Isolation and Characterization of Proteins That Bind to Galactose, Lipopolysaccharide of Escherichia coli, and Protein A of Staphylococcus aureus from the Hemolymph of Tachypleus tridentatus

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In this study, we report the isolation and characterization of three novel hemolymph proteins that are believed to be involved in the innate immune response of horseshoe crabs, Tachypleus tridentatus. They include two closely related proteins, one that binds to the protein A of Staphylococcus aureus (PAP) and another that binds to the lipopolysaccharide of Escherichia coli (LBP). PAP binds specifically to staphylococcal protein A (SpA) with a $K_D$ of $3.86 \times 10^{-5}$ M, whereas LBP binds to lipopolysaccharide (LPS) with a $K_D$ of $1.03 \times 10^{-6}$ M. Both PAP and LBP are glycoproteins with an apparent molecular mass of about 40 kDa. N-terminal sequences of PAP and LBP showed 61.9 and 72.2% identity, respectively, to tachylectin-3, a lectin isolated from the amebocyte of T. tridentatus, previously characterized by its affinity to the O-antigen of LPS and blood group A anti-ogen (Muta, T., and Iwanaga, S. (1996) Curr. Opin. Immunol. 8, 41–47). The third protein, a galactose-binding protein (GBP), was found to bind tightly to Sepharose CL-4B and could only be eluted from the column matrix with chaotropic agents, such as 4M urea or 2M guanidine hydrochloride. Further analysis indicated that GBP binds to (+)-galactose with a $K_D$ of $2.47 \times 10^{-7}$ M. N-terminal sequence analysis showed that GBP shares a 50% identity with lectin L-6, identified in the granules of amebocyte of T. tridentatus. (Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoo, Y., Wai, S. N., Iwanaga, S., and Kawabata, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10086–10091). Lectin-L6 and tachylectin-3 are nonglycosylated intracellular proteins with about half the molecular mass of PAP, LBP, and GBP. GBP also binds to PAP and LBP with $K_D$ values of $1.25 \times 10^{-7}$ and $1.43 \times 10^{-8}$ M, respectively, and this binding is enhanced about 10-fold upon the addition of SpA and LPS to form the GBP-PAP-GBP-LPS complexes, respectively.

Invertebrates have developed several germ line-encoded receptor-dependent pathways, such as proteolytic and prophenol oxidase cascade, pathogen-specific lectins, and antibiotic peptides, which work in concert for the recognition, immobilization, and elimination of the invading pathogens (1, 3–6). The pathogen-specific lectins in the hemolymph are expected to distinguish between self and non-self and to serve as the first line of defense upon the entry of pathogens. The interaction between lectins and pathogens results in the recruitment of other defense mechanisms, which ultimately are responsible for the immobilization and elimination of the invading pathogens.

The hemolymph of horseshoe crab contains three major proteins: hemocyanin, C-reactive protein, and α-2-macroglobulin. Hemocyanin functions as oxygen-carrying protein. C-reactive proteins are lectins that bind to phosphocholine of the pneumococcus C-polysaccharide (7) and to the chromatins of damaged cells (8). α-2-macroglobulin exhibits protease inhibitory activity with a broad specificity that can block the activities of proteases secreted from invading microorganisms (9, 10). The Limulus C-reactive proteins, along with the C3 homologue α-2-macroglobulin, participates in a complement-like hemolytic activity in horseshoe crab hemolymph (11).

Recently, several lectins have been identified in the amebocytes of horseshoe crab, with a broad range of specificity (2, 12, 13). These lectins have been proposed to function in concert to defend horseshoe crabs from invading pathogens. However, because these lectins are present in the granules of the hemocytes, they are unlikely to be involved in the immediate-early response of host-pathogen interaction.

In this study, we report the isolation and characterization of three novel pathogen-specific proteins from the hemolymph of Tachypleus tridentatus that interact with each other and with pathogen specific antigens, in a manner similar to the opsonization process in vertebrates. A model of interaction for these proteins has been proposed.

MATERIALS AND METHODS

Reagents—Escherichia coli O55:B5 lipopolysaccharide (LPS)$^1$ was purchased from Sigma. Sepharose CL-4B, CNBr-activated Sepharose CL-4B, molecular weight standards, staphylococcal protein A (SpA), and SpA Sepharose CL-4B were from Amersham Pharmacia Biotech. Complete protease inhibitor tablets were from Roche Molecular Biochemicals. All other chemicals were of the highest quality commercially available.

