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Ricin B chain fragments expressed in *Escherichia coli* are able to bind free galactose in contrast to the full length polypeptide

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Deleted forms of ricin B chain (RTB) containing only one of the two galactose binding sites were produced in *E. coli* and targeted to the periplasm by fusion to the *omp*A or *omp*F signal sequences. The proteins were then isolated from the periplasm and their sugar binding properties assessed. Previous studies investigating the properties of such proteins produced in *Xenopus laevis* oocytes suggested that deleted forms of RTB, when not glycosylated, retain their ability to bind simple sugars, unlike the full-length unglycosylated proteins. When produced in *E. coli* however we found that only one, EB733, of a number of deleted forms of RTB closely related to those previously produced in *Xenopus laevis* oocytes, bound to simple sugars. All of the deletion forms of RTB were found to bind in the asialofetuin binding assay; an assay which has been previously utilized to measure binding of lectins to the terminal galactose residues of glycoprotein oligosaccharides. However, in contrast to glycosylated RTB, binding of the deletion mutants could be competed to only a small degree or not at all with galactose. The only deletion mutant observed to bind to free galactose when produced in *E. coli* corresponded closely to the complete domain 2 of RTB. It is assumed that this mutant forms a stable structure similar to that of the C-terminal domain in the full-length protein. The structural integrity of EB733 was not only suggested by its sugar binding properties and solubility but also by its consistently higher level of expression and the absence of any apparent susceptibility to *E. coli* proteases.

Keywords: ricin, ricin B chain, lectin, *E. coli* expression

Abbreviations: RTA, ricin toxin A chain; RTB, ricin toxin B chain; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside.

Introduction

The cytotoxic plant lectin ricin is composed of two disulphide-linked polypeptides; ricin A chain (RTA) and ricin B chain (RTB). The toxin is initially synthesized in the castor oil seed as a preproprotein and the proricin, formed after cleavage of a signal sequence in the endoplasmic reticulum (ER), is targeted to Golgi-derived protein storage vesicles where cleavage of a central linker polypeptide takes place to create the mature A and B chains. RTA is responsible for the intracellular toxic effect on mammalian cells where it exhibits RNA-specific *N*-glycosidase activity towards the 28S rRNA in 80S ribosomes. This leads to an inhibition of protein synthesis in sensitive cells. RTB is a galactose specific lectin which in addition to facilitating the initial obligatory cell attachment appears to have a second intracellular role in the routeing of RTA to, and/or translocation of RTA from, the compartment in which transfer of RTA to the cytosol occurs. This second potentiating function has recently been shown to involve the galactose binding sites [1], but their precise role in this process is as yet unclear.

RTB is thought to be the product of a gene duplication event resulting in two closely related globular domains [2]. Analysis of the crystal structure of these domains [3, 4] reveals that they in turn are each composed of three structurally similar subdomains α, β and γ, each derived from a primordial galactose binding peptide. However, of the six subdomains present in RTB, only two, 1α amd 2γ,

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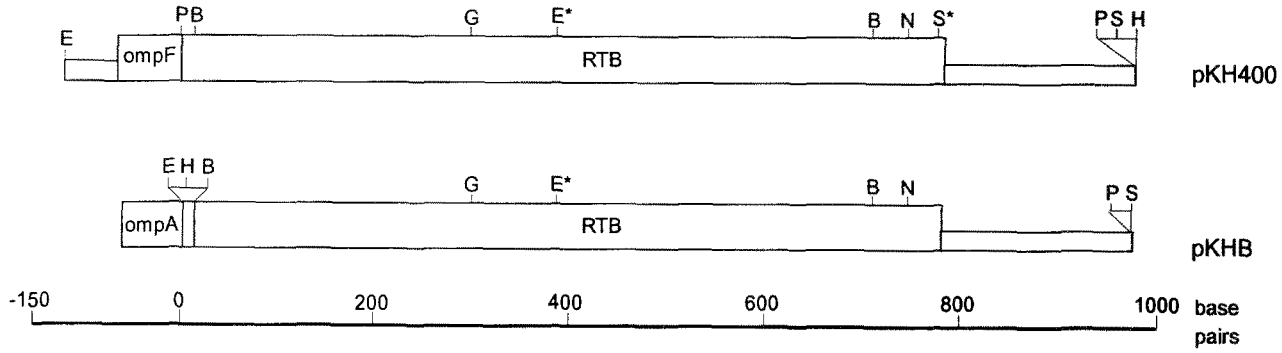
have retained their ability to bind to galactose. Mutational and deletion analysis using RTB produced in a *Xenopus* oocyte system has shown that these two galactose binding sites can function independently [5]. The two binding sites differ slightly in their specificity and affinities for sugars; the binding site on subdomain 1α being specific for galactose while the 2γ binding site will bind both galactose and *N*-acetylgalactosamine. Also there are reports that the affinity of the 2γ site for lactose is greater that that of the 1α site. The affinity of both sites for complex sugars like those found on the normal substrate for binding, i.e. cell surface glycolipids and glycoproteins, is much greater than for simple sugars [6, 7].

