Ligand specificities and structural requirements of two *Tachypleus* plasma lectins for bacterial trapping

Tun-Hsun KUO*, Shiao-Cheng CHUANG*, Sing-Yang CHANG* and Po-Huang LIANG*†¹

*Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan, and †Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan

TPL (*Tachypleus* plasma lectin)-1 was purified by using a Sepharose column and TPL-2 was purified from an LPS–Sepharose (LPS coupled to Sepharose matrix) affinity column, as described previously [Chiou, Chen, Y.-W., Chen, S.-C., Chao and Liu (2000) *J. Biol. Chem.* **275**, 1630–1634] and the corresponding genes were cloned [Chen, Yen, Yeh, Huang and Liu (2001) *J. Biol. Chem.* **276**, 9631–9639]. In the present study, TPL-1 and -2 were produced in yeast, and the recombinant proteins secreted into the media were purified and characterized. The proteins show specific PGN (peptidoglycan)- and LPS-binding activity, suggesting a role in trapping Gram-positive and Gram-negative bacteria respectively in innate immunity. Using BIAcore® assays, the dissociation constant for the TPL-1–PGN complex was measured as 8×10^{-8} M. Replacement of Asn⁷⁴, the N-glycosylation site of TPL-1, with Asp abolishes the PGN-binding affinity, whereas the un-

glycosylated TPL-2 N3D mutant retains LPS-binding activity. DTT (dithiothreitol) treatment to break disulphide linkages abrogates TPL-2 activity but does not interfere with TPL-1 function. Cys⁴ in TPL-2 may form an intermolecular disulphide bond, which is essential for activity. As a result, the TPL-2 C4S mutant is inactive and is eluted as a monomer on a non-reducing gel. TPL-2 C6S is active and forms a non-covalently linked dimer. A model describing TPL-2 binding with LPS is proposed. These two plasma lectins that have different ligand specificities can be used for the detection and discrimination of bacteria and removal of endotoxins.

Key words: horseshoe crab, host defence, innate immunity, lectin, lipopolysaccharide (LPS), peptidoglycan (PGN), *Tachypleus tridentatus*.

INTRODUCTION

Host defences can be attributed to two general immune systems: innate and adaptive. The adaptive immune system utilizes B-cells and T-cells to kill pathogens. On the other hand, the innate immune system secretes a variety of proteins which trigger immediate cellular responses against invading organisms. Although adaptive and innate immunity are present in all vertebrates, invertebrates only have an innate immune system as their host defence. The innate immune system must target the conserved molecular pattern that is present in invading microbes from the enormous variability and molecular heterogeneity in these micro-organisms. The structural components of the bacterial cell-wall such as LPS (lipopolysaccharide) in Gram-negative bacteria, PGN (peptidoglycan) and lipoteichoic acid in Gram-positive bacteria and glycolipids in mycobacteria have become the targets for host produced proteins, which they entrap. *Tachypleus tridentatus* (horseshoe crab, also known as *Limulus*) is an arthropod which relies solely on its innate immunity for host defence. This animal has survived on Earth for more than 500 million years and thus serves as a prototype for studying innate immunity. Its immune functions are mainly carried out by haemocytes (also called amebocytes), which contain coagulation factors, protease inhibitors, lectins and anti-microbial peptides acting as host defence molecules [1–4]. Several lectins have been purified from horseshoe crabs and characterized for recognition of bacterial cell-wall carbohydrates [5,6]. Among them, four types of lectins, named TL (tachylectin)-1 to -4, have been identified

in haemocytes of the Japanese horseshoe crab. TL-1 binds LPS through KDO (2-keto-3-deoxyoctonate) and also interacts with polysaccharides such as agarose and dextran with broad specificity [7]. TL-2 binds D-GlcNAc (*N*-acetylglucosamine) or GalNAc (*N*-acetylgalactosamine) and recognizes *Staphylococcal* lipoteichoic acids and LPS [8]. TL-3 and TL-4 specifically recognize O-antigen of S (smooth)-type LPS [9,10]. Moreover, TL-5 from plasma, and TL-P in the perivitelline fluid of embryos exhibit broad specificity against acetyl substances [11,12].

Previously, two lectins from the plasma of Taiwanese *T. tridentatus* were isolated [13]. One was named GBP (galactose-binding protein) since it was purified using a Sepharose CL-4B column that is a polymerized form of agarose consisting of repeated units of 1,6-linked D-galactose and an unusual 3,6-anhydro-L-galactose. The other lectin was purified using the affinity column LPS–Sepharose and was named LBP (LPS-binding protein) [13]. Subsequently, the cDNAs of GBP and LBP were cloned and they were renamed TPL (*Tachypleus* plasma lectin)-1 and -2 respectively, to be systematic with other TLs in the nomenclature [14]. The TPL-1 gene encodes an approx. 26 kDa protein (232 amino acids) containing a potential N-glycosylation site, comprising Asn-Gly-Ser, at residues 74–76 (Figure 1A). The deduced sequence of TPL-2 (approx. 15 kDa) consists of 128 amino acids with a predicted N-glycosylation site, comprising Asn-Cys-Thr, at positions 3–5 (Figure 1B). TPL-1 shows sequence similarity to previously identified TL-1 and TL-P, and TPL-2 is homologous to TL-3 (Figure 1). Unlike the amebocyte lectins, TPL-1 and TPL-2 are glycoproteins. In the present study,

Abbreviations used: BPI, bactericidal/permeability-increasing; CRP, C-reactive protein; dsDNA, double-stranded DNA; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GalNAc, *N*-acetylgalactosamine; GBP, galactose-binding protein; GlcNAc, *N*-acetylglucosamine; KDO, 2-keto-3-deoxyoctonate; LacNAc, *N*-acetylglucosamine; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; ManNAc, *N*-acetylmannosamine; MIC, minimum inhibitory concentration; MurNAc, *N*-acetylmuramic acid; NANA, *N*-acetylneuramic acid; PGN, peptidoglycan; TL, tachylectin; TPL, *Tachypleus* plasma lectin; YNB, yeast nitrogen base.

¹ To whom correspondence should be addressed (email phliang@gate.sinica.edu.tw).