Horseshoe Crab and Hemolymph—T. tridentatus specimens were captured on the beaches of Quinoi Island, Taiwan. Horseshoe crabs were bled by cardiac puncture, and hemolymph was collected in a conical tube containing equal volume of chilled sterile 3% NaCl supplemented with 2 mM propanol and 2 mM phenylmethylsulfonyl fluoride to maintain the isotonic condition and to prevent the lysis of amebocytes (14). The amebocytes were separated from plasma by centrifugation at

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1 The abbreviations used are: LPS, lipopolysaccharide; GBP, galactose-binding protein; SpA, staphylococcal protein A; PAP, protein A-binding protein; HPLC, high performance liquid chromatography; LBP, LPS-binding protein; PAGE, polyacrylamide gel electrophoresis.

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Purification of Lectins from Hemolymph—To prepare material for affinity chromatography, hemolymph was thawed and filtered through a 0.2 μm pyrogen-free filter to remove insoluble residues. Protease inhibitor tablets were added according to the manufacturer’s instructions. Five hundred milliliters of filtered, protease inhibitor-supplemented hemolymph was passed sequentially through three 10-cm tandemly linked affinity columns packed with Sepharose CL-4B, SpA-Sepharose CL-4B, and LPS-Sepharose CL-4B, respectively. The columns were pre-equilibrated with initial buffer (10 mM TrisCl, pH 7.4, 150 mM NaCl) and, at the end of sample loading, washed with at least 10 column volumes of the initial buffer until a steady baseline was obtained. The columns were then detached from each other. To recover proteins from the affinity matrix, the Sepharose CL-4B column was eluted with 6 M guanidine hydrochloride, the SpA CL-4B column with 0.1 M citric acid, and the LPS column with 4 M urea in 10 mM TrisCl, pH 7.4. The effluent fractions containing the adsorbed proteins were collected and desalted by gel filtration on a 1 × 10-cm Sephadex G-25 column, equilibrated, and eluted with 10 mM ammonium bicarbonate. The entire purification procedure was performed at 4 °C.

Reverse Phase HPLC Analysis—High performance liquid chromatography was performed on an HP1100 (Hewlett-Packard) HPLC system with a C4 column (Aquapore butyl, 0.25 mm × 10 cm; Perkin-Elmer) using a flow rate of 0.25 ml/min. The compositions of Buffer A and Buffer B were acetonitrile:water trifluoroacetic acid at 10:90:0.1 and acetonitrile:water:trifluoroacetic acid at 90:0.1:0.01, respectively. Proteins were analyzed by an isocratic elution of 0% Buffer B for 10 min followed by a linear gradient from 0 to 100% Buffer B within 40 min and finally with an isocratic elution of 100% Buffer B for 10 min.

Sugar Analysis—PAS stain was performed to assay for glycoprotein. At the end of SDS-PAGE, gel was fixed with trichloroacetic acid, oxidized with periodic acid, followed by staining with Schiff’s reagent and destaining with acetic acid as described (15). Monosaccharide contents were analyzed by GC-MS using the Hewlett-Packard gas chromograph model 6890 connected to a HP 5973 mass selective detector. Samples for analysis were subjected to methanolysis, re-N-acetylation, and trimethylsilylation (16) and dissolved in hexane prior to splitless injection on a HP-5MS fused silica capillary column (30 m × 0.32 mm inner diameter, Hewlett-Packard). The column head pressure was maintained at around 8.2 psi to give a constant flow rate of 1 ml/min using helium as carrier gas. Oven temperature was held at 60 °C for 1 min, increased to 90 °C in 1 min, and then increased to 290 °C in 25 min. The trimethylsilyl derivatives were analyzed by GC-MS on the HP system using a temperature gradient of 60–140 °C at 25 °C/minute and then increased to 300 °C at 10 °C/minute.

Protein Sequencing and Sequence Analysis—Sampling of proteins recovered from the reverse-phase HPLC and from SDS-PAGE/electroblotting were performed on an ABI 492 Procise automatic protein sequencer (Perkin-Elmer). The initial yield ranged from 10 to 20 pmol. The sequences were then analyzed by the GCG package (Genetics Computer Group Inc.).

