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Characterization of a Pattern Recognition Protein, a Masquerade-Like Protein, in the Freshwater Crayfish *Pacifastacus leniusculus*¹

So Young Lee and Kenneth Söderhäll²

A multifunctional masquerade-like protein has been isolated, purified, and characterized from hemocytes of the freshwater crayfish, *Pacifastacus leniusculus*. It was isolated by its *Escherichia coli* binding property, and it binds to formaldehyde-treated Gram-negative bacteria as well as to yeast, *Saccharomyces cerevisiae*, whereas it does not bind to formaldehyde-fixed Gram-positive bacteria. The intact masquerade (mas)-like protein is present in crayfish hemocytes as a heterodimer composed of two subunits with molecular masses of 134 and 129 kDa. Under reducing conditions the molecular masses of the intact proteins are not changed. After binding to bacteria or yeast cell walls, the mas-like protein is processed by a proteolytic enzyme. The 134 kDa of the processed protein yields four subunits of 65, 47, 33, and 29 kDa, and the 129-kDa protein results in four subunits of 63, 47, 33, and 29 kDa in 10% SDS-PAGE under reducing conditions. The 33-kDa protein could be purified by immunoaffinity chromatography using an Ab to the C-terminal part of the mas-like protein. This subunit of the mas-like protein has cell adhesion activity, whereas the two intact proteins, 134 and 129 kDa, have binding activity to LPSs, glucans, Gram-negative bacteria, and yeast. *E. coli* coated with the mas-like protein were more rapidly cleared in crayfish than only *E. coli*, suggesting this protein is an opsonin. Therefore, the cell adhesion and opsonic activities of the mas-like protein suggest that it plays a role as an innate immune protein. *The Journal of Immunology*, 2001, 166: 7319–7326.

Microorganisms have highly conserved and widely distributed signature molecules in their cell walls such as LPSs (1) or peptidoglycans of bacterial cell walls and β-1,3-glucans of fungal cell walls, which are not found in other multicellular organisms. These microbial carbohydrates can then serve as recognition molecules for different microorganisms; they are recognized by molecules of the immune or defense systems of both plants and animals that have been named pattern recognition molecules (1). To date, a number of LPS-recognition/binding molecules have been isolated and characterized from several animals, and these pattern recognition molecules have been shown to be involved in the innate immune system of both invertebrates and vertebrates (1–3). Vertebrates have developed an immune defense repertoire that includes an adaptive memory with Igs and innate immune systems, including phagocytes, NK cells, and the complement system to defend themselves against pathogenic microorganisms. Invertebrates defend themselves by using innate immune responses, because they cannot produce Igs, although proteins that contain domains belonging to the Ig superfamily have been found in invertebrates (4). The innate immune system in invertebrates consists of cellular and humoral responses. The phagocytosis and opsonization of invading microorganisms by blood cells, proteolytic cascades leading to localized blood clotting, melanin formation, and transient synthesis of potent antimicrobial peptides contribute to this resistance (3). LPS binding molecules are of particular importance because LPS binding results in markedly different functional outcomes. Some of the LPS binding molecules have opsonic effect (5, 6), can degranulate blood cells (7), can participate in hemocyte nodule formation (8), and can clear bacteria from the circulation (9). Recently, LPS or/and β-1,3-glucan binding proteins from a freshwater crayfish (10) and two species of insects, the silkworm (11) and the tobacco hornworm (12), have been shown to be involved in activation of the prophenoloxidase-activating system (proPO system).³

The proPO system is an important nonself-recognition system present in most invertebrates. It is an enzyme cascade containing several serine proteinases and their inhibitors and terminates with the zymogen, prophenoloxidase (13). Microbial polysaccharides such as LPS or β-1,3-glucans will first react with pattern recognition proteins, which then will induce activation of several serine proteinases within the proPO system. One of these terminal serine proteinases in the cascade, which is named the phenoloxidase-activating enzyme, will cleave proPO to generate the active enzyme, phenoloxidase. This enzyme can produce toxic compounds to microorganisms by oxidizing phenols to melanin (13), and it also participates in the sclerotization of the cuticle (14). Upon activation of the proPO system, not only will melanization occur, but other proteins will gain their biological function concomitant with proPO activation and can participate in cellular defense. One such molecule is a cell adhesion molecule, peroxinectin (15, 16).