Figure 1 Sequence alignment for TPL-1 and TPL-2

(A) Sequence alignment of TPL-1 with TL-1 and TL-P of Japanese *T. tridentatus*. The asparagine N-glycosylation site of Asn-Gly-Ser at positions 74–76 of TPL-1 is not conserved in TL-1 and TL-P. (B) Alignment of the TPL-2 sequence with that of TL-3 isolated from Japanese *T. tridentatus* amebocytes. Only TPL-2 possesses an N-glycosylation site, Asn-Cys-Thr, at positions 3–5. *, Indicates the amino acid residues mutated for the purposes of this study. Signal peptides prior to N-termini are not included in these sequences.

we have utilized yeast *Pichia pastoris* to express glycosylated TPL-1 and TPL-2 and characterized their functions in innate immunity.

The targets which TPL-1 and TPL-2 bind to remain to be elucidated, although previous experiments have suggested they bind *N*-acetylmonosaccharide and LPS respectively. By thoroughly examining the ligand specificity of the two TPLs using ELISA and BIAcore® assays, we have now narrowed down their potential specific ligands to the PGN repeating unit and O-antigen of LPS respectively. Ligand-induced aggregation/oligomerization of TPL-2 is reported here. Disulphide bond disruption by treatment with DTT (dithiothreitol) abolishes the activity of TPL-2 by causing the formation of an inactive monomer. The cysteine residue critical for intermolecular disulphide bond formation in TPL-2 is identified from site-directed mutagenesis studies. Moreover, we demonstrate that recombinant TPLs function to distinguish Gram-positive and Gram-negative bacteria, inhibit bacterial growth and remove endotoxin (LPS).

EXPERIMENTAL

Materials

Sepharose CL-4B was obtained from Amersham Pharmacia Biotech. LPS–Sepharose CL-4B was prepared by coupling *Escherichia coli* (O26:B6) LPS to Sepharose CL-4B as described previously [13]. The compounds, including GlcNAc, ManNAc (*N*-acetylmannosamine), GalNAc, NANA (*N*-acetylneuramic acid), LacNAc (*N*-acetylglactosamine), acetate, L-glutamine, *N*-acetylglutamine, muramyl dipeptide, lipid A from *E. coli* F583, LPS from *E. coli* O26:B6, and O55:B4, and O111:B4 were purchased from Sigma. An Ra (rough-type) LPS mutant from *E. coli*,

which lacks O-antigen but still retains a core sugar residue, was purchased from Merck. *N,N*-Dimethylacetamide, *N,N*-diacetylchitobiose and *N,N,N,N*-penta-acetylchitopentose are products of Calbiochem Co. The plasmid mini-prep kit and DNA gel extraction kit were obtained from QIAGEN. The yeast expression system including the pPICZ- α A vector and the yeast strain *Pichia pastoris* KM71 were purchased from Invitrogen. Zeocin antibiotic for selection of highly expressing clones was also from Invitrogen. All other buffers and reagents are of the highest commercial purity.

Expression of wild-type TPL-1 and TPL-2 using *Pichia pastoris*

The genes encoding TPL-1 and -2 in *T. tridentatus* were amplified by PCR from a cDNA library of hepatopancreas excised from an adult male of *T. tridentatus* captured on the beaches of Quimoi Island. We utilized a cloning strategy without the need for restriction enzymes [15]. Accordingly, four DNA primers containing suitable restriction sites were designed. There were two forward primers: A (5'-AATTCAATGGGAGTTGGATA-3') and B (5'-CAATGGGGAGTTGGATA-3'), as well as two reverse primers: C (5'-GGCCGCTATAGGAAAGGATT-3') and D (5'-GCCTATAGGAAAGGATT-3') for TPL-1. For TPL-2, primers A (5'-AATTCGAAGATAACTGCACG-3'), B (5'-GGAAGATAACTGCACG-3'), C (5'-GGCCGCGGACTTAATTATAT-3') and D (5'-GCGGACTTAATTATAT-3') were used. One PCR product was obtained using primers A and D, and the other PCR product was generated from primers B and C. The two PCR products were purified by 0.8 % agarose gel electrophoresis and were combined and annealed by incubation at 95 °C for 3 min, followed by 65 °C for 10 min, and 37 °C for 10 min. This process yielded dsDNA (double-stranded DNA) with sticky-end EcoRI

and NotI sites at the 5' and 3' ends respectively. The resulting DNA was ligated with the vector pPICZ- α A treated with the two restriction enzymes and the recombinant plasmid was used to transform *E. coli* strain JM109. The colonies grown at 37°C on an agar plate were selected for Zeocin resistance. A single colony resistant to 25 μ g/ml Zeocin was grown overnight in 5 ml of Luria-Bertani medium containing the same concentration of the antibiotic. The plasmids collected from the JM109 cells were sequenced using Prism Ready Reaction DyeDeoxy Terminator sequencing kit (PE Applied Biosystems). Samples were subjected to electrophoresis on an ABI 310 DNA sequencer and the data were recorded and read using ABI Prism Model software provided by PE Applied Biosystems. The plasmid was then transfected in to yeast expression strain *Pichia pastoris* KM71. A single colony resistant to 2 mg/ml Zeocin was selected and grown in 2 l of BMGY medium [1.34 % YNB (yeast nitrogen base, Sigma), 100 mM potassium phosphate (pH 6.0)/ 4×10^{-5} % biotin, 4×10^{-3} % histidine, and 1 % glycerol] at 30°C for 48 h. When absorbance at 600 nm reached 2, the medium was centrifuged at 4000 *g* for 10 min and the cells were resuspended in 500 ml of BMMY medium [1.34 % YNB, 100 mM potassium phosphate (pH 6.0), 4×10^{-5} % biotin, 4×10^{-3} % histidine, and 1 % methanol] containing 1 % methanol for induction.