RTB in its native form is a glycoprotein carrying two asparagine-linked carbohydrate groups. Previous reports [5, 8] have shown that in the absence of these carbohydrates and the RTA subunit, RTB rapidly aggregates and becomes insoluble. This is thought to explain the reported difficulties in the expression of RTB in *E. coli* at high levels [9]. Recently, RTB has been dissected into an amino-terminal galactose binding domain (RTB residues 1–143) and a carboxy-terminal galactose binding domain (residues 82–262). These single binding domains were expressed as fusion proteins on the surface of *fd* bacteriophage [10]. Both single-binding domains retained the ability to bind complex galactosides (asialofetuin) in a manner that could be at least partially competed with lactose, showing that *N*-glycosylation was not a prerequisite for the correct folding of these truncated RTB fusion polypeptides into a biologically active conformation. We have also reported the expression of single binding domains of RTB in *Xenopus* oocytes [11]. Both glycosylated and non-glycosylated products were formed, all of which were stable and possessed galactose binding activity. Taken together these data suggest that single binding domains of RTB might be intrinsically more stable in a non-glycosylated form than the full length RTB molecule. It was not, however, clear whether this phenomenon was a product of the expression environment or an intrinsic feature of the proteins themselves.

In this preliminary report we describe the expression of deletion mutants of RTB in *E. coli* and examine their ability to bind both complex and simple sugars.

Materials and methods

Plasmid construction. All the constructs described in this work are based upon two plasmids, pKH400 and pKHB (Fig. 1a), and the numbers used in the description of their construction refer to either the first nucleotide of the DNA



(a)

5'-CCCAGATCTGAGCGGATAACAATTTAACAGGAAACAGCTTATGATGAAGCG
*Bgl*II SD

CAATATTCTGGCAGTGATCGTCCCTGCTCTG-3'
3'-TAGCAGGGACGAGACAATCATCGTCCATGACGTTT

GCGACGTCTACAAACATACCTAGGATTTAAACAGCTGCCCTTCGAACCC-5'
»»»» *Bam*HI *Sal*I *Hin*DIII

(b)

Figure 1. (a) Structure of the constructs pKH400 and pKHB, Nucleotides are numbered with the first nucleotide of the DNA encoding the mature RTB taken as 1. Restriction enzyme sites relevant to the cloning strategies used in the creation of the deletion mutants are indicated; B, *Bam* HI; E, *Eco* RI; G, *Bgl* III; H, *Hind* III; N, *Nco* I; P, *PstI* I; S, *Sal* I. (b) Sequence of the oligonucleotides used to create SG2. The restriction endonuclease sites, Shine Dalgarno sequence (SD) and start of the mature RTB (> > > > > >) are indicated. The initiator codon at the start of the *omp*F signal is shown in boldface.

sequence encoding the mature RTB, or the first amino acid of mature RTB. The first of these plasmids, pKHB, has been described previously [11], described by the authors as pIN-III-ompA3RB) and encodes the full length RTB although the first five amino acids including the cysteine normally invovlved in forming a disulphide bridge to RTA differ from the wild type. The second source plasmid from which the deletion mutants of RTB are derived is pKH400. The initial aim of this construct was to create a wild type RTB with a correct *N*-terminus.

