A recombinant polypeptide, composed of the α-helical neck region and the carbohydrate recogniton domain of conglutinin, self-associates to give a functionally intact homotrimer

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Received 12 October 1995

Abstract **A recombinant polypeptide composed of the α-helical neck region and carbohydrate recognition domain (CRD) of bovine conglutinin was expressed in *Escherichia coli.* The recombinant protein formed inclusion bodies but could be solubilised using a denaturation-renaturation cycle based on urea and then purified by affinity chromatography on a TSK-*N*-acetylglucosamine column. The purified product behaved as a homotrimer in non-dissociating conditions, with three CRDs held together by the α-helical neck regions. The trimer, although lacking the N-terminal and collagen regions of the native conglutinin, showed the same binding carbohydrate specificities as the native molecule, for the complement fragment C3b and for lipopolysaccharides derived from Gram-negative bacteria.**

Key words: Collectin; Recombinant bovine conglutinin; Carbohydrate recognition domain; Lipopolysaccharide; C3 component of complement; C-type lectin

1. Introduction

The collectins are members of a group of oligomeric mammalian lectins composed of polypeptides with N-terminal collagen-like regions attached via α-helical neck regions to C-terminal, C-type, lectin domains. The collectins include the serum proteins, mannose-binding protein (MBP), bovine conglutinin (BK), collectin-43 (CL-43) and the lung surfactant proteins SP-A and SP-D [1]. Conglutinin, which was first described as a factor which agglutinates complement-reacted erythrocytes [2] and has only been fully characterised from bovine serum [3]. Conglutinin is composed of 12 identical chains of 44 kDa, each chain contains 351 amino acid residues and consists of a short N-terminal cysteine-containing region of 25 residues followed by a 171-residue-long collagenous region and then a short (28 residues) α-helical neck region which is connected to the, C-terminal, globular carbohydrate recognition domain (CRD) [4]. Alignment of the amino acid sequences of bovine conglutinin and bovine SP-D shows 78% identity between the two proteins [5,6], despite their quite different carbohydrate specificities. As judged from electron microscopy and protein chemistry studies, conglutinin is composed of four subunits radiating from a central hub. Each subunit being composed of a triple-helical, collagen-like, rod connected by an α-helical 'neck' region to three, C-terminal, C-type lectin domains [7].

Bovine conglutinin is able to mediate the agglutination of serum-reacted erythrocytes. The ligand recognised by conglutinin in this reaction has been shown to be iC3b derived from C3b deposited on the cells. The specific site on iC3b to which conglutinin binds is an exposed carbohydrate structure on the α′-chain of iC3b [8]. By use of carbohydrate ligands as inhibitors of agglutination of serum-reacted erythrocytes, it can be shown that conglutinin binds preferentially to terminal *N*-acetylglucosamine [9]. Conglutinin can also cause opsonisation by binding to iC3b on the surface of microorganisms [10] and It has been shown to bind, via its collagenous region, to the widely distributed C1q receptor on phagocytes and other cells [11].

In the present study, a polypeptide composed of the neck region plus the CRD of bovine conglutinin has been expressed in *Escherichia coli.* This polypeptide, which lacked both the N-terminal and collagenous regions of conglutinin, was found to form a homotrimer composed of the neck region and CRD domains of the protein. The trimeric recombinant material showed the same, calcium-dependent, sugar-binding specificity as the native protein and also the same affinity for lipopolysaccharides (LPS) from Gram-negative bacteria. Also, like native conglutinin, the recombinant material bound to C3b in a calcium- and saccharide-dependent fashion. These results are consistent with the previous hypothesis that the neck region in each of the collectins is essential for trimerisation of the three collagenous polypeptide chains and in maintenance of multivalency of the binding to microorganisms [12–14].