Mutation of TPL-1 and -2 N-glycosylation sites

Using PCR, TPL-1 and -2 mutants were constructed by replacing the Asn with Asp to alter the potential N-glycosylation sites. The mutagenic primers for the N74D mutation in TPL-1 were: forward 5'-GTGGATGGTGTATGGGAGT-3' and reverse 5'-ACTCCCATCACCATCCAC-3'. The two mutagenic primers in combination with primers A and C as described above were used to generate the PCR products. For the N3D mutation in TPL-2, the mutagenic primers were: 5'-AATTCGAAGATGACTGCACGTGT-3' and 5'-CGAAGATGACTGACTGCACGTGT-3'. Both primers were used as forward primers and the primers C and D described above were used as reverse primers to generate DNA with restriction sites at both ends. The dsDNA construct was ligated to the vector that was treated with the same restriction enzymes and the correct colony was selected for transfection in to yeast as described above.

Expression of mutant TPL-2 with disrupted disulphide bonds

TPL-2 mutants, in which the cysteine was replaced with serine to break a disulphide bond, were constructed using PCR. The four TPL-2 DNA primers (A–D) described above containing suitable restriction sites were used for cloning. Since Cys⁴ and Cys⁶ are close to the N-terminus, one PCR product was obtained using primer C4S-A (5'-AATTCGAAGATAACAGCACG-3') for C4S, or C6S-A (5'-AATTCGAAGATAACTGCACGAGT-3') for C6S and primer D, and the other PCR product was generated from primer C4S-B (5'-CGAAGATAACAGCACG-3') for C4S, or C6S-B (5'-CGAAGATAACTGCACGAGT-3') for C6S and primer C. For generating the other C32S, C51S, C62S, C64S and C102S mutants the following mutagenic forward primers were used: C32S, 5'-TTGGATGGGAGTCGGCGA-3'; C51S, 5'-TGGGAAAGTATTCAATCA-3'; C62S, 5'-CTCTTGAGTAAATGTGAT-3'; C64S, 5'-TGTAAGATGATTCTCTT-3'; C102S, 5'-GGCTTTAGTTTCGACTGG-3', the respective reverse primers were used in combination with primers A and D or B and C as described above, to generate the relevant PCR products. The dsDNA constructs were ligated to the vector that was treated with the same restriction enzymes. The correct colony was selected for yeast transfection as described above.

Purification of recombinant wild-type and mutant TPL-1 and TPL-2

The recombinant proteins contained α -factor signal peptide so that they could be secreted into the medium. A single colony was used to inoculate 500 ml of BMGY medium in a 2 l Baffled flask. The cells were grown at 30°C in an incubator with gentle agitation until A_{600} reached 2–6. After 2 days, the cells were then harvested by centrifugation at 2500 *g* for 5 min. To induce protein expression, the cell pellet was resuspended in 250 ml of BMMY media and continued to grow at 30°C. Methanol was added to a final concentration of 1 % every 24 h to maintain induction. After 9 days of induction, the cells were removed by centrifugation at 8000 *g* for 20 min and the medium containing TPL-1 was filtered through a 0.45 μ m filter before loading onto a 30 ml Sepharose CL-4B column which was pre-equilibrated with 25 mM Tris/HCl (pH 7.4), 150 mM NaCl and 10 mM CaCl₂. At the end of sample loading, the column was washed with the same buffer for 10 column-volumes and the protein was then eluted with the buffer containing 0.4 M GlcNAc.

A mixture containing the TPL-2 mutant was loaded on to a 30 ml Sepharose-LPS CL-4B column, which was pre-equilibrated with 25 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 10 mM CaCl₂. This affinity column was prepared as described previously [13]. After sample loading, the column was washed with the same buffer for 10 column-volumes and the protein was then eluted with buffer containing 7 M urea.

The procedure for purification of the active TPL-2 N3D mutant was the same as for the wild-type protein. Since TPL-1 N74D was inactive and unable to bind to Sepharose CL-4B, a 1.5 cm $\times$ 30 cm size column (Sephadex G-75) was utilized to purify the mutant protein from the medium. To simplify the purification, the minimal medium BMGH [1.34 % YNB, 100 mM potassium phosphate (pH 6.0), 4×10^{-5} % biotin, 4×10^{-3} % histidine and 1 % glycerol] and BMMH [1.34 % YNB, 100 mM potassium phosphate (pH 6.0), 4×10^{-5} % biotin, 4×10^{-3} % histidine and 1 % methanol] that did not contain yeast extract and peptone was utilized for cell culture. After centrifugation, the supernatant was dialysed against 1 litre of buffer containing 25 mM Tris/HCl (pH 7.4), 150 mM NaCl and 10 mM CaCl₂, three times and the sample was filtered through a 0.45 μ m pore filter and concentrated to 0.2 mg/ml for loading on to the column.

Inactive TPL-2 C4S was purified from the minimal medium using a pre-packed Sephadex G-200 column (1 cm $\times$ 20 cm, Amersham Pharmacia Biotech). The other active cysteine to serine TPL-2 mutants were purified using the same procedure as for the wild-type protein.

Preparation of bacterial PGN

To obtain cell-wall PGN, *Bacillus subtilis* obtained from A.T.C.C. was grown in nutrient broth (Difco) at 37°C with shaking under aerobic conditions. The extraction of PGN from this bacterium was performed as described previously [16]. Cells were collected by centrifugation (14000 *g* for 8 min at 4°C) and boiled in a water bath for 7 min. Heated 5 % (w/v) SDS was added to the cell pellet and the mixture was resuspended and boiled for 25 min. Insoluble material was recovered by centrifugation (14000 *g* for 8 min at 20°C) and boiled again in 4 % (w/v) SDS for 15 min. The resulting insoluble cell-wall component was then washed with hot distilled water (60°C) at least 5 times to remove SDS. Covalently attached proteins were removed by treatment with protease K (50 μ g/ml) for 1 h at 60°C. The cell wall was recovered by centrifugation (14000 *g* for 8 min at 4°C), washed once in distilled water and suspended in hydrofluoric acid [400 μ l of a 48 % (v/v) solution]. The insoluble material containing PGN was collected by centrifugation (14000 *g* for 8 min at 4°C).

and washed repeatedly with Tris/HCl (50 mM, pH 7) and with cold distilled water until the pH was neutral. PGN was lyophilized and stored at -20°C before use. To prepare the PGN repeating units, which contain GlcNAc and MurNAc (*N*-acetylmuramic acid), PGN (2 mg/ml) was hydrolysed with purified peptidoglycan hydrolase (LytG, 200 $\mu\text{g}/\text{ml}$) at 37°C [17]. This muramidase enzyme was cloned by PCR using its genomic DNA as a template and using forward primer 5'-GGTATTGAGGGTCGCATGGCCCGTAAAAAACTTAAAAAC-3' and reverse primer 5'-AGAGGAGAGTTAGAGCCGGTTGCCTCCTTTATTCAACAGCT-3'. The protein was expressed in *E. coli* strain BL21 (DE-3) with the pET32Xa/LIC vector and was purified using an NiNTA (QIAGEN, Germany) column.