Interestingly, several serine proteinase homologues are present in animals, and they have a variety of biological functions, such as a cell adhesion activity, e.g., *Pacifastacus* masquerade (mas)-like protein (17), *Drosophila* mas (18), glactatin (19), and neurotactin.

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³ Abbreviations used in this paper: proPO system, phenoloxidase-activating system; mas, masquerade; HLS, hemocyte lysate supernatant; LB, Luria Bertoni; HGF, hepatocyte growth factor.
(20); antimicrobial and LPS binding activity, e.g., human azurocidin (21–24) and horseshoe crab factor D (25); to function as a growth factor, e.g., human hepatocyte growth factor (HGF) (26); a component of proPO system, e.g., 45-kDa serine proteinase homologue protein of coleopteran insect, H. diomphalia (27); and an immune molecule, e.g., mosquito infection-responsive serine protease-like protein (ispl5) (28). These molecules show homology to serine proteinases, except for a substitution(s) within the catalytic triad that will render them without proteinase activity. Although these molecules do not exhibit any proteinase activity, their biological functions in animals suggest that these serine proteinase homologues serve as important immune-related molecules.

We here describe the structure of the mas-like protein from the hemocytes of the freshwater crayfish Pacíficastacus leniusculus and its binding ability to Gram-negative bacteria and yeast and to their corresponding signature molecules. These results demonstrate that this mas-like protein from an invertebrate is a pattern recognition molecule.

**Materials and Methods**

**Animals**

Freshwater crayfish, *Pacíficastacus leniusculus*, were purchased from Berga Kräftodling (Södermanland, Sweden) and were maintained in tanks with aerated water at 10°C. Only intermolt crayfish were used in this study.

**Purification of the mas-like protein**

Hemocyte lysate supernatant (HLS) was prepared by collecting hemolymph from 30 crayfish in anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citic acid, and 10 mM EDTA, pH 4.6) (29) and centrifuged at 4°C and 800 g for 10 min. The hemocyte pellets were homogenized in crayfish PBS (10 mM Na$_2$HPO$_4$, 10 mM KH$_2$PO$_4$, 0.15 M NaCl, 10 mM CaCl$_2$, 10 mM MnCl$_2$, and 2.7 mM KCl, pH 8.0) and then centrifuged at 4°C and 16,000 g for 30 min. The protein concentration of HLS from 30 crayfish is ~4 mg. The resulting supernatant was used to purify the mas-like protein by using in vitro bacterial binding affinity precipitation as described by Koizumi et al. (9). Briefly, *Escherichia coli* D21 was cultured in 9 ml Muria Bertoni (LB) medium (10 g peptone, 5 g yeast extract, 5 g NaCl, and 1 g glucose/liter distilled water) for 6 h at 37°C. The cells in the logarithmic growth phase were fixed with 1 ml 37% formaldehyde by gently shaking at 37°C to destroy the proteinase activity of the bacteria. After 1 h the fixed cells were harvested by centrifugation at 4°C and 2000 × g for 10 min, washed twice with crayfish PBS, and then finally resuspended in crayfish PBS. A bacterial suspension of 0.5 ml (final concentration, 3.5 × 10$^5$ cells) and 0.5 ml crayfish HLS (final concentration, 0.75 mg proteins) were mixed, incubated with gentle shaking at 4°C for 30 min, and then centrifuged at 2000 × g and 4°C for 10 min. The pellets were removed, and the cells were washed twice with crayfish PBS. Bound proteins were subsequently eluted with 100 μl 50 mM sodium citrate, pH 3.5, by centrifugation at 2000 × g and 4°C for 10 min, and the eluates were immediately neutralized with 1 M Tris base. As a control, *E. coli* D21 incubated with crayfish PBS was used as above. The eluates were run on SDS-PAGE and subjected to immunoblotting using the anti-mas-like protein Ab.