Surface Plasmon Resonance Analysis—Surface plasmon resonance technology was used to analyze the affinity between the purified lectins and their analytes. Three to 6 μg of hemolymph lectins were immobilized on sensor chips CM5 (BIAcore AB, Uppsala, Sweden) by the amine coupling method according to the manufacturer’s instructions. After immobilization, the sensor chips were washed with assay buffer (10 mM TrisCl, pH 7.4, 150 mM NaCl) until a stable baseline was obtained. The binding experiments were performed at 25 °C with a flow rate of 35 μl/min. Binding constant of the lectins was obtained by calculation of data in sensogram with the BIAevaluation program (Version 3.0, BIAcore AB, Uppsala, Sweden).

Peptidase Activity Assay—Peptidase activities were assayed in 0.1 M Tris-Cl, pH 8.0, containing a given amount of enzyme source and 2 mM 4-methoxybenzyl-Val-Pro-Arg-p-nitroanilide as substrate in a total volume of 220 μl. The reaction was allowed to proceed at 37 °C for 1 h. At the end of the incubation period, the reaction was stopped by adding 0.8 ml of 0.6 N acetic acid. Absorbency at 405 nm was measured for the release of p-nitroanilide as described (17).
mollymph varied according to the season of the year when the animal was captured. It was estimated to be 0.01–0.02% and 0.02–0.04% of the total hemolymph protein for PAP and LBP, respectively.

GBP, PAP, and LBP Are Glycoproteins—The presence of glycans in the three proteins was examined by PAS (data not shown), because microheterogeneties were detected in both SDS-PAGE and HPLC analyses (Fig. 1). All three proteins gave a positive signal confirming that these three proteins are glycoproteins. Further analysis of sugar composition showed that although all three proteins are glycosylated, PAP and LBP contain a higher amount of galactose than GBP (Table I).

N-terminal Sequence Analysis of Hemolymph Lectins—The results of N-terminal residue analyses of GBP, PAP, and LBP are summarized in Fig. 2. The N-terminal residues 1–26 of GBP contain a higher amount of galactose than GBP (Table I).

Table II

| Ligand/analytes | $k_a$ | $k_d$ | $K_D$ |
|-----------------|------|------|------|
| GBP/galactose$^a$ | $1.08 \pm 0.05 \times 10^{-4}$ | $2.67 \pm 0.04 \times 10^{-4}$ | $2.47 \times 10^{-7}$ |
| PAP/SpA$^b$ | $5.35 \pm 6.95 \times 10^{-3}$ | $3.62 \pm 0.11 \times 10^{-4}$ | $3.86 \times 10^{-5}$ |
| GBP/LPS$^c$ | $3.32 \times 10^{-3}$ | $3.42 \times 10^{-3}$ | $1.03 \times 10^{-6}$ |
| GBP/PAP$^d$ | $4.33 \times 10^{-3}$ | $5.43 \times 10^{-3}$ | $1.25 \times 10^{-5}$ |
| GBP/LBP$^e$ | $9.9 \times 10^{-5}$ | $1.41 \times 10^{-3}$ | $1.43 \times 10^{-5}$ |
| GBP · PAP/SpA$^{d,e}$ | $3.73 \times 10^{-3}$ | $4.62 \times 10^{-5}$ | $1.24 \times 10^{-9}$ |
| GBP · LBP/LPS$^{d,h}$ | $6.39 \times 10^{-3}$ | $3.12 \times 10^{-3}$ | $4.95 \times 10^{-7}$ |

$^a$ Calculated from binding assays using 1, 0.9, 0.8, 0.7, 0.6, 0.1, and 0.05 M (±)-galactose as analytes.
$^b$ Calculated from binding assays using 119, 59.5, 29.8, 14.9, and 7.44 M SpA as analytes.
$^c$ Calculated from binding experiments using 20 pm LPS as analytes.
$^d$ Constants obtained from average of independent duplicated experiments.
$^e$ Calculated from binding experiments using 17.5 μM PAP as analytes.
$^f$ Calculated from binding experiments using 17.5 μM LBP as analytes.
$^g$ Calculated from binding experiments using 100 μM SpA as analytes, which binds to the preformed complex between GBP and PAP (GBP · PAP).
$^h$ Calculated from binding experiments using 20 μM LPS as analytes, which binds to the preformed complex between GBP and LBP (GBP · LBP).