Bacteria- and ligand-binding experiments using ELISA

The bacteria-binding assays for wild-type and mutant TPL-1 or TPL-2 were performed as described previously [13,14]. In brief, an *E. coli* cell suspension in a mixture of chloroform and ethanol (1:9, v/v) was added to 96-well microplates and the solvent was evaporated by warm air. Serially diluted TPL-1 or TPL-2 samples in buffer containing 1% BSA were added to each well and incubated at 25°C for 2 h. The rabbit antiserum against TPL-1 or TPL-2 was added after washing each well. Subsequently, horseradish peroxidase-linked anti-(rabbit IgG) antibody was added and incubated for 2 h followed by the addition of 0.1 ml of 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (Sigma) in substrate buffer and the absorbance was recorded at 450 nm using a microplate reader (Molecular Devices). In examining the ligand-binding for the lectins, TPL-1 or TPL-2 was first incubated with each of the compounds, including saccharides (glucose, galactose, mannose, fructose, maltose and lactose), *N*-acetylsaccharides (GlcNAc, ManNAc, GalNAc, NANA and LacNAc), acetate, *L*-glutamine, *N*-acetylglutamine, *N,N*-dimethylacetamide, *N,N*-diacetylchitobiose, *N,N,N,N,N*-penta-acetylchitopentaose, PGN and muramyl dipeptide, as well as lipid A from *E. coli* F583 and LPS from *E. coli* 026:B6, 055:B4 and 0111:B4 at different concentrations before addition to the wells in the microplate. The absorbance at 450 nm for each sample was determined.

BIAcore® assays

BIAcore® X (BIAcore® AB, Uppsala, Sweden) apparatus (surface plasmon resonance technology) was used to analyse the affinity between the recombinant TPL-1 and its ligand. TPL-1 (4.2 μg) was immobilized on sensor chip CM5 (BIAcore®) by the amine coupling method, according to the manufacturer's instructions. After immobilization, the sensor chip was washed with assay buffer [10 mM Tris/HCl (pH 7.4), 150 mM NaCl] until a stable baseline was obtained. The binding experiments were performed by injecting different concentrations of ligand (50, 100, 150 and 200 nM PGN unit or 100, 200, 300 and 500 nM muramyl-dipeptide) at 25°C with a flow rate of 30 $\mu\text{l}/\text{min}$, and the sensogram data were collected. The k_{on} , k_{off} , and K_D were obtained by fitting the data with the BIAcore® evaluation program (Version 3.2). The K_D and the binding stoichiometry were also determined from the Scatchard plot.

Determination of molecular mass by gel-filtration

The polymeric status of recombinant wild-type and mutant TPL-2 was determined by size-exclusion chromatography on a Blue Dextran 2000 calibrated pre-packed Superdex-75 column (1 cm $\times$ 20 cm, Amshera Pharmacia Biotech). The molecular mass was estimated from the plot of K_{av} against log molecular mass of the protein using molecular mass standards, which were albu-

min (67 000 Da), ovalbumin (43 000 Da), chymotrypsinogen A (25 000 Da) and ribonuclease A (13 700 Da). A buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl and 10 mM MgCl_2 was used to elute the protein at a flow rate of 0.5 ml/min. The K_{av} values were calculated using the equation $K_{\text{av}} = (V_e - V_o)/(V_i - V_o)$ where V_e is elution volume of the protein, V_o is the elution volume of Blue Dextran 2000 and V_i is total-gel bed-volume.

DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] titration

DTNB stock solution containing 50 mM sodium acetate (pH 5) and 2 mM DTNB was prepared. The denaturing buffer contained 6.4 M GuHCl in 0.01 mM sodium phosphate buffer (pH 5), 0.5 mM EDTA and 100 mM KCl. A TPL-2 solution (20 μl) containing wild-type, C4S, C6S or other mutants was mixed with 980 μl of DTNB solution (20 μl of DTNB stock solution plus 960 μl of denaturing buffer) and incubated for 60 min at 25°C in a quartz cuvette. The absorbance was measured at 412 nm and free thiol content in the solution was estimated using the molar absorption coefficient $13\,600\text{ M}^{-1} \cdot \text{cm}^{-1}$ of DTNB.

Bacterial-growth inhibition and endotoxin removal

E. coli (Gram-negative) or *B. subtilis* (Gram-positive) growth was monitored until absorbance reached 650 nm, when TPL-1 or TPL-2 was added in to the culture to a concentration of 0, 50 nM, 100 nM or 500 nM. After inoculation, each well on the ELISA plate contained approximately 5×10^4 CFU (colony forming units)/well. The growth was continuously monitored for up to 8 h for *E. coli* and up to 14 h for *B. subtilis*, at 37°C .

To remove endotoxin (LPS), 20 μg of TPL-2 was coupled to NHS (*N*-hydroxysuccinimide)-activated Sepharose resin purchased from Amersham Biosciences Inc. and used in accordance with the manufacturer's instructions (prepared by GlycoNex Inc., Taiwan). The resin was mixed with 10 μg of LPS for 10 min. After centrifugation (13 000 *g* for 1 min), the concentration of unbound LPS in buffer was measured using an LAL kit (Limulus Amebocyte Lysate Pyrochrome™ Chromogenic Test Kit, Associates of Cape Cod, Inc., MA, U.S.A.). The resin was washed with 7 M urea or 500 mM NaCl for 10 min and then removed by centrifugation (13 000 *g* for 1 min) three times. The resin was regenerated in buffer (10 mM Tris/HCl, 150 mM NaCl and 10 mM CaCl_2) for 10 min. The resin was continuously reloaded with 10 μg of LPS and regenerated to test its LPS-binding capacity after repeated use.

