [16]. One of the two susceptible oligosaccharide chains of RCA B-chain was protected by lactose against removal by PNGase F in similar fashion to ricin B-chain. This suggests that the non-fucosylated chains of RCA B-chain are located at the glycosylation sites common to both B-chains. Furthermore, the results suggest that the binding of lactose to ricin B-chain and to RCA B-chain helps to maintain a protein conformation in which the *N,N'*-diacetylchitobiose core of one oligosaccharide chain is protected by the polypeptide backbone.

Ricin B-chain that had been deglycosylated at high pH in the absence of lactose differed from native B-chain in two important respects. First, consistent with complete removal of both oligosaccharide chains, it was unable to bind to concanavalin A. Since all the mannose residues have been removed, this enzymically deglycosylated B-chain will not be recognised in animals by the carbohydrate receptors of the reticuloendothelial system. Secondly, deglycosylation promoted aggregation of ricin B-chain. Complete removal of both the oligosaccharide chains may expose surface regions of the B-chain polypeptide which are normally shielded from the solvent and which induce aggregation under physiological conditions. Alternatively, since cleavage with PNGase F converts the oligosaccharide substituted asparagine residues into aspartic acid residues [13], the resulting change in the p*I* of the modified protein could affect its pH stability. It may prove possible to prevent aggregation from occurring by attaching the B-chain to a monoclonal antibody or by chemically modifying the B-chain to improve solubility.

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