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Two carbohydrate recognition domains of *Hyphantria cunea* lectin bind to bacterial lipopolysaccharides through O-specific chain

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Abstract We previously identified a novel lectin cDNA from the fall webworm [Shin et al. (1998) Insect Biochem. Mol. Biol. 28, 827–837], which encodes two carbohydrate recognition domains (CRD-N and CRD-C) and is up-regulated following bacterial challenge. The lipopolysaccharide (LPS) binding activities of the recombinant CRD-N and CRD-C (rCRD-N and rCRD-C) were investigated by enzyme-linked immunosorbent assay. The LPS binding of rCRD-N and rCRD-C was pH-dependent: at pH below 6.0, they show a higher binding ability to LPS. The binding of the rCRD-N was inhibited by both D-mannose and *N*-acetyl-D-glucosamine, whereas the binding of the rCRD-C was inhibited only by D-mannose. The binding of both rCRD-N and rCRD-C to *Escherichia coli* was mainly mediated through the O-specific chain.

Key words: Insect immunity; Carbohydrate recognition domain; Lipopolysaccharide binding; *Hyphantria cunea* lectin

1. Introduction

Injury and subsequent bacterial infection in insects stimulate a complex battery of defense responses, similar to the acute inflammatory response of mammals [1]. In most cases, hemocytes attach to pathogens and remove them by phagocytosis, nodule formation or encapsulation. Invasion of foreign pathogens may be recognized by plasma or hemocyte surface proteins that bind specifically to microbial cell wall components. Lectins are possible recognition molecules and may be involved in innate insect immunity. Previously, we identified a cDNA from a lepidopteran insect, *Hyphantria cunea*, that encodes a novel member of the C-type lectin superfamily with two different carbohydrate recognition domains (CRDs), namely CRD-N and CRD-C [2]. The deduced amino acid sequence of *H. cunea* lectin showed a significant similarity with the lipopolysaccharide (LPS) binding protein of the American cockroach, that is related in sequence to the C-type lectins of vertebrates [3,4]. Recently, the studies of BmLBP (LPS binding protein from *Bombyx mori*) and immunolectin revealed that they contain two different CRDs [5,6]. The amino acid sequence alignment of *H. cunea* lectin, BmLBP and immunolectin shows that C-type lectins with two CRDs are conserved in three lepidopterian insects, *H. cunea*, *B. mori* and *Manduca sexta* (Fig. 1).

While most members of the C-type lectins contain only a single carbohydrate binding domain, macrophage mannose receptor, phospholipase A2 receptor and DEC receptor contain either eight or 10 lectin domains [7]. This raises the question as to how three insect lectins, *H. cunea* lectin, BmLBP and immunolectin with two carbohydrate binding domains, relate to the multiple carbohydrate binding domains found in higher animals. It is speculated that an increased number of binding domains in the same molecule work cooperatively to enhance ligand avidity [8,9]. As part of the functional analysis of *H. cunea* lectin, we expressed the genes encoding each of CRD-N and CRD-C domains of *H. cunea* lectin in *Escherichia coli*, and characterized the binding specificity of the recombinant CRD-N and CRD-C (rCRD-N and rCRD-C).

2. Materials and methods

2.1. Reagents

The following reagents were purchased from Sigma: LPSs from *E. coli* 026/B6 (L8274), *E. coli* O111/B4 (L2630), *E. coli* EH100 (L9641), *E. coli* F583 (L6893), *Klebsiella pneumoniae* (L4268), *Pseudomonas aeruginosa* (L9143) and *Serratia marcescens* (L6136); yeast mannan (M7504), D-glucose (G8270), D-mannose (M4625), D-galactose (G0750), D-fructose (F0127), *N*-acetyl-D-glucosamine (A8625), *N*-acetyl-D-galactosamine (A2795), L-fucose (F2252) and L-rhamnose (R3875).