RESULTS

Expression and purification of TPL-1, TPL-2 and their mutants

In a previous study [13], native TPL-1 and TPL-2, purified from the plasma of Taiwanese *Tachypleus tridentatus* by using affinity chromatography, were shown to contain a variety of different glycosylated and partially-protease-cleaved forms, making it difficult to identify which form has bacterial binding activity. In the present study, TPL-1, TPL-1 N74D (non-glycosylated TPL-1 mutant), TPL-2, TPL-2 N3D (non-glycosylated TPL-2 mutant) and all seven TPL-2 cysteine to serine mutants were expressed in yeast, secreted into the extracellular milieu and purified for characterization. From 500 ml of medium, approx. 5.2 mg of TPL-1 can be purified via Sepharose CL-4B column chromatography (53% yield) and 0.6 mg of purified TPL-2 can be obtained by LPS-Sepharose CL-4B column chromatography (66% yield). Their analyses by SDS/PAGE are described below. Approx. one-third of the amount of expressed TPL-1 was lost because it was

Figure 2 Ligand recognition specificity of TPL-1 and TPL-2

In (A), GlcNAc (■) serves as a moderate ligand, whereas glucose (○) does not inhibit the bacterial-binding activity of TPL-1. In particular, the PGN unit GlcNAc-MurNAc-pentapeptide (●) shows the best inhibitory activity. (B) Ligand recognition of TPL-2 with LPS (■), delipidized LPS (○), Ra mutant LPS (□) and lipid A (△). Entire LPS and delipidized LPS show good inhibitory activity of the bacterial-binding activity of TPL-2, whereas Ra mutant LPS and lipid A do not inhibit binding, indicating that the O-antigen of the LPS is the component responsible for binding with TPL-2. All experiments included 2 μ M TPL-1 or TPL-2. At higher concentrations of the ligand that were tested, the binding of the lectin to the bacteria was inhibited as revealed by the reduction of absorbance at 450 nm in ELISA.

bound to the yeast cell-surface. The purified recombinant TPL-1 and TPL-2 were first subjected to a study of ligand specificity.

Ligand specificity of TPL-1 and TPL-2 examined by ELISA

Inhibition of the binding of recombinant wild-type TPL-1 and TPL-2 to immobilized *E. coli* cells by a number of potential ligands was examined by ELISA as described previously [13]. A representative study indicated that GlcNAc served as a modest ligand, whereas glucose lacking an N-acetyl group did not inhibit the bacterial binding activity of TPL-1 (Figure 2A). The MIC (minimum inhibitory concentration) values for the compounds, at which over 90 % of the bacterial binding of TPL-1 was inhibited, are summarized in Table 1. The data indicate the requirement for an acetyl moiety attached to an amino sugar to function as a ligand for TPL-1. N-acetylmonosaccharides such as GlcNAc, ManNAc, GalNAc, NANA, and LacNAc, but not monosaccharides, disaccharides, acetate, glutamine, N-acetylglutamine and N,N-dimethylacetamide inhibited the binding of TPL-1 to the *E. coli* cells. Larger N-acetylated saccharides such as N,N-diacyltchitobiose and N,N,N,N,N-pentaacetylchitopentose inhibited the binding with similar MIC to that of GlcNAc. However, the PGN unit (GlcNAc-MurNAc-pentapeptide) showed 100-fold (in molar ratio) better affinity than GlcNAc alone in binding with TPL-1 (see Table 1). Muramyl dipeptide was found to be 6-fold weaker in affinity than GlcNAc-MurNAc-pentapeptide. This observation defines the physiological role of TPL-1 in trapping Gram-positive bacteria via PGN on the cell wall.

Table 1 MICs of ligands required for inhibiting TPL-1 and TPL-2 activity in bacterial binding by ELISA

ND, not determined.

Ligand	MIC (mM or *mg/ml)	
	TPL-1	TPL-2
Glucose	> 50	> 50
Galactose	> 50	> 50
Mannose	> 50	> 50
Fucose	> 50	> 50
Maltose	> 50	> 50
Lactose	> 50	> 50
GlcNAc	8.4	> 50
ManNAc	12.3	> 50
GalNAc	18.5	> 50
NANA	32	> 50
LacNAc	14.3	> 50
Acetic acid	> 50	> 50
L-Glutamine	> 50	> 50
N-Acetylglutamine	50	> 50
N,N-Dimethylacetamide	> 50	> 50
N,N-Diacyltchitobiose	9.3	> 50
N,N,N,N,N-Penta-acetylchitopentose	7.2	50
GlcNAc-MurNAc-pentapeptide	0.078	ND
Muramyl-dipeptide	0.4	ND
Lipid A <i>E. coli</i> F583 (Rd)*	> 1	> 1
Ra mutant LPS <i>E. coli</i> EH100*	ND	> 1
Delipidized LPS O128:B8*	ND	0.16
LPS <i>E. coli</i> K-235*	ND	0.3
LPS <i>E. coli</i> O26:B6*	> 1	0.22
LPS <i>E. coli</i> O55:B4*	> 1	0.15
LPS <i>E. coli</i> O111:B4*	> 1	0.28
GlcNAc-MurNAc-pentapeptide*	0.1	> 1
MurNAc-dipeptide*	0.6	> 1
Mannan*	> 1	> 1
Lipoteichoic acid*	> 1	> 1

The binding of TPL-2 to *E. coli* cells was not inhibited by any of the compounds that bound to TPL-1, but was inhibited most effectively by LPS (Table 1), indicating a completely different ligand specificity for TPL-2. Delipidized LPS still served as a good ligand, but Ra mutant LPS (containing lipid A and core sugars but not O-antigen) did not inhibit the binding of TPL-2 to bacteria, neither did lipid A (Figure 2B). These results suggest that O-antigen of LPS is the specific ligand for TPL-2 in binding with LPS, which is the major cell-wall component of Gram-negative bacteria.