GBP, PAP, and LBP share a 80% sequence identity with each other (Fig. 2D), and residues 1–27 of PAP share a 62% identity with residues 47–73 of tachylectin-3, a 14-kDa protein isolated from the hemocytes of T. tridentatus (18), as shown in Fig. 2B. Residues 1–40 of LBP share a 72% sequence identity with residues 47–86 of this lectin (Fig. 2C).

Binding Specificity of GBP, PAP, and LBP—The fact that GBP, PAP, and LBP were selectively adsorbed and eluted from their respective affinity column is a strong indication that each of the protein binds specifically to its own ligand. Table II summarizes the measurement of surface plasmon resonance responses of the immobilized GBP, PAP, and LBP against their respective analytes. (±)-galactose bound to GBP with a $K_D$ of
under these conditions, it was expected that both GBP and LBP would bind to this affinity column. The column was washed with the initial buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl) until a stable baseline was obtained and subsequently eluted with 4 mM urea, in 10 mM Tris-Cl, pH 7.4. After removal of urea by dialysis or gel filtration, the recovered proteins were subjected to SDS-PAGE/electroblotting sequence analysis. Unlike GBP, PAP, and LBP recovered from the three tandemly connected affinity columns, proteins recovered from the direct passage of hemolymph through the LPS-Sepharose CL-4B column showed a complex mixture of protein bands (Table III and Fig. 4). The N-terminal sequences showed that they represented proteolytically degraded forms of GBP (Table III). LBP or its degraded derivatives, if present, were too low to be detected.

The presence of peptidase activity in the effluent from the LPS-Sepharose CL-4B column was examined, using a typical thrombin substrate, 4-methoxybenzyl-Val-Pro-Arg-pNA. The results presented in Table IV indicate that although only a negligible peptidase activity was detected in the freshly collected hemolymph, the activity increased nearly 10-fold after the hemolymph had passed through the LPS-Sepharose CL-4B column. Addition of LPS to the hemolymph enhanced its peptidase activity by nearly 100-fold. Notably, the specific activity of the 4 mM urea eluate was about 1600 times higher than the hemolymph spiked with LPS. Neither GBP, PBP, nor PAP isolated in the presence of protease inhibitors (as described above) exhibited any significant peptidase activity. The peptidase activity associated with the 4 mM urea eluate from the LPS-Sepharose CL-4B column in the absence of protease inhibitors, therefore, is most likely due to the presence of a peptidase or its precursor activated by LPS. The amount of peptidase eluted by 4 mM urea was estimated to be less than 0.01% (w/w) of the proteins eluted by 4 mM urea. This estimation is based on the assumption that the putative peptidase possesses a specific activity (milliunits/g of protein) similar or identical to that of trypsin (Table IV).

**DISCUSSION**

An invertebrate depends solely on innate immunity, consisting of three major coordinately working systems (pattern recognition, enzymatic cascade, and antimicrobial peptide) to protect itself against microbial invasion. Molecular structures that are integral to the Gram− bacteria, such as LPS, are not subjected to changes during the evolutionary process and are highly conserved among these microorganisms. Molecules that are able to recognize these structures would thus be able to identify a wide spectrum of bacteria. In this study, we have isolated and characterized such a protein from the hemolymph of *T. tridentatus*, the pattern recognition molecules that specifically interact with the LPS of Gram− bacteria. Independently, GBP, a protein that binds to SpA of a Gram + bacterium, *S. aureus*, was isolated and characterized. Based on the finding that GBP and PAP share an 80% identity in their N-terminal sequences (Fig. 2D), GBP and PAP must be very similar, yet each recognizes a different ligand.

The N-terminal amino acid sequences (residues 1−19) determined for GBP and for PAP share 47−50% identity with residues 2−20 of lectin L-6, an LPS-binding protein isolated from the large granules of amebocyte (Fig. 2A) and C). Significant sequence homologies were not found with any other proteins for which the sequences have been elucidated. Whereas the plasma GBP is a glycosylated protein, lectin L-6 is a nonglycosylated amebocyte protein (12). The existence of two similar lectins, an intracellular one and an extracellular one, in the

| TABLE III | Protein sequence from amino termini of GBP derivatives |
|-----------|------------------------------------------------------|
| Sample    | Protein sequence                                     |
| GBP, 30 kDa (I) | EWTH1 NRKL HSHTVT PRFV                              |
| GBP, 30 kDa (II)| EWTH1 NRKL HSHTVT PRF                              |
| GBP, 30 kDa (III)| EWTH1 NRKL HSHTVT PRF                              |
| GBP, 18 kDa | EWTH1 NRKL                                              |
| GBP, 16 kDa | EWTH1 NRKL                                              |

* Proteins sequences obtained from microsequencing of electroblots of the respective bands designated in Fig. 4.