2.2. Expression of CRDs in E. coli

The cDNA fragments encoding each of CRD-N and CRD-C domains were PCR-amplified with a pair of specific primers flanking each domain. The PCR products were cloned into the *Eco*RI and *Sal*I sites of the pET-21a(+) containing T7·Tag (Novagen, Madison, WI, USA). Plasmids with correct inserts were transformed into *E. coli* strain BL 21 (DE3)pLysS (Novagen). The recombinant rCRDs were expressed as insoluble inclusion bodies. The purified inclusion bodies were applied to a T7·Tag agarose affinity column. The eluents were subjected to refolding process by step-dialysis (urea concentration = 1 M, 0.5 M, 0.25 M and 0 M) against TBS buffer (20 mM Tris–HCl, 140 mM NaCl, 0.05% (w/v) NaN_3, pH 7.5) containing 0.05% (v/v) Tween-20 and 10 mM $CaCl_2$. The refolded proteins were dialyzed three times against distilled water at 4°C.

2.3. The enzyme-linked immunosorbent assay (ELISA)

ELISA for C-type lectin-carbohydrates binding assay [10,11] was used to detect LPS binding activity of the rCRDs. Polysorp microtiter plates (Nunc, Denmark) were coated with LPS overnight in 100 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% (v/v) Tween-20, pH 9.5) at 4°C. The plates were washed three times with washing buffer (TBS buffer containing 0.05% Tween-20, pH 5.0) after each subsequent step. After coating, the plate was blocked with 1% (w/v) BSA in washing buffer for 1 h. The rCRDs (100 μl in washing buffer), T7·Tag antibody (100 μl of a 1:5000 dilution in washing buffer, Novagen), α-mouse IgG alkaline phosphatase conjugate (100 μl of a 1:5000 dilution in washing buffer, Sigma) were added sequentially while washing between each step. All incubation steps were carried out at room temperature for 1 h. Alkaline phosphatase substrate,

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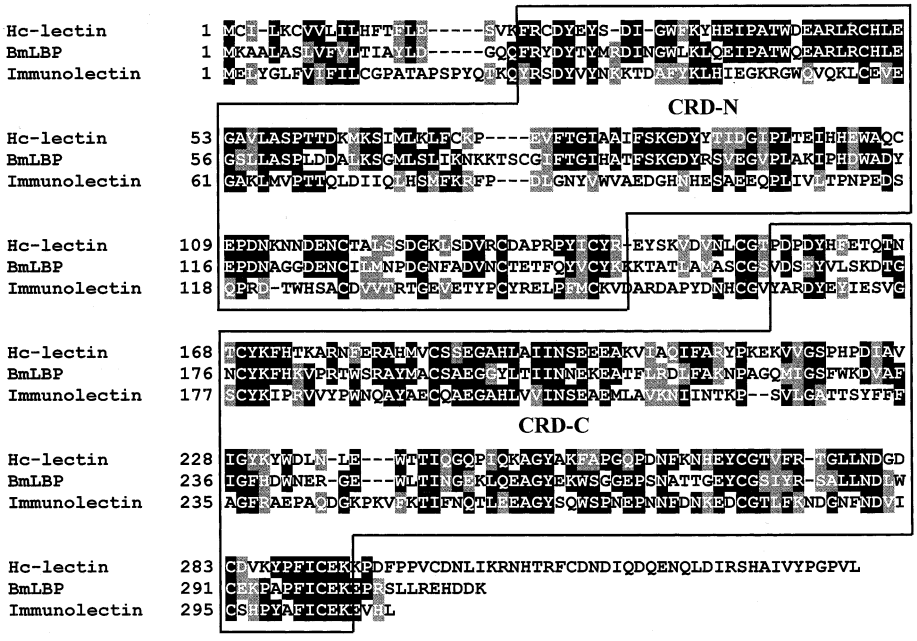

Fig. 1. Amino acid sequence alignment between *H. cunea* lectin, BmLBP and immunolectin. Identical amino acids are indicated by a dark background and similar amino acids by a gray one. The numbering is based on the sequence of each protein.