BIAcore® binding study

Surface plasmon resonance technology was further used to measure the binding constants of TPL-1 with its ligands. According to the sensogram shown in Figure 3(A), TPL-1 bound the PGN unit (digested by LytG as described in the Experimental section) with a k_{on} of $5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_{off} of $4 \times 10^{-2} \text{ s}^{-1}$, so that the $K_D = 8 \times 10^{-8} \text{ M}$. Scatchard plot analysis gave a K_D that was $7.8(\pm 0.2) \times 10^{-8} \text{ M}$ and a stoichiometry of 1:1 for the ligand/TPL-1 monomer (not shown). The binding curves of muramyl-dipeptide with TPL-1 measured by BIAcore® assay yielded a k_{on} of $3.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_{off} of $1 \times 10^{-1} \text{ s}^{-1}$, so that the K_D was $2.9 \times 10^{-7} \text{ M}$ (Figure 3B). Scatchard plot analysis gave a K_D of $3.1(\pm 0.3) \times 10^{-7} \text{ M}$ and a binding stoichiometry of 1 (results not shown). Consistent with the ELISA described above, muramyl-dipeptide bound to TPL-1 with 6-fold weaker affinity than

Figure 3 BIAcore® sensogram of TPL-1 binding with PGN unit and muramyl dipeptide

(A) Sensogram of TPL-1 binding with GlcNAc-MurNAc-pentapeptide (PGN unit). (B) The sensogram of TPL-1 association with MurNAc-dipeptide. The lack of GlcNAc and three amino acids from the PGN unit decreases the TPL-1 binding affinity 6-fold.

the PGN unit. TPL-2 bound LPS with a K_D of 6.3×10^{-8} M in the BIAcore® assay experiments (results not shown).

Glycosylation is important for TPL-1 binding, but not for TPL-2 binding

Unlike TLs in amoebocytes, TPL-1 and TPL-2 are glycoproteins. On reducing SDS/PAGE, TPL-1 purified from a Sepharose CL-4B column showed two bands at approx. 30 kDa (Figure 4A, lane 2), representing two differently glycosylated forms. The TPL-1 N74D mutant (Figure 4A, lane 3) showed a single band at approximately the same position as the lower band of the wild-type TPL-1. This result confirmed the glycosylation site of TPL-1 at Asn⁷⁴, as predicted from the sequence. ELISA showed that TPL-1 N74D lost its bacteria-binding activity, indicating the importance of glycosylation for the function of TPL-1 (Figure 5A, left panel). The CD spectra of wild-type and non-glycosylated TPL-1 N74D are similar (results not shown), indicating that the loss of the activity for the mutant is not owing to misfolding. On non-reducing SDS/PAGE, TPL-1 was found mostly as a monomer (approx. 30 kDa) and a small amount of dimer (approx. 60 kDa) (Figure 4B, lane 2). Inactive TPL-1 N74D mutants exclusively showed a single band at molecular mass of approx. 30 kDa (monomer) (Figure 4B, lane 3).

On reducing SDS/PAGE gels, TPL-2 purified from an LPS affinity column was observed as two bands (Figure 4A, lane 4), whereas the TPL-2 N3D mutant (Figure 4A, lane 5) was observed as a single band that migrated as the lower band of the wild-type. This also confirmed Asn³ as the glycosylation site of TPL-2, as predicted from the sequence. On non-reducing SDS/PAGE gels, TPL-2 was found exclusively as a doublet at the dimer position (Figure 4B, lane 4), and TPL-2 N3D also migrated as a dimer. As shown in the right panel of Figure 5(A), this mutant that is without glycosylation retained bacteria-binding activity. This suggests that glycosylation is not important for the function of TPL-2.

Figure 4 TPL-1 and TPL-2 on reducing and non-reducing SDS/PAGE

(A) Reducing SDS/PAGE analysis of recombinant TPL-1 and TPL-2 purified using Sepharose CL-4B and LPS-Sepharose CL-4B columns respectively. Lanes 1–8 represent molecular mass standards, TPL-1, TPL-1 N74D, TPL-2, TPL-2 N3D, TPL-2 C4S, TPL-2 C6S and TPL-2 C64S respectively. Purified TPL-1 and TPL-2 show two bands on SDS/PAGE gels due to differences in glycosylation. TPL-1 N74D and TPL-2 N3D are unglycosylated (a single band on the SDS/PAGE gel). It is notable that the C4S and C6S mutants are also unglycosylated probably because mutations are located at or near the glycosylation site. (B) SDS/PAGE analysis of the above proteins under non-reducing conditions. Lanes 2 and 3 display a mixture of dimer and monomer forms for TPL-1 and a monomer for TPL-1 N74D under non-reducing conditions, whereas both wild-type TPL-2 and TPL-2 N3D (lanes 4 and 5) are dimeric proteins. The TPL-2 C4S mutant exists as a monomer under non-reducing conditions (lane 6), as does TPL-2 C6S (lane 7). However, C64S (lane 8) and other cysteine to serine mutants (results not shown) are dimers under non-reducing conditions. Molecular mass (MM) markers used are myosin (220 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).

Disulphide bonds are required for binding of TPL-2 to LPS

Since TPL-2 formed a dimer on the non-reducing gel, this indicates that dimer linkage by a disulphide bond may be essential for its activity. MS measurements also show that TPL-2 exists as a covalent dimer (results not shown). This was confirmed by the observation that DTT treatment abolished TPL-2 activity by converting it into an inactive monomer (Figure 5B, right panel). On the other hand, DTT treatment did not affect the activity of TPL-1 (Figure 5B, left panel). To identify which cysteine is involved in the dimerization of TPL-2, seven mutants, C4S, C6S, C32S, C51S, C62S, C64S and C102S, were prepared and assayed for their bacteria-binding activity. TPL-2 C4S and TPL-2 C6S mutants became monomers (Figure 4B, lanes 6 and 7), whereas all other mutants remained dimeric on the non-reducing gel as in the wild-type protein (Figure 4B, TPL-2 C64S in lane 8 as a representative). However, only TPL-2 C4S lost its activity, indicating that Cys⁴ may be involved in forming the intermolecular disulphide bond. This is consistent with the possibility that all six cysteine residues except Cys⁴ form three intramolecular disulphide bonds in TL-3 [9], which shares an almost identical

Figure 5 Evaluation of binding of TPL-1 N74D and TPL-2 N3D to the immobilized bacteria by using ELISA