To determine the saturation of binding of the mas-like protein to *E. coli* cells, different concentrations of *E. coli* (1 × 10$^4$, 10$^5$, 5 × 10$^5$, 2 × 10$^6$, 10$^6$, 1 × 10$^7$, 5 × 10$^7$, and 2.5 × 10$^8$) were incubated in 1 ml crayfish HLS containing 2.5 mg protein. Also, different concentrations of HLS from 30 crayfish is ~4 mg. The resulting supernatant was subjected to SDS-PAGE and stained with 0.2% Coomassie blue in 50% methanol. The bands were excised and treated with 37% TCA and 10% methanol, pH 11.0. The bands on the membrane were excised after staining with Coomassie blue and subjected to Applied Biosystems 476A automated protein sequencer for amino acid sequence. For determination of the internal amino acid sequence of the bound proteins, the bands were subjected to SDS-PAGE and stained with 0.2% Coomassie blue in 50% methanol. The bands were excised and treated with lysylendopeptidase according to the method described by Wilm et al. (32). The resulting peptides were separated by HPLC (Pharmacia Smart chromatography system) on a phase μRPC C$_2$/C$_18$ column (MIC-15-03-MRP; Amershams Pharmacia Biotech). The most prominent peak was sequenced using an Applied Biosystems 476A sequencer (Foster City, CA).

**Assay of specific binding activity to microorganisms, LPS, and β-1,3-glucans**

The Gram-negative bacteria (*E. coli* D21, *E. coli* Y1088, *Proteus vulgaris* OX19 ATCC 6380 (American Type Culture Collection, Manassas, VA), *Shigella flexneri* ATCC 203 (American Type Culture Collection), and *Pseudomonas aeruginosa* OT 97), the Gram-positive bacteria (*Bacillus megaterium* Bm 11, *Staphylococcus aureus* JC 1, and *Micrococcus luteus* M1 11), and the yeast, *Saccharomyces cerevisiae* W1134-1D were used for testing the specific binding property of the mas-like protein. Various bacterial and yeast extracts were cultured in 9 ml LB medium or YPD medium (10 g yeast extract, 20 g peptone, 20 g glucose/liter distilled water) for 6 h at 37°C, respectively. Then the purification method of the bound protein described above was followed. The resulting eluates and supernatants were run on 10% SDS-PAGE under reducing conditions and then electrotransferred to nitrocellulose membranes in transfer buffer (25 mM Tris-HCl, 190 mM glycine, and 20% methanol) for 2 h at 280 mA on ice. All the following steps were performed at room temperature. The membrane was subsequently blocked in TTBS (0.1% Tween 20 in 20 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 3% BSA for 1 h and incubated with Ab in TTBS containing 0.1% BSA overnight. An affinity-purified Ab (10 μg/ml) was used for the immunoblotting. Then the membrane was washed with TTBS once for 15 min and three times for 5 min each time. The anti-rabbit IgG peroxidase-conjugated IgG diluted 1/20,000 with TTBS containing 0.1% BSA was incubated for 1 h and washed with TTBS once for 15 min and four times for 5 min each time. For detection, the ECL Western blotting reagent kit (Amershams Pharmacia Biotech) was used.

**Determination of amino acid sequences**

To determine the N-terminal amino acid sequences, the 65, 63, 47, and 33 kDa, were subjected to SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid and 10% methanol, pH 11.0. The bands on the membrane were excised after staining with Coomassie blue and subjected to Applied Biosystem 476A automated protein sequencer for amino acid sequence. For determination of the internal amino acid sequence of the bound proteins, the bands were subjected to SDS-PAGE and stained with 0.2% Coomassie blue in 50% methanol. The bands were excised and treated with lysylendopeptidase according to the method described by Wilm et al. (32). The resulting peptides were separated by HPLC (Pharmacia Smart chromatography system) on a phase μRPC C$_2$/C$_18$ column (MIC-15-03-MRP; Amershams Pharmacia Biotech). The most prominent peak was sequenced using an Applied Biosystems 476A sequencer (Foster City, CA).