PNPP (100 μl, 1 mg/ml) in developing solution (100 mM Tris–HCl, pH 9.6, 100 mM NaCl, 5 mM $MgCl_2$), was applied to each well, and the OD at 405 nm was read in an ELISA plate reader.

3. Results

3.1. The expression of rCRDs in E. coli

As part of the functional analysis of the *H. cunea* lectin, we expressed the cDNA fragments encoding each of CRD-N and CRD-C domains in *E. coli*. The T7·Tag sequence was added to the N-terminus of the recombinant proteins for affinity purification and LPS binding assay. The expressed rCRD-N, which had a T7·Tag fused to the N-terminus and a histidine-rich peptide chain fused to the C-terminus, carried residues 22–147 of the *H. cunea* lectin, corresponding to the CRD-N domain. The rCRD-C contains a T7·Tag in the N-terminus and the CRD-C domain, residues 156–299 of *H. cunea* lectin. When the rCRD-N and rCRD-C were expressed in *E. coli*, bands with a molecular mass of about 20 kDa were shown on sodium dodecyl sulfate (SDS) gel electrophoresis. The calculated molecular weights of rCRD-N and rCRD-C based on the amino acid sequences were 17 294.58 and 18 080.53, respectively. The expression and purification of the recombinant proteins are shown in Fig. 2. The T7·Tag fusion proteins were insoluble in *E. coli* lysis buffer (lanes 3 and 7). The insoluble proteins were solubilized with a denaturing buffer containing 6 M urea following centrifugation. The diluted fusion proteins with 2 M urea were bound to the T7·Tag affinity column. After washing out impurities with wash/bind buffer, the rCRDs were eluted with an elution buffer (lanes 4 and 8) and refolded by step-dialysis. When the refolded rCRD-N and rCRD-C were respectively applied to the reverse phase high performance liquid chromatography (YMC-Pack PROTEIN-RP, YMC, Japan), they appeared as one major peak, showing that most of rCRD-N and rCRD-C were folded as a major conformation.

3.2. The specific LPS binding of rCRDs

The binding ability of rCRDs to smooth LPS from *E. coli*, under a variety of different conditions, was tested by ELISA. Negative controls which lacked rCRDs gave base values of $OD_{405} \approx 0.065 \pm 0.05$. Since the binding without LPS coating showed near base values regardless of the concentration of rCRDs and since they increased with the amount of LPS (1 and 10 μg/ml) coated on the plate, the signals did not result from aggregation or non-specific binding of recombinant proteins to the ELISA plates (Fig. 3a). The coating of microtiter plates with smooth LPS at a higher concentration than 10 μg/ml did not increase the value of OD_{405}. This may be caused by micelle formation of LPS at higher concentration. Micelle formation may reduce the free LPS concentration of coating buffer and then may decrease the LPS coating of microtiter plates. The binding was found to be dependent on pH, higher

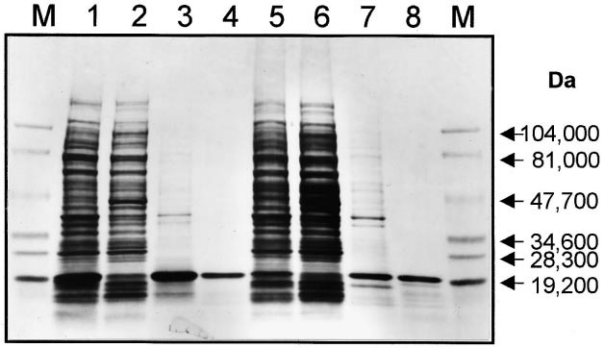

Fig. 2. Expression and purification of rCRD-N and rCRD-C. Lanes 1–4 (rCRD-N) and lanes 5–8 (rCRD-C) show the purification steps: total cell lysate with ITPG induction (1, 5); soluble fraction (2, 6); insoluble fraction after sonication (3, 7); proteins retained on the T7·Tag affinity column (4, 8). All samples were run on 4–20% (w/v) gradient SDS–polyacrylamide gel electrophoresis under reducing conditions.