pkH400: The expression vector pKK223.3 (12, Pharmacia Biotech Ltd) was cut with *Eco* RI and *Hind* III and a stuffer fragment cloned in between these sites to create pKH10. Following this, pKH10 was cut with *Bam* HI and *Sal* I, and after removal of the overhangs was self-ligated to create pKH50. Two oligonucleotides were designed such that when annealed and extended to create dsDNA they encoded a Shine Dalgarno sequence followed by DNA encoding the *omp*F signal sequence [13] fused to the *N*-terminus of RTB (Fig. 1b). The oligonucleotides also encoded a series of restriction sites to facilitate further cloning steps. The dsDNA fragment (SG2) resulting from the extended oligonucleotides was restricted with *Bgl* II and *Hind* III and cloned into M13mp 19 digested with similar enzymes. This clone, M13SG2, was then used to create single stranded DNA and sequenced to check the structure of the 'synthetic gene' created. Following this, M12SG2 was cut with *Eco* RI and *Hind* III and the SG2-containing fragment cloned into the *Eco* RI and *Hind* III sites of pKH50, replacing the stuffer fragment and creating pKH200. The remainder of the RTB encoding DNA was incorporated by a cloning a 1229bp *Bam* HI-*Sal* I fragment encoding RTB from Asp6 to the C-terminus, derived from a partial digest of pKHB into the *Bam* HI and *Sal* I sites of pKH200 thus creating the construct pKH400.

Construction of deletion mutants: To provide extra restriction sites within the RTB-encoding DNA sequence the *Eco* RI-*Sal* I fragment of pKHB encoding this region was cloned into M13mp19 to create mp19KH. Oligonucleotide directed mutagenesis was carried out on this plasmid to introduce into the RTB DNA sequence an *Eco* RI site (nt 389–394), and a *Sal* I site (nt 780–785) immediately prior to the natural RTB termination codon, thus creating mp19KHM5. Also constructed were serially modified versions of pKH400. The *Eco* RI site upstream of the *omp*F–RTB gene was removed by cleavage with *Eco* RI followed by S1 nuclease treatment and self ligation to create pKH400E, from which *Sal* I site downstream of the RTB gene removed by cleavage with *Sal* I, treatment with Klenow fragment and self ligation, resulting in pKH400ES. The *Bgl* II-*Hind* III fragment of mp19KHM5 encoding the C-terminus of RTB was then used to substitute the analogous fragment in pKH400ES to create pKH400ESM5.

Plasmid pEB701 was created by cleavage of pKH400ESM5 with *Nco* I, treatment with S1 nuclease, followed by cleavage with *Sal* I, end-filling with Klenow fragment and subsequent self ligation. The domain I-encoding plasmid pEB712 was also derived from pKH400ESM5, by cleavage with *Eco* RI and *Sal* I, end filling with Klenow fragment and self ligation. The final plasmid derived from pKH400ESM5 was pEB723. Here pKH400ESM5 was restricted with *Bgl* II followed by partial end-filling through the use of Klenow fragment in the presence of only dGTP and dATP and subsequent digestion with S1 nuclease. This was followed by cleavage with *Sal* I, end filling with Klenow fragment and subsequent self-ligation.

Plasmid pEB733 was derived from a modified form of pKHB in which the *Bgl* II-*Nco* I fragment of the RTB encoding region of pKHB was replaced by that of mp19KHM5 to create pKHBM5. Cleavage of pKHBM5 with *Eco* RI followed by self ligation in the absence of the RTB domain I-encoding *Eco* RI fragment then led to the creation of pEB733.

All junction regions of the deletion mutants were checked by DNA sequencing to ensure the correct structure prior to further analysis.

Expression of constructs in E. coli. *E. coli* strain TG2 (Δ(*lac-pro*), *sup*E, *thi*, *hsdD5*, *F'tra*D*36*, *proAB*, *LacIq*, *lacZ*Δ *M15*) [14] was used for all the expression studies in this work and the medium used in all experiments was M9 medium [15] supplemented with 1% casamino acids and 100 μg ml^{-1} ampicillin. Overnight cultures were used to inoculate 500 ml of media to give an initial OD_{600} (Shimadzu UV-160A spectrophotometer) of 0.1 and these cultures shaken at 250 rpm at 37 °C until at OD_{600} of approximately 0.6 was reached. At this point cultures were transferred to a temperature of 18°C and when the OD_{600} reached 0.7–0.8 expression was induced by the addition of IPTG to a final concentration of 1 mM. Growth was then allowed to continue overnight at 18 °C. Cells were harvested by centrifugation at 1000 × **g** for 30 min at 4 °C and this was followed by the isolation of the periplasm fraction as described previously [9]. Periplasm fractions were stored at 4 °C with no addition other than sodium azide to 0.02%. The total protein content of the periplasm extracts was determined using the Bio-Rad protein assay system.