**Electrophoresis**

Ten percent and 6% SDS-PAGE were conducted by the method of Laemmli (31). Samples were denatured by heating them for 4 min at 95°C in SDS-PAGE sample loading buffer with or without 0.1% DTT. The gels were stained according to the method of Fairbank et al. (31). A low molecular mass calibration kit for electrophoresis (Amershams Pharmacia Bio-tech, Arlington Heights, IL) was used for size markers: rabbit muscle phosphorylase b (94 kDa), BSA (67 kDa), egg white OVA (43 kDa), and bovine erythrocyte carbonic anhydrase (30 kDa). The high molecular mass marker was purchased from Sigma (St. Louis, MO); rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), BSA (66 kDa), egg white OVA (45 kDa), and bovine erythrocyte carbonic anhydrase (29 kDa).

**Ab and immunoblotting**

An antiserum against the crayfish mas-like protein was made by first preparing a synthetic peptide, CFTPQDLRVRWVSGRSTS, corresponding to a portion of the 33-kDa band (the serine proteinase-like domain) of the crayfish mas-like protein as described by Huang et al. (17). Briefly, the synthetic peptide was coupled to OVA (Sigma) using sulfo-N-maleimide benzoyl-N-hydroxysulfosuccinimide ester (Calbiochem, La Jolla, CA) as a coupling agent and then was used for production of a rabbit antiserum. An affinity-purified Ab using the synthetic peptide coupled to cyanogen bromide-activated Sepharose 4B (Amershams Pharmacia Biotech) was used for immunoblotting.

For immunoblotting, the proteins were subjected to 10% SDS-PAGE under reducing and nonreducing conditions and then electrotransferred to nitrocellulose membranes in transfer buffer (25 mM Tris-HCl, 190 mM glycine, and 20% methanol) for 2 h at 280 mA on ice. All the following steps were performed at room temperature. The membrane was subsequently blocked in TTBS (0.1% Tween 20 in 20 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 3% BSA for 1 h and incubated with Ab in TTBS containing 0.1% BSA overnight. An affinity-purified Ab (10 μg/ml) was used for the immunoblotting. Then the membrane was washed with TTBS once for 15 min and three times for 5 min each time. The anti-rabbit IgG peroxidase-conjugated IgG diluted 1/20,000 with TTBS containing 0.1% BSA was incubated for 1 h and washed with TTBS once for 15 min and four times for 5 min each time. For detection, the ECL Western blotting reagent kit (Amershams Pharmacia Biotech) was used.
eluted by SDS-PAGE sample loading buffer (60 mM Tris-HCl, pH 6.8, containing 2% SDS, 1% glycerol, 0.01% bromophenol blue, and 0.1% DTT) after 8 h and treated by heating at 95°C for 4 min. The resulting supernatant and eluted proteins were applied to 10% SDS-PAGE under reducing conditions and subsequently developed for immunoblotting using the anti-mas-like protein Ab.

Whether the mas-like protein purified by its \( E. coli \) D21 binding property has retained its binding activity, its binding activity to microorganisms, LPS, and laminarin was investigated. The purified protein (4 \( \mu g/20 \)\( \mu l \)) was again incubated with microorganisms, LPS, or laminarin in 1 ml crayfish PBS and eluted using the conditions described above. The resulting eluates and supernatants were applied to 10% SDS-PAGE under reducing conditions and subsequently subjected to immunoblotting using the anti-mas-like protein Ab.

**Determination of the structure of the mas-like protein**

To detect the intact (nonprocessed) form of the mas-like protein, crayfish HLS was prepared in crayfish PBS or CAC buffer. The crayfish HLS and the mas-like protein were subjected to 6 and 10% SDS-PAGE under reducing conditions or nonreducing conditions. The anti-mas-like protein Ab was used for immunoblotting to compare the intact mas-like protein in HLS with the processed form of the mas-like protein after \( E. coli \) recognition.

For analysis of the structure of the processed mas-like protein, the protein was purified by its \( E. coli \) binding activity and then run on 6% SDS-PAGE under nonreducing conditions, and the 134- and 129-kDa bands were eluted using the Electro-Eluter (model 422; Bio-Rad, Hercules, CA) according to the manufacturer’s instruction. The 134- and 129-kDa bands were obtained by electrophoresis from this 6% SDS-PAGE and then subjected to immunoblotting.