2. Materials and methods

2.1. DNA construct encoding the neck plus CRD domain of bovine conglutinin (recombinant BK-neck-CRD)

A cDNA clone for conglutinin, previously isolated from bovine liver λgt11 cDNA library (Cambridge Biosciences, Cambridge, UK) [3], was subcloned in Bluescript-plasmid and used as a template for polymerase chain reaction (1 μl/100 μl PCR). The two terminal primers, 5′-ATTAGGATCCGCAGAGGTCAATGCTCTCA-3′ (forward primer) and 5′-TGCTAGTTATTGCTCAGCGG-3′ (T7 primer) were used to amplify a cassette which encoded for the neck plus lectin domain of bovine conglutinin. The PCR product was cleaved with *Bam*H1 and *Eco*R1 and ligated to the *Bam*H1-*Eco*R1 cleaved backbone of a modified pET vector [15] and transformed into competent *E. coli* DH5α cells. The resultant recombinant plasmid, pBK was introduced into the expression host of *E. coli* BL21 (λDE3) which carries the chromosomally integrated T7 RNA polymerase gene under the control of the *lac* UV5 promoter

2.2. Expression and purification of the recombinant neck region plus lectin domain of bovine conglutinin (BK-neck-CRD)

E. coli BL21 (λDE3), containing plasmid pBK, was grown to an OD of 0.8 at 700 nm in 1 l LB medium in the presence of ampicillin (100 μg/ml) at 37°C, with vigorous aeration. IPTG (isopropyl-β-D-thiogalac-

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SSDI 0014-5793(95)01232-X

topyranoside, NOVA Biochem) was added to a final concentration of 0.4 mM to induce the T7 RNA polymerase gene in the host chromosome. The cells were shaken under these conditions for 3 h and harvested. The wet cell pellet was suspended in ice-cold lysis buffer [20 mM Tris-HCl/500 mM NaCl, pH 8.0, containing 0.25% (v/v) Tween 20, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, Poole, UK) and 200 μg/ml of lysozyme (Sigma) for 30 min and the lysate was then sonicated at 60 Hz for 2 min with an interval of 1 min (15 cycles) to shear the chromosomal DNA. After sonication and centrifugation at 16,000 rpm for 30 min, most of the expressed recombinant protein was present in the pellet. Therefore, a denaturing and refolding procedure was carried out on the cell pellet