Solubility of RTB mutants. This was examined by the centrifugation of periplasmic extracts at 108 000 × **g** for 1 h followed by harvesting and recentrifugation of the supernatant fraction, resuspension of the pellet in 1 mM Tris-HCl pH 7.5 and subsequent recentrifugation. The respun supernatant fraction and washed pellet were then taken to represent the soluble and insoluble fractions respectively and the distribution of RTB related proteins between these fractions was determined by SDS-PAGE, Western blotting and immunodetection using sheep-derived RTB-specific antisera.

Sugar binding activity. Binding to complex sugars was determined using the asialofetuin plate assay described previously [16] with modifications according to Wales *et al.* [5]. Binding to lactose was assessed using 0.5 ml columns of lactose immobilized on to polyacrylamide (SeLectin, Pierce Co) as described previously [5]. Elution of bound polypeptides from the columns was achieved with 50 mM galactose in 1 mM Tris-HCl pH7.5 and the distribution of RTB-related proteins between fractions determined as for the solubility assay.

Miscellaneous. All DNA manipulations and purification, SDS-PAGE and Western blotting was carried out by standard techniques. The detection of sheep anti-RTB antibodies bound to proteins immobilized on nitrocellulose filters was carried out using donkey anti-sheep antibodies conjugated to alkaline phosphatase (Sigma). Site directed mutagenesis was carried out using an 'Oligonucleotide directed *in vitro* mutagenesis kit' (Amersham) and all DNA fragments subjected to the procedure were sequenced prior to subsequent cloning.

Results

Plasmids encoding a number of deleted forms of RTB were constructed to facilitate the production of these mutant proteins in *E. coli* (Fig. 2). The constructs, based upon two previously described expression vectors pKK223.3 [12] and pIN-III-*ompA*3 [17], were used to express the RTB mutants in *E. coli* strain TG2 and the expressed proteins were targeted to the periplasmic space by either the *ompA* or the *ompF* signal sequences. A periplasm fraction was prepared by an osmotic shock procedure and any RTB-related proteins identified by SDS-PAGE followed by Western blotting and probing of the blot with RTB specific antisera.

The solubility and stability of the RTB fragments was examined by high speed centrifugation followed by the location of the RTB related proteins to the supernatant or pellet fractions using SDS-PAGE and Western blotting. All the forms of RTB produced in this work were found to be in a soluble form and remained so during storage at 4 °C for at least 17 days (Fig. 3). It can be observed that expression of a number of the constructs results in the production of more than one form of RTB. In the case of pKH400 and pKHB, the two largest polypeptides are thought to represent mature RTB plus the preRTB form which has retained its bacterial periplasm targeting sequence. A similar situation is found for the protein produced using the pEB701 construct, which lacks the C-terminal 12 amino acids. Further smaller products observed from the expression of these three constructs are thought to be the result of proteolytic degradation, as the use of protease inhibitors was deliberately avoided in these studies. In some cases the constructs encoding smaller forms of RTB expressed here, also gave rise to a number of products. Once again these are thought to be the result of proteolytic degradation and incomplete signal cleavage. The relative amounts of the various RTB forms obtained varied between preparations as can be seen from the two time points used in Fig. 3, which were from two separate periplasm preparations.

Examination of the ability of the complete and deleted forms of RTB produced in this work to bind to complex glycoproteins was studied using the asialofetuin binding assay. Equal amounts of periplasm (total protein) from *E. coli* expressing the various constructs were loaded into microtitre plate wells previously coated with the glycoprotein asialofetuin; the carbohydrate groups of which terminate in galactose residues. The amount of bound RTB-related protein was then determined using sheep