The mas-like protein was run on 6% SDS-PAGE under nonreducing conditions and stained with Coomassie blue, and then two bands, 134 and 129 kDa, were cut out. The gel pieces were subjected to 10% SDS-PAGE under reducing conditions to confirm the bands obtained derived from the 134- and 129-kDa proteins, respectively.

**Opsonic activity of the mas-like protein**

In vivo bacterial clearance was tested as described by Koizumi et al. (9).

Briefly, \( E. coli \) D21 was cultured in LB broth until the logarithmic growth phase. The cells were harvested by centrifugation and washed twice with cryfish PBS, then the cells (2 \( \times 10^8 \) cells) were incubated in 1 ml cryfish HLS containing 2.5 mg protein at 4°C for 1 h to obtain \( E. coli \) coated with the mas-like protein (mas-\( E. coli \)). The supernatant was then removed, and the cells were washed three times with cryfish PBS. Fifty microliters of non-treated \( E. coli \) (control) or mas-\( E. coli \) containing 1 \( \times 10^7 \) cells were injected together with 50 \( \mu l \) LPS (1 \( mg, E. coli \) serotype 026:B6 (Sigma) suspended in 1 ml cryfish PBS) into cryfish to activate the cryfish cells. Animals receiving \( E. coli \) coated with mas-like protein and LPS had much higher clearance rates than those animals receiving mas-like protein-coated \( E. coli \). Treated cryfish were kept at 10°C in aerated water, and the he-molymph from six cryfish was collected at different time periods (30 min, 1 h, 3 h, and 6 h). Five hundred microliters of hemolymph was collected from each cryfish; 100 \( \mu l \) of this hemolymph was plated immediately on an LB agar plate and incubated overnight at 37°C, and the number of viable bacteria was determined.

**Results**

**Isolation and purification of the mas-like protein**

The cryfish mas-like protein was isolated by incubating HLS of \( P. leniusculus \) with formaldehyde-fixed \( E. coli \) D21. After incubation, bound proteins were eluted using an acidic solution (50 mM sodium citrate, pH 3.5) and then neutralized. The eluate was analyzed by 10% SDS-PAGE under both nonreducing and reducing conditions. Two bands with molecular masses of 134 and 129 kDa were observed in SDS-PAGE under nonreducing conditions. Under reducing conditions these bands gave rise to five subunits, 65, 63, 47, 33, and 29 kDa (Fig. 1). As a control, \( E. coli \) and cryfish PBS were incubated instead of the cryfish HLS. No protein could be eluted from bacteria itself using sodium citrate, pH 3.5 (data not shown). This result demonstrates that the \( E. coli \) binding proteins are from cryfish HLS and not from \( E. coli \) itself. About 10 \( \mu g \) purified bacterial recognition protein could be isolated from the HLSs from 30 cryfish containing 4 mg protein.

**Analysis of the HLS proteins bound to \( E. coli \) D21**

\( E. coli \) D21 cells were treated with \( P. leniusculus \) hemocyte lysates and then eluted with sodium citrate as described in Materials and Methods. Twenty microliters of the eluate was subjected to 10% SDS-PAGE under nonreducing (lanes 1–3) and reducing (lanes 4–6) conditions. Lanes 1 and 6, Size marker; lanes 2 and 4, 15 \( \mu g \) crude HLS; lanes 3 and 5, 20 \( \mu l \) purified protein after incubation with \( E. coli \).

**Determination of amino acid sequences**

The N-terminal amino acid sequences and the partial internal amino acid sequences of the 65-, 63-, 47-, 33-, and 29-kDa proteins were determined by Edman degradation and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. All the amino acid sequences could be matched exactly to the previously cloned and characterized cryfish mas-like protein (Fig. 2A) (17). The N-terminal amino acid sequence of the 65- and 63-kDa proteins had identical amino acid sequences from T102 to A106. The N-terminal amino acid sequences of the 47- and 33-kDa proteins were from F407 to A421 and from I714 to E728, respectively. Internal amino acid sequences of the 65- and 63-kDa proteins were determined on peptides obtained after lysylendopeptidase treatment, and the sequences were consistent with the deduced sequence between residues N110 and K120. A second internal amino acid sequence from residues I395 to R406 was determined using another fragmented peptide of the 65-kDa protein. Partial amino acid sequences of the 45- and 33-kDa proteins were also determined, and amino acid residues from P628 to K639 and residues from D851 to K863, respectively, corresponded exactly with the deduced amino acid sequences of the mas-like protein.