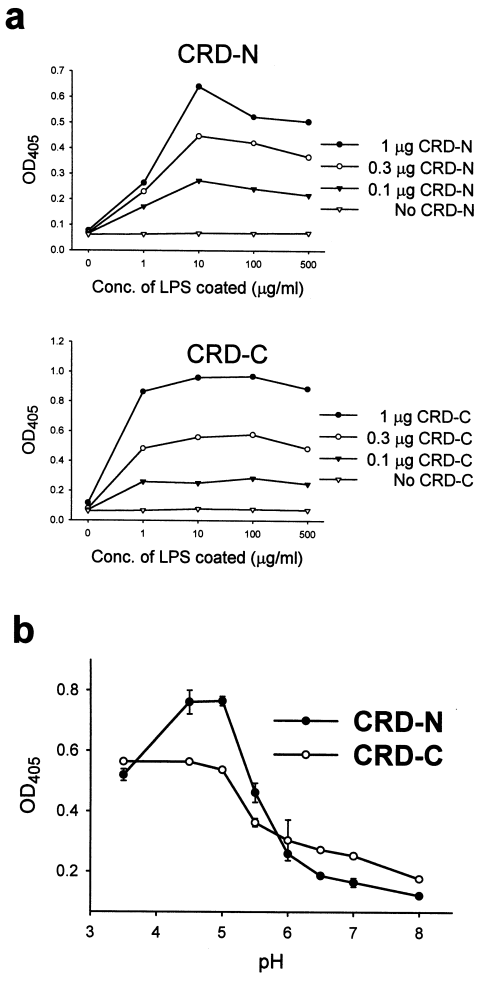

Fig. 3. Demonstration of the binding to *E. coli* LPS and pH dependence of the binding. 100 μl (0–500 μg/ml) LPS of *E. coli* 026/B6 was coated on the Polysorb ELISA plate, and the rCRD-N and rCRD-C were tested for their binding to LPS (a). 100 μl (10 μg/ml) of LPS was coated and the binding was tested with a change of buffer and sample pH (b).

at acidic pH below 6.0 (Fig. 3b). The binding pattern as shown in Fig. 3a was exactly the same for pH 5.0 and pH 7.0. Like BmLBP, the calcium dependence of the binding was not clear, because the binding activity was not affected by excessive dialysis against distilled water and addition of EDTA.

3.3. Sugar specificity of LPS-rCRDs binding

To examine the sugar specificity of the rCRDs, sugar inhibition was tested. As shown in Fig. 4a, the LPS binding of rCRD-N was significantly inhibited by D-mannose and *N*-acetyl-D-glucosamine at the concentration of 100 mM, whereas the binding of rCRD-C was inhibited only by D-mannose at 100 mM. To definitely explain the sugar specificity, the LPS binding was tested with increasing sugar concentration of up to 250 mM, we confirmed that only rCRD-N showed sugar specificity to *N*-acetyl-D-glucosamine (Fig. 4b).

3.4. LPS binding specificity of rCRDs

The LPS of Gram-negative bacteria is composed of three parts: the lipid A region, core polysaccharide region and protruding O-specific chain. To know which part of the LPS is recognized by the rCRDs, an inhibition assay was done with LPS from *E. coli* R-mutants lacking the O-specific chain (Ra) and the additional core region (Rd_2) (Fig. 5). At a concentration of 1 μg/ml free LPS, the LPS binding of rCRD-N was almost completely inhibited by smooth LPS from *E. coli* 026/B6 strain and only slightly by Ra LPS from *E. coli* EH100. The LPS binding of the rCRD-C also showed the same results but at a higher concentration of 5 μg/ml free LPS. The inhibition by Rd_2 LPS from *E. coli* EH100 was negligible for both rCRDs, similar to the inhibition by yeast mannan. These results indicate that the binding of two rCRDs to *E. coli* LPS is mainly mediated through the O-specific chain.