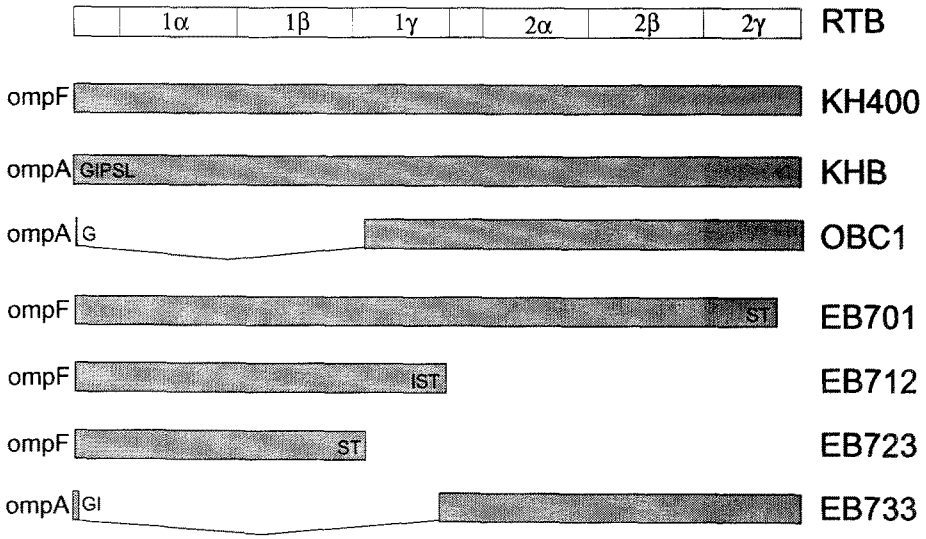


Figure 2. Forms of RTB produced from the clones indicated and their relationship to the wild-type mature RTB. The signal sequence used in each case is indicated and also any amino acid changes at the termini of the proteins introduced by the cloning procedures.

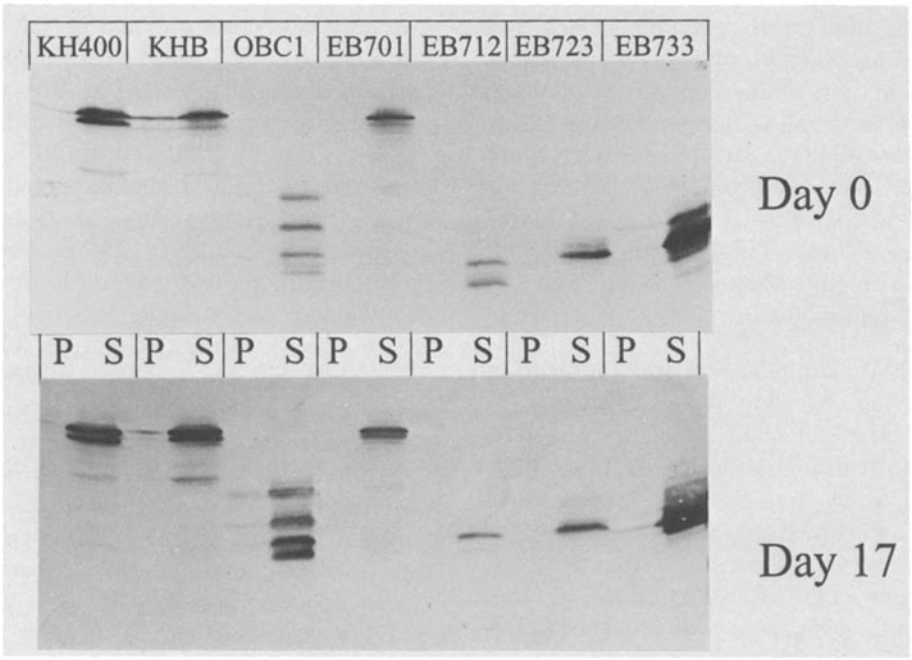


Figure 3. Solubility of the recombinant forms of RTB expressed in *E. coli*, immediately after preparation of the periplasm extract or after storage at 4 °C for 17 days. Equal volumes of the periplasm extracts (100 µl) were centrifuged at 108 000 × **g** for 1 h followed by separation of the pellet and supernatant fractions. The supernatant fraction and the resuspended pellet were then centrifuged for a further 1 h at 108 000 × **g**. The recentrifuged supernatant and washed pellet were then subjected to SDS-PAGE and Western blotting and the RTB-related proteins detected using antibodies against RTB. P, pellet fraction; S, supernatant fraction.

antisera specific for RTB and ^{125}I-labelled protein G. Periplasm preparations from *E. coli* expressing the constructs described here were all found to contain RTB-related proteins that bound to asialofetuin (Fig. 4) although these same proteins did not bind to fetuin (data not shown). In competition studies, the binding of 10 ng per well of plant-derived RTB was inhibited by 80% in the presence of 50 mM galactose whereas decreased competition was observed when full-length *E. coli*-produced (non-glycosylated) KH400 (decreased by up to 40%) or KHB (decreased by 30%) were used in the assay. Galactose competition decreased binding to asialofetuin of the C-terminal half of RTB encoded by EB733 by 30% (results not shown). No competition for the binding to asialofetuin was observed at all with the other RTB deletion mutants.