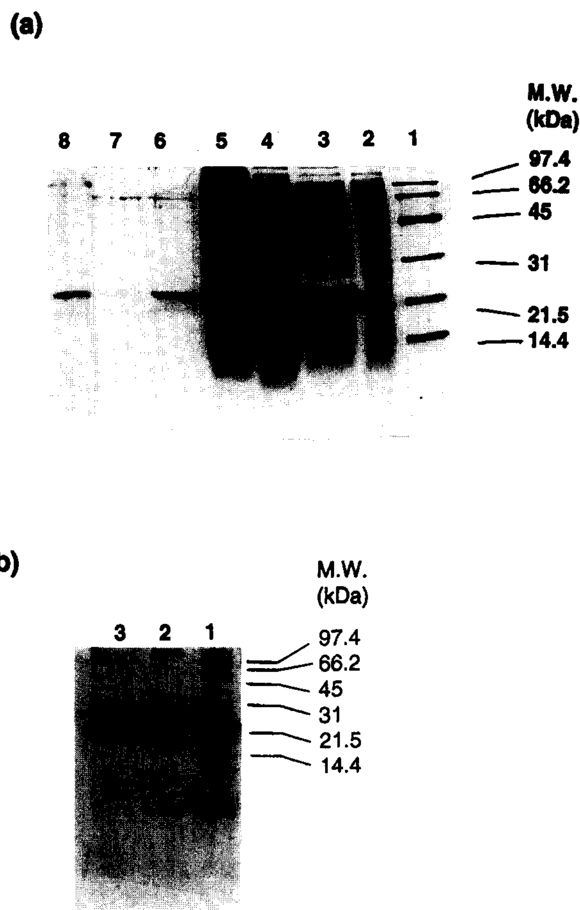

Fig. 1. (a) SDS-PAGE analysis in different steps of purification of the recombinant bovine conglutinin. Lane 1 contains molecular weight standards. From top to bottom are 97.4 kDa, 66.2 kDa, 45.0 kDa, 31.0 kDa, 21.5 kDa and 14.4 kDa. Lanes 2 to 7 show various steps in the purification: lane 2, total cell lysate without IPTG induction; lane 3, total cell lysate after IPTG induction; lane 4, insoluble fraction after centrifugation of the IPTG-induced and sonicated cell lysate; lane 5, final soluble fraction of cell lysate after denaturation and then refolding by dialysis against decreasing concentrations of urea; lane 6, the major peak eluted, with the NaCl gradient, from the DEAE-cellulose column; lane 7, recombinant protein eluted from the TSK-GluNac column by TBS-NTC buffer containing 10 mM *N*-acetylglucosamine; lane 8, recombinant protein eluted from TSK-GluNac column by TBS buffer containing 10 mM EDTA. All the samples were run on 15% (w/v) SDS-PAGE under reducing conditions. (b) Immunoblotting of recombinant BK-neck-CRD. Lane 1, the total cell lysate (10 μg/ml) after induction with IPTG; lane 2, recombinant protein eluted from the TSK-GluNac column by TBS-NTC buffer containing 10 mM *N*-acetylglucosamine; lane 3, recombinant protein eluted from the TSK-GluNac column by TBS buffer containing 10 mM EDTA. Samples were transferred onto a Hybond-C membrane. The blot was probed using a biotinylated rabbit anticonglutinin IgG (1:1000 dilution)/streptavidin alkaline phosphatase detection system.

by using repetitive dialysis against decreasing concentrations of urea

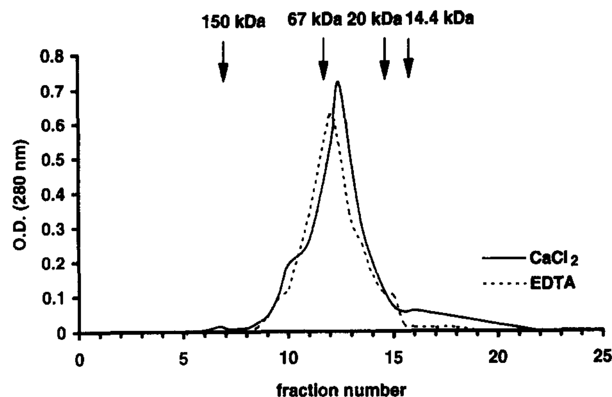

Fig. 2. Elution profile of recombinant bovine conglutinin from an FPLC Superose 12 column (HR 10/30). Approximately 0.1–0.2 mg (in 0.2 ml) of recombinant material was applied to the column. The column was eluted with TBS-NTC buffer (solid line) or TBS-EDTA buffer (dotted line), at a flow rate of 0.2 ml/min. The absorbance at 280 nm was measured and 0.5-ml fractions were collected and examined by SDS-PAGE. Calibration of the column was carried out using the following marker proteins: alchohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), soybean trypsin inhibitor (20.3 kDa), cyctochrome *c* (12.4 kDa). The elution positions of molecular weight markers are shown above the elution profiles.

(starting at 6 M and reducing to zero). The starting buffer, containing 20 mM Tris-HCl/100 mM NaCl, pH 8.0, 5% (v/v) glycerol and 6 M urea, was used to resuspend the cell pellet and repetitive dialysis performed against 1000 vols. of the same buffer containing decreasing concentrations of urea. The final supernatant containing soluble recombinant BK-neck-CRD protein was dialysed against ion-exchange starting buffer (20 mM Tris-HCl/50 mM NaCl, pH 7.4) and loaded to DEAE column (20 ml) and eluted on a Pharmacia HighLoad FPLC (Pharmacia, Milton Keynes, UK). The recombinant BK-neck-CRD was eluted by Tris buffer containing 250 mM NaCl. The eluant was pooled and dialysed against three changes of TBS-NTC buffer (50 mM Tris-HCl/150 mM NaCl, 0.05% (w/v) NaN_3, 0.05% (v/v) Tween 20 and 5 mM $CaCl_2$, pH 7.4). The dialysate was applied to a TSK-*N*-acetylglucosamnie (TSK-GluNac) column. After washing with 5 bed volumes of TBS-NTC, bound protein was eluted with TBS-NTC buffer containing 10 mM GluNac. The purity and identity of the recombinant BK-neck-CRD was assessed by 15% (w/v) SDS-PAGE and detected by Western blotting, using rabbit antibovine conglutinin IgG (gift from Dr. U. Holmoskov, University of Odense, Denmark).

2.3. *Characterisation of the recombinant BK-neck-CRD*

The gel-filtration chromatography was performed on a FPLC system (Pharmacia, Milton Keynes, UK) using a Superose 12 HR 10/30 (Pharmacia Biotech, Sweden) column. Approximately 0.1–0.2 mg (in 0.2 ml) of recombinant protein was applied to the column. For each application, the column was eluted with 20 mM Tris-HCl/100 mM NaCl, pH 7.4, containing 5 mM $CaCl_2$ or 10 mM EDTA, at flow rate of 0.5 ml/min.