Interestingly, there are several arginine residues in the cryfish mas-like protein, but only three cleavage sites were present at the specific residues, LDL/TR (Fig. 2, A and B). This sequence, LDL/TR, should be accessible to proteolytic enzyme cleavage of the mas-like protein and should generate its cell adhesion activity (17). The protein, which had been cleaved to generate the 65-, 63-, 47-, 33-, and 29-kDa subunits, is referred to as the processed form of the mas-like protein.

**Analysis of structure of the mas-like protein and immunoblotting**

To further investigate the structure of the processed mas-like protein, the 134- and 129-kDa proteins were separated on 6% SDS-PAGE under nonreducing conditions (Fig. 3A, lane 4). The proteins were excised, and then each gel piece was reloaded onto 10% SDS-PAGE under reducing conditions. The 134-kDa protein gave rise to 65-, 47-, 33-, and 29-kDa bands, whereas the 129-kDa protein produced 63-, 47-, 33-, and 29-kDa bands (Fig. 3B, lanes 3 and 4). These results show that the cryfish mas-like protein exists as a heterodimer, and some of the cysteine residues are likely to be involved in interdisulfide bonding (Fig. 2B).

The mas-like protein purified by its binding affinity to \( E. coli \) was applied to 10 or 6% SDS-PAGE under both reducing and...
nonreducing conditions, respectively. The anti-mas-like protein Ab could detect only the 33-kDa subunit (Fig. 3B, lane 5) under reducing conditions, because the Ab was made to a synthetic peptide corresponding to residues from F764 to S780 present in the 33-kDa band. However, under nonreducing conditions both 134- and 129-kDa proteins (Fig. 3A, lanes 2 and 3) were detected by the anti-mas-like protein Ab, because these two bands contain the 33-kDa protein (Fig. 3B). This result shows that neither the 65-, 63-, 47-, nor 29-kDa protein contained the 33-kDa protein. Based upon these results and numerous sequences of the different subunits, we can predict a putative structure of the mas-like protein (Fig. 2B).

**Binding specificity and characterization of the mas-like protein**

The anti-mas-like protein Ab was used to detect the specific binding activity to several kinds of microorganisms, LPS, curdlan, and laminarin, as well as to detect the intact form of the mas-like protein within the crayfish blood cell lysates (HLS). The crayfish HLS was mixed with various microorganisms, sodium citrate eluates obtained from them were subjected to 10% SDS-PAGE under reducing conditions (lane 1) and nonreducing conditions (lane 2).
The specific binding activity of the crayfish mas-like protein under nonreducing conditions, probably because the high amount of disulfide bonds prevents exposure of epitopes to this Ab (Fig. 2C). The band detected by the anti-mas-like protein Ab shows identical mobility to the processed form of the mas-like protein on SDS-PAGE under reducing conditions. The purified mas-like protein was subjected to 6% SDS-PAGE under nonreducing conditions and then stained with Coomassie blue. After the 134- and 129-kDa bands were cut out, these two bands were reloaded on 10% SDS-PAGE and then run under reducing conditions. Lane 1, Size marker; lane 2, E. coli binding protein as a control; lane 3, reloaded 134-kDa protein; lane 4, reloaded 129-kDa protein; lane 5, immunoblotting using the anti-mas-like protein Ab.