The binding of the various forms of RTB to simple sugars was also analysed. Equal amounts of periplasm extract (total protein) were passed several times through a 0.5 ml column of lactose immobilized on to polyacrylamide, and after washing with 3 ml of buffer the bound proteins were eluted with 50 mM galactose. Samples from the flow through were dot blotted on to a nitrocellulose filter, undiluted and also at dilutions of 0.1 and 0.01, as were equivalent amounts of the galactose eluate. The nitrocellulose filter was blocked and then probed with RTB-specific antisera. It can be seen (Fig. 5) that despite the universal binding to asialofetuin, only two of the constructs produce proteins that bind to lactose; binding being observed by the RTB forms in the

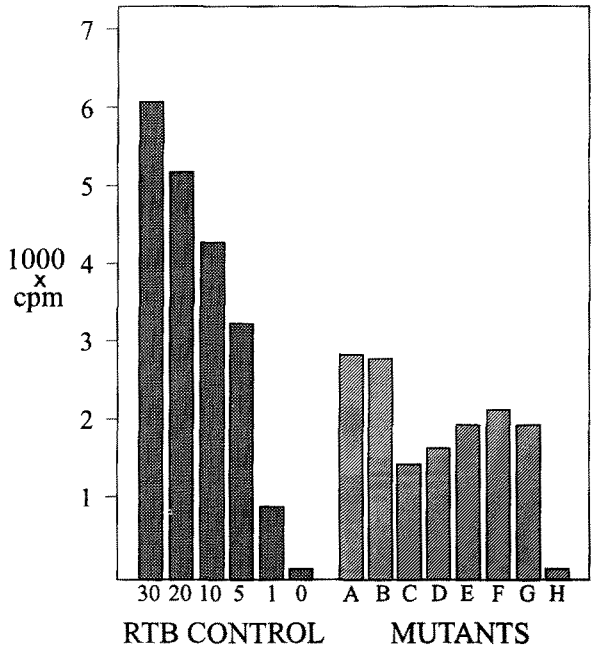


Figure 4. Binding of RTB deletion mutants to asialofetuin. Equal amounts of periplasm-derived protein (200 µg) from *E. coli* TG2, expressing proteins encoded by the indicated plasmids, or purified plant RTB, was added to microtitre plates wells coated with asialofetuin. Bound RTB-related proteins were detected using sheep antibodies against RTB and ^{125}I-protein G. The amounts of control RTB added are indicated in ng per well. All results are the mean of triplicate samples. A, pKH400; B, pKHB; C, pEB701; D, pEB712; E, pEB723; F, pEB733; G, pOBC1; H, vector pKK233.3.

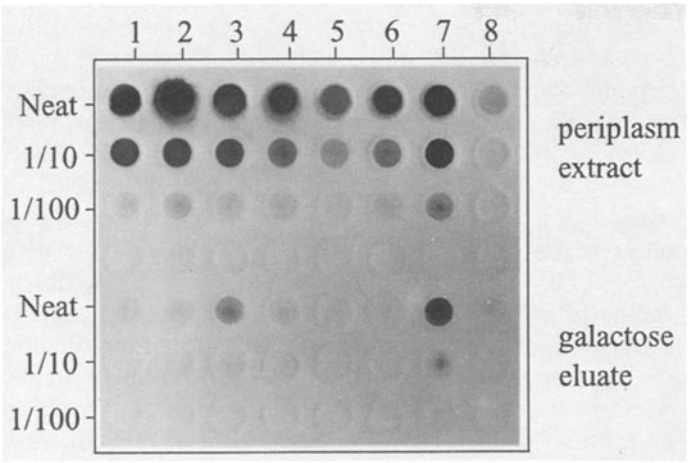


Figure 5. Ability of recombinant wild-type RTB and deletion mutants to bind to immobilized lactose. 400 µl of periplasm extract (840 µg protein) prepared from *E. coli* TG2, expressing proteins encoded on the indicated plasmids, were dot-blotted on to nitrocellulose membrane along with similar volumes after dilution to 1:10 and 1:100. Equivalent amounts of the lactose eluate from an immobilized lactose column through which the extract had been passed were also blotted. RTB-related proteins were then detected using antibodies against RTB and an alkaline phosphatase-linked second antibody. 1, pKH400; 2, pKHB; 3, pOBC1; 4, pEB701; 5, pEB712; 6, pEB723; 7, pEB733; 8, vector pKK223.3.