2.4. *Saccharide-binding specificities of recombinant BK-neck-CRD*

The relative potencies of a number of monosaccharides and disaccharides at inhibiting the binding of biotinylated recombinant BK-neck-CRD to mannan were estimated. Recombinant BK-neck-CRD (200 μg in 2 ml 0.1 M $NaHCO_3$ buffer, pH 8.0) was biotinylated by incubation with biotinyl-*N*-hydroxy succinimide ester (BNHS; 10 μl of a 5 mg/ml solution (Sigma)) for 3 h at room temperature. The biotinylated protein was dialysed 3 × against TBS buffer (pH 7.4) to remove unbound labelling reagents. The sugar-binding characteristics were tested in a solid-phase carbohydrate assay as previously described [12]. In this assay, dilutions of recombinant BK-neck-CRD were incubated alone, or with dilutions of different saccharides, on mannan-coated plates. The bound recombinant BK-neck-CRD was detected immunochemically.

2.5. Binding of the recombinant BK-neck-CRD to C3b and to LPS

Complement component C3 was purified from normal human serum as described elsewhere [16] and was digested with 1% (w/w) trypsin (Sigma; type T2271) for 90 s at 37°C in order to generate C3b [8]. The analysis of binding of recombinant BK-neck-CRD to fluid-phase C3b was carried out as described by Laursen et al. [17], Briefly, purified recombinant BK-neck-CRD (1 μg/well) was coated on microtitre plates and, after incubation and washing, the plates were incubated with various concentrations of purified C3b in either TBS-NTC alone, TBS-NTC containing 100 mM GluNac, or TBS-NTE (50 mM Tris-HCl/150 mM NaCl, 0.05% (w/v) NaN_3, 0.05% (v/v) Tween-20 and 10 mM EDTA, pH 7.4). After 2 h of incubation at 4°C, the plates were washed and biotinylated rabbit anti-C3b antibody in 0.1 μg in 100 μl was added. After incubation for 2 h, the microtitre wells were developed using an extravidin-alkaline phosphatase-alkaline phosphatase substrate system.

The binding of LPS to native and recombinant BK-neck-CRD was measured by sandwich ELISA methods as previously described [12]. Lipopolysaccharides from *E. coli* (L8274), *Klebsiella pneumoniae* (L4268) and *Pseudomonas aeruginosa* (L9143) were all purchased from Sigma. TBS buffers containing 5 mM $CaCl_2$, or 10 mM EDTA, or 5 mM $CaCl_2$ plus 100 mM of saccharide (mannose or GluNac), were used to determine if the binding to LPS was calcium-dependent and lectin-specific.

3. Results and discussion

3.1. Bacterial expression of a fragment of human bovine conglutinin: BK-neck-CRD

The expressed recombinant fragment of conglutinin, contains residues 197–351 of the mature protein, corresponding to 28 amino acids of the α-helical neck region (residues 197–224) and the 127-amino-acid-long, C-terminal, CRD (residues 225–351). Most of the expressed protein formed inclusion bodies was recovered from the cell pellet (Fig. 1a, lane 4). The insoluble recombinant material was denatured and gradually refolded [18] by dialysis against decreasing concentrations of urea-TBS buffer (Fig. 1a, lane 5). The BK-neck-CRD was purified by ion exchange and affinity chromatography and gave a major band of 23 kDa on reducing, and non-reducing, conditions on SDS-PAGE (Fig. 1a, lane 8). The identity of the recombinant protein was confirmed by immunoblotting, using rabbit antibovine conglutinin IgG as a probe (Fig. 1b, lanes 2 and 3).