(A) Left panel shows that wild-type TPL-1 binds to bacteria (■) but TPL-1 N74D does not (●). Right panel shows that TPL-2 N3D retains the binding affinity for bacteria (■) as in wild-type protein. At high concentrations of TPL-1 or TPL-2, the binding level of the lectin to the bacteria reached a plateau. (B) The binding affinity of TPL-1 and TPL-2 for bacteria in the presence (■) and absence (●) of DTT. Left panel shows that in the presence of DTT, fully reduced TPL-1 retains full bacteria-binding activity. Right panel demonstrates that DTT treatment abolishes the activity of TPL-2. Higher concentrations of TPL-1 or TPL-2 saturated the binding sites on immobilized bacteria.

sequence with TPL-2 but lacks the first cysteine residue (Figure 1B). Unexpectedly, however, TPL-2 C6S was found as a monomer on non-reducing SDS/PAGE gels but still retained its activity.

TPL-2 C6S retained its activity by forming a non-covalent dimer

It was surprising that TPL-2 C6S was a monomer under non-reducing condition since Cys⁴ was present and presumably formed the intermolecular disulphide bond. DTNB titration revealed that unlike other mutants, which contained one free thiol from the unpaired cysteine, TPL-2 C6S contained no free thiol group, the same as in TPL-2 C4S. We speculated that Cys⁴ may replace Cys⁶ to form the intramolecular disulphide bond in the C6S mutant since they are next to each other so that Cys⁴ could not be involved in forming the intermolecular disulphide bond. However, TPL-2 C6S formed a non-covalent dimer, whereas TPL-2 C4S was a monomer, according to the size-exclusion column chromatography (Figure 6). Wild-type TPL-2 was also a dimer (Figure 6). Apparently, dimerization is required for the function of TPL-2. TPL-2 C4S cannot form a non-covalent dimer as shown by gel filtration data. TPL-2 C4S and TPL-2 C6S have different secondary structures judged by CD, and different stabilities against protease digestion (results not shown). Moreover, LPS induced the formation of tetrameric and even hexameric TPL-2 in a dose-dependent manner (Figure 6). We could not determine whether this oligomerization could occur for TPL-1 in the presence of PGN since TPL-1 was bound to the matrix of the gel filtration column.

A working model for LPS binding to TPL-2

Our results described above can be explained by the model illustrated in Figure 7. Wild-type TPL-2 is dimeric and becomes oligomeric (tetramer and hexamer) upon addition of higher concentrations of LPS. The DTT treatment disrupts the intermolecular

Figure 6 Gel filtration experiments to determine the oligomerization status of TPL-2 in solution

Wild-type TPL-2 is a dimer and becomes a tetramer or hexamer by incubation with higher concentrations of LPS. The TPL-2 C4S is a monomer but TPL-2 C6S is a dimer, as in the other mutants.

disulphide bond and destroys LPS-binding activity in TPL-2 (Figure 7A). TPL-2 C4S fails to form a dimer and loses its LPS-binding activity. On the other hand, TPL-2 C6S remains active by forming a non-covalent dimer (Figure 7B).

Detection of bacteria by TPL-1 and TPL-2

From the above studies, we have established that TPL-1 recognizes PGN in Gram-positive bacteria and TPL-2 specifically binds to LPS of Gram-negative bacteria. Using an ELISA for the two lectins, the detection of serially diluted Gram-positive

Figure 7 Model of TPL-2 binding with LPS

(A) TPL-2 is shown as a pentangle linked by the intermolecular disulphide bond. An oligomer is formed on binding with LPS. In the presence of DTT, all the disulphide bonds are reduced and the protein is inactive. As shown in (B), TPL-2 C4S lacks intermolecular disulphide linkage and is inactive since it becomes monomeric according to the size exclusion determination. By contrast, TPL-2 C6S forms a non-covalent dimer to maintain its activity.

or Gram-negative bacteria in solutions was tested. As shown in Figure 8(A), TPL-1 could detect the Gram-positive bacteria (*B. subtilis*) at a concentration as low as 100 bacteria/ml (measured by visible absorbance at 650 nm). On the other hand, TPL-2 could detect Gram-negative bacteria (*E. coli*) in a solution containing 100 bacteria/ml (Figure 8B). However, TPL-1 was less sensitive in the detection of Gram-negative bacteria and TPL-2 was almost unable to detect Gram-positive bacteria (results not shown).

Bacterial killing activity and endotoxin-removal capability of TPL-2

TPL-2 was tested for its bactericidal activity. As shown in Figure 9(A), *E. coli* growth was inhibited by TPL-2 in a dose-dependent manner. However, the growth-curve was not retarded by adding TPL-1 (results not shown). This indicated that TPL-2 (LBP) could inhibit the growth of Gram-negative *E. coli*. However, TPL-1 did not have the ability to inhibit the growth of Gram-positive *B. subtilis* (results not shown), although it did bind PGN. Next, the capacity of TPL-2 to remove endotoxin (LPS) from a protein sample was examined. The resin coupled with TPL-2 was incubated with an excess of LPS. After centrifugation, the concentration of unbound LPS in solution was determined as described in the Experimental section. According to the calculations used, one LPS monomer is bound with two TPL-2 monomers. This stoichiometry is consistent with the model proposed in Figure 7. After repeated use, the binding capacity of TPL-2 was gradually decreased. Overnight regeneration could recover the binding-capacity.

Figure 8 Detection of bacteria in serially diluted solutions by using TPL-1 and -2

(A) The ELISA was performed to detect certain concentrations (indicated on the x-axis) of Gram-positive bacteria (*B. subtilis*) using TPL-1. (B) TPL-2 was used to detect specified concentrations (indicated on x-axis) of Gram-negative bacteria (*E. coli*). In the presence of 100 bacteria/ml, the decrease of A_{450} was noticeable. The concentrations of bacteria were determined from absorbance at 600 nm.