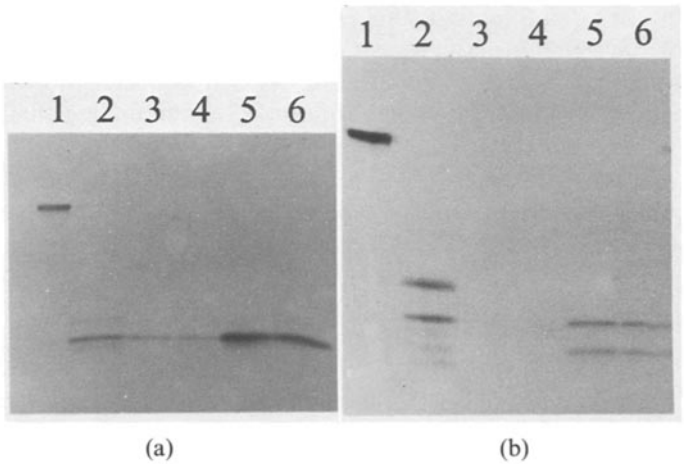


Figure 6. Binding of EB733 (a) or OBC1 (b) to immobilized lactose. Periplasm extracts from *E. coli* TG2 expressing EB733 or OBC1 were passed through an immobilized lactose column and the column washed three times with 1-ml of PBS. Bound proteins were then eluted with two 0.5 ml washes of 10 mM Tris-HCl pH7.5 with 50 mM galactose. 100 µl of each fraction was then subjected to SDS-PAGE and Western blotting the RTB-related proteins detected using antibodies against RTB and an alkaline phosphatase-linked second antibody. 1, wt RTB; 2–4 10 mM Tris-HCl pH7.5 washes; 5 and 6, 10 mM Tris-HCl pH7.5 with 50 mM galactose washes.

periplasm extracts of *E. coli* transformed with pEB733, and to a lesser degree pOBC1. To investigate this binding, a further 1 ml of the periplasm extracts from *E. coli* transformed with pOBC1 or pEB733 was passed through immobilized lactose columns as described above. The washes from the columns and the eluate fractions were then subject to SDS-PAGE, and the proteins blotted on to nitrocellulose filters and the location of RTB-related polypeptides determined using RTB-specific antisera. The single main product of expression from pEB733 binds clearly and reversibly to lactose (Fig. 6a). However, the situation with pOBC1 is more complex (Fig. 6b). The largest form of RTB observed shows no binding to lactose whereas smaller forms, assumed to be the result of proteolytic degradation, do bind reversibly to lactose. Such a situation has been observed previously in certain preparations of the KH400 protein where no binding to immobilized lactose is observed by the full length RTB, but a proteolytic fragment of approximately half the size of RTB is seen to bind lactose (results not shown).

Discussion

The contrasting results obtained here with regard to the solubility of the full length KHB product compared with previous results showing insolubility of the same protein [9] are difficult to explain. One possibility is that it is related to the expression levels, which were approximately 100-fold lower in the preparations obtained here. Results of the sugar binding properties of the full length constructs do, however, agree with earlier work. They demonstrate that the previously observed lack of binding to immobilized lactose and the reduced ability of simple sugars to compete with complex carbohydrate groups for the binding sites of RTB encoded by pKHB [8] cannot be due to the mutated *N*-terminus of the mature KHB protein but appears to be a general property of any non-glycosylated full-length form of RTB.