These results show that recombinant BK-neck-CRD can be overexpressed in *E. coli* and that after denaturing and refolding

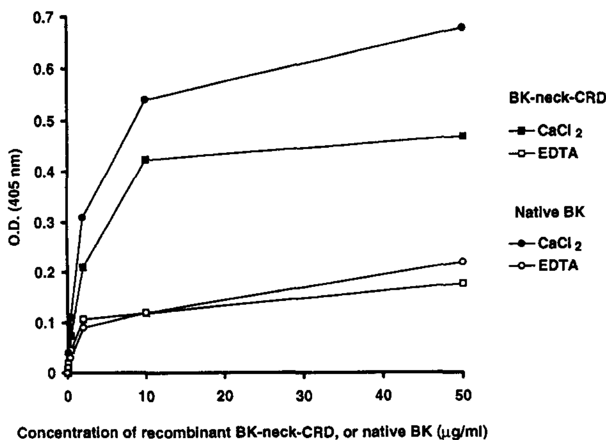

Fig. 3. Binding of native or conglutinin, or BK-neck-CRD, to immobilized mannan. Details are given in section 2. Binding was carried out in buffers containing $CaCl_2$ (●,■) or buffers containing EDTA (○,□).

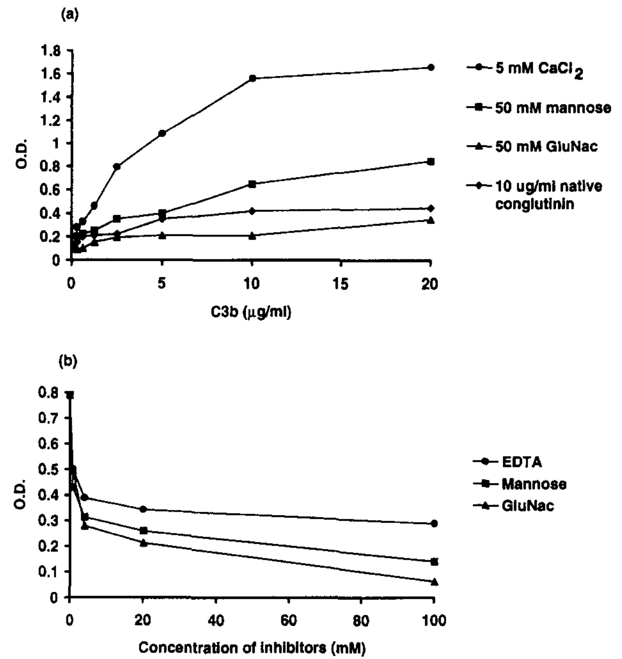

Fig. 4. (a) The binding of C3b to the recombinant BK-neck-CRD-coated microtitre plates in the presence of various inhibitors. Details are given in section 2. Binding was carried out in buffers containing: 5 mM $CaCl_2$ (●); 50 mM mannose (■); 50 mM GluNac (▲); 10 μg/ml native conglutinin (♦). (b) Sugar inhibition of C3b binding to recombinant BK-neck-CRD. Details are given in section 2. Binding was carried out in the presence of EDTA (●); Mannose (■); GluNac (▲).

the purified recombinant protein refolds correctly, as judged by its retention on a TSK-GluNac lectin-affinity column.

3.2. Characterisation of recombinant BK-neck-CRD

The molecular size of the recombinant BK-neck-CRD, in non-dissociating conditions, was determined by gel filtration on Sepharose 12 chromatography at room temperature in the presence of TBS buffer containing EDTA (10 mM), or $CaCl_2$ (5 mM). In Fig. 2, it can be seen that the 155-residue-long peptide of the expressed fragment, BK-neck-CRD, behaved as a single peak having apparent molecular weight of approximately 64 kDa. This retention profile was not significantly changed when the eluting buffer contained EDTA (10 mM) (dotted line) instead of calcium ions (solid line). The peak fractions were collected and shown to give a major band of 23 kDa when examined by SDS-PAGE, under reducing and non-reducing conditions. Thus, residues 197–351 of conglutinin are sufficient to form a homotrimer of the 23-kDa recombinant peptide in non-dissociating conditions and this trimerisation of the recombinant BK-neck-CRD is not affected by Ca^{2+} or EDTA. Although we have not expressed the CRD domain of BK on its own, the studies by Hoppe et al. [13] on the α-helical neck region and unpublished work in this laboratory on the recombinant fragments of human SP-D (neck region with CRD domain and CRD domain alone) have shown that the neck region of human SP-D is essential for the trimerisation and orientation of the CRDs of human SP-D. Also the crystal structures of the neck region plus CRDs of human and rat, MBP are consistent with this structural role of the neck region being common to all three of these collectins [19–21].