The binding inhibition by smooth LPS from *E. coli* O111/B4, *P. aeruginosa*, *K. pneumonia* and *S. marcescens* was tested. The inhibitory effect of *P. aeruginosa* LPS is as strong as that of smooth LPS from *E. coli* 026/B6. In contrast, the inhibitory effects of smooth LPS from *E. coli* O111/B6, *K. pneumonia* and *S. marcescens* are as weak as that of Ra LPS from *E. coli* EH100. The difference of binding affinity to smooth LPS from different bacterial species is additional evidence that the binding is mainly mediated through the O-specific chain. The O-specific chain is the immunodominant structure exposed to the environment and is highly variable among bacterial strains, whereas the core polysaccharides and lipid A moieties are relatively conserved [12].

4. Discussion

Koizumi and co-workers [13] reported that BmLBP is a

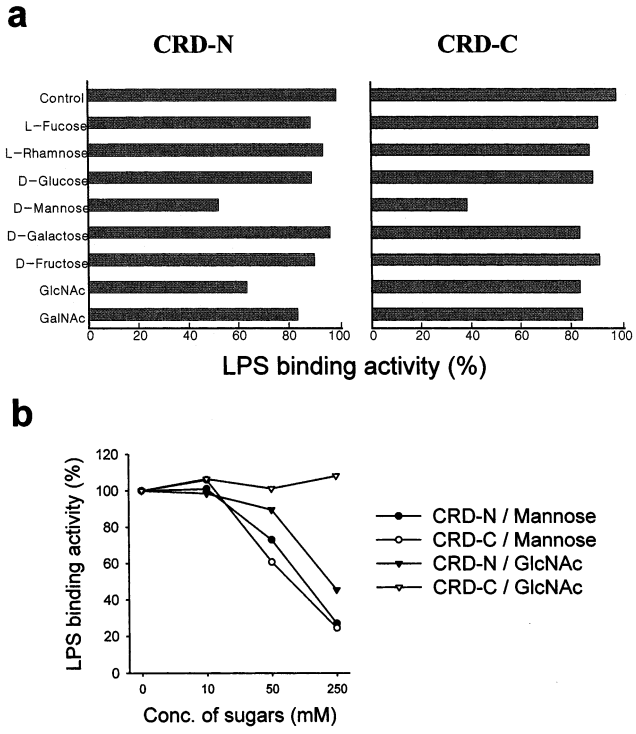

Fig. 4. Sugar specificity of rCRDs. The binding inhibition assays by 100 mM concentration of several sugars (a) and by 0–250 mM concentration of D-mannose and *N*-acetyl-D-glucosamine (b) were done. The plates were coated with 100 μl (10 μg/ml) LPS of *E. coli* 026/B6100 and 1 μg of each rCRD was used. The data represent a typical experiment which was performed three times.

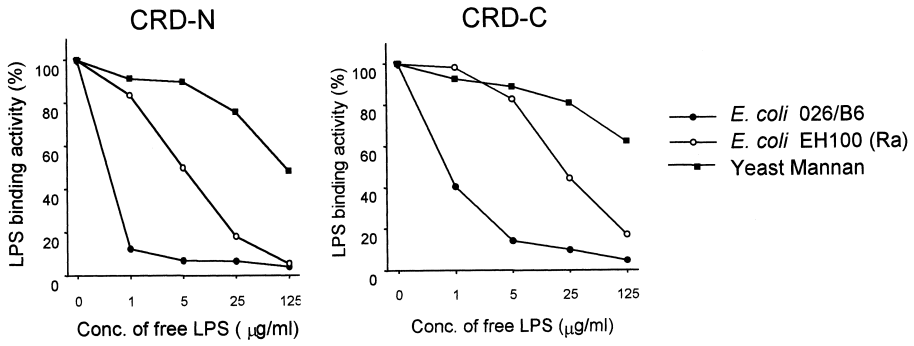

Fig. 5. Binding properties of rCRDs to O-specific chain of *E. coli* LPS. Binding inhibition assays by 0–125 mM concentration of smooth LPS (*E. coli* 026/B6), Ra LPS (*E. coli* EH100) and yeast mannan were done. The plates were coated with 100 μl (10 μg/ml) LPS of *E. coli* 026/B6100 and 1 μg of each rCRD was used. The data represent a typical experiment which was performed three times.