SDS-PAGE under reducing conditions (data not shown), whereas no band was detected by anti-mas-like protein Ab under nonreducing conditions, probably because the high amount of disulfide bonds prevents exposure of epitopes to this Ab (Fig. 2C). The band detected by the anti-mas-like protein Ab shows identical mobility to the processed form of the mas-like protein on SDS-PAGE under nonreducing conditions. Therefore, these results suggest that the intact and processed forms of the mas-like protein both exist as heterodimers consisting of 134- and 129-kDa proteins and that a proteolytic cleavage is likely to be involved in generating the processed form of the mas-like protein. The supernatant solution obtained after incubation with Gram-positive bacteria contained the intact form of the mas-like protein, demonstrating that it does not bind to Gram-positive bacteria (Fig. 4A).

Fig. 5 shows the specific binding activity of the mas-like protein to LPS, curdlan, and laminarin. These polysaccharides were incubated with crayfish HLS in PBS or CAC buffer and centrifuged after different time intervals. The supernatants and the eluted protein from each pellet were subjected to SDS-PAGE under reducing conditions and subsequently examined using the anti-mas-like protein Ab. The amount of the mas-like protein started to decrease in the supernatant fractions incubated for >2 h for LPS and 4 h for laminarin (Fig. 5A) and was detected by the anti-mas-like protein Ab in the eluted protein fractions of LPS, laminarin, or curdlan pellets (Fig. 5C). These results clearly demonstrate that the mas-like protein can bind both to LPS and β-1,3-glucans and, therefore, seems to play a role as a recognition protein for stimulation of innate immune system in crayfish. However, crayfish HLS prepared in CAC buffer containing 100 mM CaCl₂ did not exhibit any binding activity to LPS and β-1,3-glucans. In this case, a 27-kDa protein was detected by the anti-mas-like protein Ab instead of the 33-kDa subunit of the mas-like protein (Fig. 5B) (17).

The processed form of the mas-like protein was incubated with several bacteria, yeast, LPS, and β-1,3-glucans in crayfish PBS for testing its binding activity. It could not bind to any microorganism, LPS, or β-1,3-glucan (Fig. 6). These results show that the mas-like protein to various microorganisms. Crayfish HLS was incubated with various microorganisms in crayfish PBS, and the mixtures were centrifuged. Fifteen micrograms of supernatant protein was taken and precipitated with TCA. The precipitated proteins were subjected to 10% SDS-PAGE under reducing conditions and analyzed by immunoblotting using the anti-mas-like protein Ab. The pellets of each microorganism were eluted with TCA. The precipitated proteins were subjected to 10% SDS-PAGE under reducing conditions and then stained with Coomassie blue. After the 134- and 129-kDa bands were cut out, these two bands were reloaded on 10% SDS-PAGE and then run under reducing conditions. Lane 1, Size marker; lane 2, E. coli binding protein as a control; lane 3, reloaded 134-kDa protein; lane 4, reloaded 129-kDa protein; lane 5, immunoblotting using the anti-mas-like protein Ab.

![FIGURE 3. A, SDS-PAGE (6%) and immunoblotting analysis of the mas-like protein under nonreducing conditions. The proteins were separated by electrophoresis as described in Materials and Methods. Immunoblotting (lanes 1–3) with an Ab against a synthetic peptide of the mas-like protein. Lane 1, E. coli binding protein under nonreducing conditions; lane 2, purified 134-kDa protein; lane 3, purified 129-kDa protein; lane 4, SDS-PAGE pattern of E. coli binding protein after the Coomassie blue staining. B, SDS-PAGE (10%) and immunoblotting analysis of the mas-like protein under reducing conditions. The purified mas-like protein was subjected to 10% SDS-PAGE under reducing conditions and then run under reducing conditions. The purified mas-like protein was subjected to 6% SDS-PAGE under nonreducing conditions and then stained with Coomassie blue. After the 134- and 129-kDa bands were cut out, these two bands were reloaded on 10% SDS-PAGE and then run under reducing conditions. Lane 1, Size marker; lane 2, E. coli binding protein as a control; lane 3, reloaded 134-kDa protein; lane 4, reloaded 129-kDa protein; lane 5, immunoblotting using the anti-mas-like protein Ab.](http://www.jimmunol.org/)