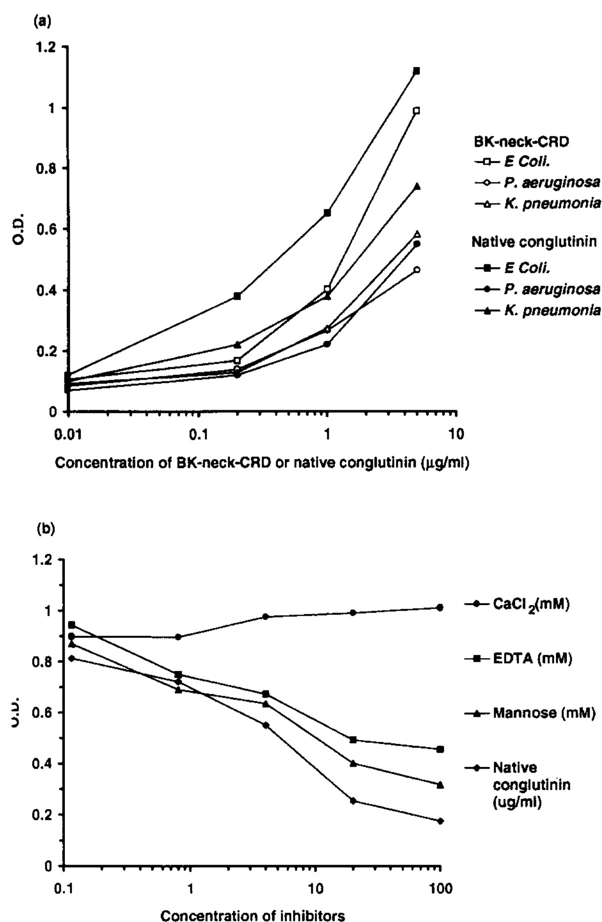

Fig. 5 (a) Dose-dependent binding of native conglutinin and BK-neck-CRD to LPS from *E. coli* (□,■), *P. aeruginosa* (○,●) and *K pneumonia* (△,▲). Details given in section 2. (b) Inhibition of the binding of recombinant BK-neck-CRD to LPS (*E. coli*). 100 µl of recombinant BK-neck-CRD (10 µg/ml) were co-incubated with various concentrations of inhibitors in LPS (*E. coli*)-coated microtitre wells: buffer containing $CaCl_2$ (●), buffer containing EDTA (■), buffer containing mannose (▲) and buffer containing native conglutinin (♦). The bound recombinant BK-neck-CRD protein was detected with biotinylated rabbit antibovine conglutinin IgG followed by alkaline phosphatase-avidin and the substrate for alkaline phosphatase. Colour development was measured by reading the OD at 405 nm.

3.3. Sugar specificities of the recombinant BK-neck-CRD

To examine the sugar-binding specificity of the recombinant BK-neck-CRD protein, a sugar inhibition assay was carried out. Recombinant BK-neck-CRD bound, in a calcium-dependent manner, as strongly as natural conglutinin to mannan-coated plates (Fig. 3), whereas neither protein showed any significant binding to maltosyl-BSA-coated plates (data not shown). The use of a variety of monosaccharides and disaccharides as inhibitors of the binding of recombinant BK to mannan-coated microtitre plates indicated that recombinant BK-neck-CRD has a similar carbohydrate-binding specificity to that of native conglutinin (Table 1), which suggests that the CRD in the recombinant BK-neck-CRD was correctly folded.