pattern recognition molecule that recognizes the lipid A portion of LPS and participates in a cellular defense reaction, hemocyte nodule formation. Immunolectin has a binding site with affinity for bacterial LPS, the binding specificity to LPS was not clearly defined [6]. Although two rCRDs of *H. cunea* lectin weakly bind to LPS from *E. coli* R-mutants lacking the O-specific chain (Ra and Rd_2 mutants), it is evident from our results that they bind to bacterial LPS mainly through O-specific chain. It is unlikely that native *H. cunea* lectin may recognize the lipid A portion rather than the O-specific chain of LPS. When considered that C-type lectin genes are so diverse in an invertebrate genome [14] and that many genes similar to BmLBP existed in the *B. mori* genome [5], *H. cunea* lectin may have a different LPS binding specificity from BmLBP.

The pH dependence is interesting because the ligand binding activity of animal C-type lectins is readily demonstrable at pH above approximately 6.5 [15]. It is reasonable that the rCRDs show higher LPS binding at acidic pH, considering that the physiological pH of *H. cunea* hemolymph is about 5.5–6.0. The binding activity rapidly changed between pH 5.0 and pH 6.0. The high binding sensitivity to pH change at physiological pH may provide a regulatory mechanism for LPS-lectin interaction during insect immune response.

The concentrations of D-mannose and *N*-acetyl-D-glucosamine that show an inhibitory effect on LPS binding are quite high compared to other well characterized C-type lectins, in which sugars that inhibit at concentrations in the range of 1 mM or less are considered to be significant inhibitors. Also yeast mannan, mainly composed of D-mannose, inhibits activity less than 50% at the concentration of 125 μg/ml, whereas free LPS from *E. coli* 026/B6 inhibits activity more than 50% at the concentration of 1 μg/ml (Fig. 4). The O-specific chains of *P. aeruginosa* LPS are composed of derivatives of *N*-acetyl-D-mannosamine, *N*-acetyl-D-fucosamine and guluronic acid [16]. The O-specific chain of *E. coli* O26/B6 LPS is composed of trisaccharide repeating units of L-rhamnose, *N*-acetyl-L-fucosamine and *N*-acetyl-D-glucosamine [17]. The O-specific chains of both LPS, which show a strong inhibitory effect against LPS-rCRDs binding, do not share any specific sugar. These results indicate that a specific sugar moiety may be less important in the recognition of LPS by rCRDs. These assumptions may coincide with the characteristics of BmLBP that none of the monosaccharides tested so far is a hapten that specifically inhibits BmLBP binding to LPS [15]. Also several mono- and disaccharides (mannose, glucose, galactose and the core disaccharide of the lipid A component of LPS) lacked the ability to inhibit immunolectin's binding to bacteria [6].

The difference of sugar specificity between rCRD-N and rCRD-C may enhance ligand avidity and/or ligand affinity of *H. cunea* lectin by recognizing different sugar moieties of LPS. Moreover, two CRDs may be related to the clustering of CRDs, which has been shown to be important in several other C-type lectins. These lectins contain only a single CRD in each polypeptide and form homo- or hetero-oligomers, indicating that clustering of CRDs determines the specificity of the interaction, as well as the affinity [18–20]. The two CRDs of *H. cunea* lectin may be arranged spatially to match the geometric configurations of bacterial LPS.

The mRNA expression of *H. cunea* lectin was clearly induced by bacterial challenge [2]. Our study reveals that both CRDs of *H. cunea* lectin specifically bind to bacterial LPS, an elicitor of insect immune response against Gram-negative bacteria, and the binding was mainly mediated through the O-specific chain. These results strongly suggest that *H. cunea* lectin might be involved in insect immune response against invading Gram-negative bacteria as a recognition molecule.

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