DISCUSSION

Invertebrates have only evolved innate immunity. In this paper, *T. tridentatus* (a living fossil) was used as a model of innate immunity and the ligand specificities and structural requirements of the two host defence molecules, TPL-1 and TPL-2 in plasma, were characterized. These TPLs co-exist in the haemolymph with the CRPs (C-reactive proteins) previously identified from *L. polyphymus* [18], and the TLs frequently isolated from the amoebocytes of *Tachypleus tridentatus* that act as pattern-recognition molecules in innate immunity [19]. In contrast with homologous TL-1 and TL-3, which are intracellular non-glycosylated proteins and recognize LPS through KDO and O-antigen respectively [7,9], TPL-1 and TPL-2 are glycoproteins that are secreted into plasma and recognize PGN and LPS respectively. TL-P found in the perivitelline fluid of the embryo of the Japanese horseshoe crab, which has sequence identity with TPL-1, also lacks a glycosylation site and recognizes acetyl-group-containing substances [12]. The non-glycosylated TPL-1 N74D mutant is inactive, indicating that functional TPL-1 is dependent on glycosylation. By contrast, the TPL-2 N3D mutant without glycosylation retains LPS-binding affinity, indicating the unimportance of glycosylation for TPL-2 activity.

Previously, we were able to elute native TPL-1 from a Sepharose CL-4B column with GlcNAc but not glucose, suggesting that the N-acetyl group represents a key structural feature of TPL-1 ligands [13]. In the present study we report that TPL-1 specifically recognizes PGN units. Although the MIC values of

Figure 9 Bacterial-growth inhibition and endotoxin removal by TPL-2

(A) TPL-2 inhibited *E. coli* growth in a dose-dependent manner at 0 (○), 50 (□), 100 (●) and 500 nM (■) TPL-2 in the culture medium. (B) Capacity (%) of TPL-2 for removing endotoxin (LPS) after repeated use. Either 500 mM NaCl (●) or 7 M urea (■) was used to regenerate the column and the column was then washed with the buffer three times before re-use. The capacity decreased with repeated use, but can be almost regenerated after overnight (O.N.) incubation with buffer.

GlcNAc in inhibiting the binding of TPL-1 and TL-5 (which also recognizes N-acetyl sugars) to bacteria are of the same order of magnitude [11], TPL-1 shows 100-fold better binding affinity with the GlcNAc-MurNAc-pentapeptide of the PGN than the N-acetylsaccharide. It is still unknown whether the TL-P that recognizes the N-acetyl group can better recognize PGN. Apparently, TPL-1 represents the pattern-recognition molecule in plasma of *T. tridentatus* that recognizes PGN units which exist in both Gram-positive and Gram-negative bacterial cell-walls, but are enriched in Gram-positive bacteria. On the other hand TPL-2 which is also found in plasma shows ligand specificity toward LPS, particularly O-antigen. TL-3 (a homologue of TPL-2) was also identified as recognizing the O-antigen of LPS with comparable activity (MIC = 0.01 mM) [20]. However, TL-3 is a non-glycosylated lectin located in amebocytes and it does not require intermolecular disulphide bonds for activity.

Despite similar primary structures, TLs and TPLs have different properties. TPL-1 and -2 found in plasma with completely different ligand specificities are the defence molecules that first encounter invading organisms and represent the essential components in innate immunity of *T. tridentatus*. Polymorphic ligand-specificity was also seen for CRP, which was found to consist of a mixture of closely related family-proteins with sequence identities encoded by multiple genes [21]. The interaction of pattern-recognition molecules with a somewhat different sequence/structure could result in combinational possibilities that could diversify the immune system of *T. tridentatus* to cope with a broad spectrum of invading pathogens [22].

Oligomerization in the presence of ligand has been observed for these pattern-recognition molecules. In the present study, we have identified that dimerization via intermolecular disulphide linkage is required for TPL-2 function. TL-3 (a TPL-2 homologue) also exists as a dimer in solution as judged from ultracentrifugation-analysis, but the cysteine corresponding to Cys⁴ in TPL-2 involved in forming the particular disulphide-bridge is missing [9]. Based on TL-3 in which the three intramolecular disulphide bonds have been identified, corresponding to Cys⁶, Cys³², Cys⁵¹, Cys⁶², Cys⁶⁴ and Cys¹⁰² in TPL-2, it is likely that Cys⁴ in TPL-2 forms the essential intermolecular disulphide bond. As shown in the present study, TPL-2 C4S is inactive and is a monomer. TPL-2 C6S exists as an active non-covalent dimer. Apart from Cys⁴, all other cysteine residues, which may be involved in forming the intramolecular disulphide bonds, are not important for the function of TPL-2. LPS further induces tetramerization and hexamerization of TPL-2 in a dose-dependent manner.

TPL-1 and TPL-2 have shown no sequence identity with any protein found in humans, including the Nod proteins and PGN recognition proteins which bind with the muramyl tripeptide [23,24], and BPI (bactericidal/permeability-increasing) protein which binds LPS [25] in innate immune functions. Unlike these proteins, TPL-1 prefers GlcNAc-MurNAc-pentapeptide as a ligand. These horseshoe crab lectins are the prototype of PGN and LPS recognition proteins. Although the crystal structures of GlcNAc/GalNAc- and KDO-binding TLs (TL-2 and TL-5A) are available [26,27], the structural information for PGN- and O-antigen-mediated LBPs from *T. tridentatus* is lacking. The previous crystal structure of a human BPI protein was solved with bound phospholipids rather than bound LPS [25]. A lectin isolated from *Limulus* was also found to bind phosphocholine [28]. These findings are consistent with their binding activity with lipid A moiety in LPS. The data presented here not only lead to the working model for lectin binding, but also serve as the basis for X-ray crystallographic characterization to elucidate the molecular interactions of LPS with its binding protein TPL-2 through O-antigen. For example, homogeneous and active TPL-2 N3D may be used and higher concentrations of LPS, which induce oligomerization, could be avoided in order to obtain homogeneous crystals. Moreover, these lectins may be constructed for use in PGN/Gram-positive bacteria and LPS/Gram-negative bacteria detection. In addition, TPL-2 may be of value for anti-septic shock therapy and a previous study has demonstrated that Factor C recombinant protein (an LBP from horseshoe crab) can neutralize this endotoxin [29]. Moreover, TPL-2 is able to inhibit bacterial growth and remove endotoxin from a sample, suggesting a possible commercial application.

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