The degree of binding to asialofetuin cannot be accurately quantified by comparison to that observed using the plant-derived RTB (control lanes of Fig. 4) for a number of reasons. First, the antisera were raised against plant-derived RTB which carries two carbohydrate groups. It is likely that a significant portion of the antibodies in this polyclonal sera are specific for such epitopes. Second, peptide epitopes present in the control RTB are likely to be absent in the deleted RTB forms. Although no precise quantitation can be derived from the asialofetuin binding, it is clear that the full-length and deleted forms of RTB produced in *E. coli* do retain the ability to interact with complex sugars. This is in agreement with previous studies on *E. coli* produced RTB using the clone described here as pKHB (previously pIN-III-ompA4RB) [8]. However, studies using a nonglycosylated form of RTB (in which the glycosylation sites had been removed by site-directed mutagenesis) produced in *Xenopus laevis* oocytes showed

it not to bind to asialofetuin. The reason for this difference is unclear but may be related to the amino acid changes resulting from the mutagenesis. Also in agreement with previous studies in our laboratory [8] is the finding that, unlike glycosylated RTB produced in *Xenopus laevis* oocytes or in plants [5, 17], the binding to asialofetuin of full length RTB produced in *E. coli* cannot be competed to the same degree with lactose.

The binding of the EB733 polypeptide to immobilized lactose agrees with the results observed for deleted forms of RTB produced in *Xenopus laevis* oocytes where the non-glycoslated 'half RTB' proteins were shown to bind simple sugars [11]. However, the results obtained with the other deleted forms of RTB observed in the present study are at odds with our previous work in *Xenopus laevis* oocytes. When produced in *Xenopus laevis* oocytes, products essentially analogous to the EB701. EB712 and OBC1 proteins were all observed to bind lactose. Although it is possible that slight differences between the constructs used in the oocyte work and those used here many be responsible, it seems more likely that the oocyte homogenate provides a more stable environment for the partly folded proteins. A ramification of this is that in *E. coli*, only those deletion mutants which contain all the sequences necessary to form compact, correctly folded domains result in functional lectins. Indeed this is what may have occurred fortuitously with EB733 utilized here. We may speculate that any regions of the polypeptide not intrinsically involved in the major domains that form and fold in the *E. coli* periplasm may be left exposed to proteolytic degradation; this being a possible interpretation of the fate of the OBC1 polypeptide. The ability of the EB733 protein, which correlates essentially to domain 2 of the ricin B chain, to form an almost native galactose binding conformation appears also to be reflected in the higher level of expression consistently observed.

There remains a possibility that the lack of lactose binding observed here by proteins carrying the domain 1 galactose binding site of RTB might be due to one or more features common to these constructs. First, all of these constructs, pE701, pEB712, pEB723, carry a Met76 to Ile mutation introduced to combat secondary initiation of translation [9]. Second pEB701 carries a Trp131 to Ile mutation. Initial experiments with the constructs encoding EB712 and EB701, in which these mutations have been converted back to encode wild type amino acids, show that the expressed proteins do not regain the ability to bind lactose (data not shown). Further, in support of the proposal that the stability and function of the deleted forms of RTB can be enhanced in *Xenopus laevis* oocyte homogenates, we have conducted preliminary experiments revealing that the protein BSTOP (domain 1 of RTB) does not bind to simple sugars when expressed in *E. coli* with the *ompF* signal (data not shown) though it clearly does so when expressed in oocytes [11].

Although there are a number of areas which have not been addressed in this study, such as confirmation of signal peptide cleavage, we feel a number of important points emerge. First, use of asialofetuin binding as a measure of the sugar binding activity of the recombinant forms of RTB here, is not by itself conclusive, especially when used in competition studies. Although asialofetuin binding is observed, its characteristics differ from the binding by native plant RTB. The fact that binding to asialofetuin can be competed with galactose has previously been considered to show that at least a portion of the protein is binding through its galactose binding sites [10]. However, if this were so here, one might also expect to see that same portion of protein binding to the immobilized lactose columns. Here, and in previous reports from our group [8], this has clearly not been the case. While full-length RTB produced in *E. coli* has been found to interact with asialofetuin and to be competed to approximately 40% with lactose, no binding to immobilized lactose has ever been observed. However, it is difficult to explain these data as merely a consequence of non-specific binding to asialofetuin. If binding to the complex oligosaccharides were truly non-specific, then we might also expect some interaction of non-glycosylated RTB with fetuin. This we did not observe (data now shown). We have not, however, used asialogalactofetuin to resolve this anomaly. The second point to emerge is that *E. coli* does appear able to provide a vehicle for the expression of RTB fragments with an almost native function in the absence of glycosylation, however the requirements imposed on the design of the construct are stringent. It remains to be seen if deleted forms of RTB such as EB733 can be expressed at high level in *E. coli* and whether, with more attention to the details of the construction, and further deleted forms with sugar binding ability can be produced.

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