**Fig. 4. Bands used to obtain protein sequences.** See Table III for details.

| TABLE IV | Association of protease activity with GBP·LBP complex |
|-----------|------------------------------------------------------|
| Sample    | Protein amount | Activity | Specific activity | Relative activity |
|-----------|----------------|----------|-------------------|-------------------|
| Trypsin   | 0.1            | 3.3 x 10^2 | 3.3 x 10^5        | 9.43 x 10^3       |
| Hemolymph | 79.1           | 2.7 x 10^2 | 3.5 x 10^5        | 1                 |
| Flow-through | 526.3        | 1.7 x 10^1 | 3.2 x 10^4        | 9                 |
| GBP·LBP complex | 1.5       | 8.2 x 10^2 | 5.5 x 10^2        | 1.57 x 10^2       |
| Hemolymph + LPS | 50.0      | 1.5 x 10^2 | 2.9 x 10^2        | 83                |

* One milliunit is defined as the amount of enzyme that catalyzes the release of 1 nmol of 4-nitroaniline from Moz-Val-Pro-Arg-pNA per min at 37°C, pH 8.0, as measured by the increase in A280 during a period of 60 min. Extinction coefficient of 4-nitroaniline (ε280) equals 9.62 cm²/µmol.
  * Effluent eluted by 4 mM urea, 10 mM Tris·Cl, pH 7.4. Urea in the sample was removed as described under "Materials and Methods."
horseshoe crab is reminiscent of the mammalian system (19, 20).

The principal reactivity of SpA is binding with IgG at the Fc site and affinity chromatography on SpA-Sepharose is the preferred method of isolating IgG from serum of animals (21). Although a number of proteins with IgG-like motif and property have been reported to be present in invertebrates, sequences that are homologous to the Fc site of IgG have not been reported, nor has their binding to SpA been demonstrated (22). The binding site of PAP to SpA remains to be elucidated.

Notably, the peptidase activities induced by exposure of the plasma to a catalytic amount of LPS were almost quantitatively co-adsorbed with LBP-GBP to the LPS-Sepharose CL-4B affinity column and were co-eluted with GBP with 4 M urea. The origin of the peptidase activity is not known, but judging from the specific activity, which is less than \( \frac{1}{1000} \) that of trypsin, it is highly unlikely that it is the intrinsic property of GBP.

Data obtained from surface plasmon resonance indicated that GBP also binds with PAP and LBP. Binding of PAP and LBP to GBP significantly enhances the affinity of PAP and LBP to their ligands, SpA and LPS, respectively (Table II). Based on these observations, it is suggested that GBP forms a complex with PAP and LBP in the hemolymph of horseshoe crab. Both PAP and LBP contain a higher amount of galactose than GBP (Table I). It is tempting to speculate that the binding of GBP to PAP and to LBP might be mediated by the galactosyl moiety on PAP and LBP.

When pathogens carrying SpA or LPS ligand enter into horseshoe crabs, GBP-anchored LBP or PAP docks onto the pathogens to form the GBP-PAP-SpA or GBP-LBP-LPS complex, as illustrated in Fig. 3. This complex formation could result in the recruitment of other defense mechanism in the amebocytes for the elimination of the invading pathogens.

Nature has evolved a series of galactose recognition proteins that are ubiquitously distributed throughout invertebrates and vertebrates (23–27). Anti-Gal IgG is the most abundant IgG in invertebrates and the most striking feature of this IgG is its ability of discriminating between self and non-self. In this study, we have identified a galactose-binding protein from the hemolymph of horseshoe crabs, which could be a counterpart of anti-Gal IgG in invertebrates.

It would seem therefore, that upon the entry of Gram – bacteria, GBP-LBP complex binds to LPS on the bacterial cell surface and at the same time recruit LPS-dependent plasma peptidase to initiate proteolytic attack on the microbes. The affinity of PAP for SpA, as demonstrated by its selective adsorption to SpA-affinity column and the pervasive presence of SpA as a membrane component of Gram + bacterium, such as \( S. aureus \), could signify its importance in the recognition of such microorganisms upon their entry into horseshoe crab.

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