3.4. The binding of recombinant BK-neck-CRD to C3b

Hirani et al. [8] reported that the conglutinin-binding site on the third component of human complement is the carbohydrate moiety located on the N-terminal 27-kDa polypeptide of the α′-chain. The mechanism of conglutination and the high affinity shown by conglutinin for iC3b bound to cell surfaces or to iC3b-coated immune complexes is not fully understood and it has been suggested that protein-protein interaction, rather than protein-carbohydrate interaction, between conglutinin and C3b may play a role [22]. The ELISA procedures used here showed that the binding of fluid-phase C3b to immobilised recombinant BK-neck-CRD was dose- and calcium-dependent and that this binding was inhibited by GluNac, mannose and also by native, full-length, bovine conglutinin (Fig. 4a). To determine the inhibitory capacity of GluNac and mannose, the binding of a constant amount of C3b to recombinant BK-neck-CRD in the presence of various concentrations of the sugars was tested (Fig. 4b). Approximately 70% inhibition was achieved with 20 mM GluNac and 55% inhibition was observed with 20 mM mannose. Thus, these results are in agreement with the view that the binding site for conglutinin on the C3 molecule is the carbohydrate moiety on the α′-chain of C3 and that this interaction requires calcium and can be inhibited by *N*-acetylglucosamine and to a lesser degree by mannose. The finding that purified fluid-phase C3b bound to the recombinant BK is consistent with the results by Hirani et al. [8] and Laursen et al. [17] who found that purified C3, C3b, iC3b and C3c all were bound by native conglutinin in a similar type of ELISA to the one used in this study. If C3, or C3 split products, are used in the form of whole serum, or activated whole serum, then only iC3b was found to bind to natural conglutinin coated onto ELISA plates [17]. This indicates that the binding of purified C3, C3b or C3c to conglutinin may be due to some conformational change taking place, during the purification procedures employed, which allows exposure of the carbohydrate target, on the C3α′-chain, which is recognised by conglutinin [17].

3.5. LPS-binding assays

The abilities of the recombinant BK-neck-CRD or native conglutinin to bind LPS from Gram-negative bacteria were tested by ELISA. As seen in Fig. 5a, both native conglutinin

Table 1
Sugar specificities of the BK-neck-CRD recombinant material and native conglutinin

Sugar inhibitors	Recombinant BK-neck-CRD (this study)	Native conglutinin [25]
Maltose	38 (1.73)	>50
Glucose	15 (0.68)	16 (0.8)
ManNAc	29 (1.32)	23 (1.2)
ManN	6 (0.28)	7 (0.35)
GluN	27 (1.23)	15 (0.75)
D-fucose	N.D.	31 (1.55)
GalN	>50	20 (1.0)
Mannose	22 (1.0)	20 (1.0)
Galactose	>50	>50
L-fucose	32 (1.45)	15 (0.8)
GluNAc	7 (0.32)	0.75 (0.04)
GalNAc	>50	>50
Lactose	N.D.	>50

The values given are IC_{50} (mM), i.e. the concentration required for 50% inhibition of binding of biotinylated lectin to mannan. The values in parentheses denote IC_{50} concentration relative to mannose. GalN, (*N*-galactosamine); GalNAc, (*N*-acetyl-D-galactosamine); GluN, (*N*-glucosamine); GluNAc, (*N*-acetyl-D-glucosamine); ManN, (*N*-Mannosamine); ManNAc, (*N*-acetyl-D-monosamine). N.D. (not performed). Conditions for the assay are described in section 2.

and recombinant BK-neck-CRD were able to bind to the lipopolysaccharides from *K. pneumonia*, *P. aeruginosa* and *E. coli* in a similar manner. Moreover, the binding was inhibited by 100 mM of mannose, or by 10 mM EDTA, indicating that the binding was mediated through typical C-type lectin activity (Fig. 5a,b).

Conglutinin has been shown in vitro to have antibacterial activity against *Salmonella typhimurium* and *E. coli*, with the activity requiring the presence of an intact complement system and macrophages [10]. Conglutinin and MBP also appear likely to be the β-inhibitors present in bovine serum that have been reported to inhibit the infectivity and hemagglutinating activity of different influenza viruses [23]. Moreover, conglutinin binds, in a calcium-dependent and lectin fashion, to the recombinant form of the gp160 envelope glycoprotein of HIV-1 and inhibits the binding of the gp160 to the CD4 receptor on CEM 13 cells [24]. It has been suggested that conglutinin plays a cross-linking role between the iC3b-coated microorganisms and complement/collectin receptor [11]. In the present study, the behaviour of the recombinant BK-neck-CRD fragment of conglutinin on size-exclusion chromatography, and its binding to the TSK-GluNac affinity column, indicated that it is a homotrimer containing three CRD domains which are functionally intact and bind in a multivalent fashion to LPS from Gram-negative bacteria and to C3b. The trimerisation of CRDs, by the neck region sequences forming an α-helical bundle, is an important structural feature in determining the multivalency of binding shown by the collectins.

Acknowledgements: J.-Y. Wang is studying at University of Oxford supported by a scholarship from Minister of Education, Taiwan, Republic of China.

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