![FIGURE 4. The specific binding activity of the crayfish mas-like protein to various microorganisms. Crayfish HLS was incubated with various microorganisms in crayfish PBS, and the mixtures were centrifuged. Fifteen micrograms of supernatant protein was taken and precipitated with TCA. The precipitated proteins were subjected to 10% SDS-PAGE under reducing conditions and analyzed by immunoblotting using the anti-mas-like protein Ab. The pellets of each microorganism were eluted with 100 µl of 50 mM sodium citrate, pH 3.5. Fifteen microliters of eluted protein was subjected to 10% SDS-PAGE under reducing conditions and analyzed by immunoblotting (B). Lane 1, Crude crayfish HLS; lane 2, E. coli D21; lane 3, E. coli Y1088; lane 4, P. vulgaris OX19 ATCC 6380; lane 5, S. flexneri ATCC 203; lane 6, P. aeruginosa OT 97; lane 7, S. cerevisiae W1134-1D; lane 8, B. megaterium Bm 11; lane 9, S. aureus JC-1; lane 10, M. luteus ATCC 203; lane 11.](http://www.jimmunol.org/)

![FIGURE 5. LPS and β-1,3-glucan binding activity of the mas-like protein. LPS or laminarin was incubated with crayfish HLS in crayfish PBS (A) or CAC buffer (B). After different incubation times (0, 1, 2, 4, 8, and 12 h), the mixtures were centrifuged, and the supernatants were precipitated with TCA. The resulting pellet was dissolved with SDS-PAGE sample loading buffer and subjected to 10% SDS-PAGE under reducing conditions followed by immunoblotting analysis. C. The pellets of LPS, curdlan, or laminarin were recovered from the incubated solution by high speed centrifugation after 8 h. The bound proteins were then eluted by SDS-PAGE sample loading buffer from each pellet, and these eluates were heat treated at 95°C for 4 min. The eluates were analyzed by SDS-PAGE and immunoblotting under reducing conditions. Lane 1, LPS pellet; lane 2, curdlan pellet; lane 3, laminarin pellet.](http://www.jimmunol.org/)
protein exists as an intact form in crayfish HLS, and this protein can recognize bacteria or yeasts by binding to their cell walls or their constituent carbohydrate, LPS, or β-1,3-glucan. Once the mas-like protein has been cleaved by a proteolytic enzyme and processed, it can no longer bind to microorganisms and only acts as a cell adhesion protein, the activity of which resides in the 33-kDa subunit (17).

**Biological function of crayfish mas-like protein**

The saturation of binding of the mas-like protein was determined by binding to *E. coli* cells. Various concentrations of *E. coli* cells were incubated with crude HLS, or different concentrations of crude HLS were incubated with *E. coli* cells, then the mas-like protein was detected in both the supernatant solution and eluted protein from *E. coli* by immunoblotting using the anti-mas-like protein Ab. The saturation of binding of the mas-like protein for coating 5 × 10^8 *E. coli* cells was 5 μg (Fig. 7). Accordingly, to ascertain the biological function of the mas-like protein in crayfish, 2 × 10^8 *E. coli* cells were incubated in 1 ml crayfish HLS containing 2.5 mg protein to obtain bacteria coated with the mas-like protein. These bacteria were tested under in vivo conditions in crayfish for their clearance rates compared with that of control bacteria not coated with this protein, and the results obtained clearly demonstrate that the mas-like protein functions as an opsonic protein (Fig. 8).

**Discussion**

In a previous study we showed that a mas-like protein from the crayfish, *P. leniusculus*, has cell adhesion activity and that its primary structure is similar to other known serine proteinase homologue proteins. These groups of proteins have a mutation in one or two of the three amino acids forming the catalytic triad, which results in a loss of the serine proteinase activity (17). In the present investigation we now show that the crayfish mas-like protein recognizes Gram-negative bacteria, i.e., *E. coli*, *P. vulgaris*, *S. flexneri*, and yeasts *S. cerevisiae*, but not Gram-positive bacteria, and also can participate in the clearance of bacteria in crayfish. The protein was isolated by its ability to bind to formaldehyde-fixed *E. coli* D21, and it consists of two isomers with masses of 134 and 129 kDa on SDS-PAGE under nonreducing conditions. Upon reduction, these two bands gave rise to five subunits (65, 63, 47, 33, and 29 kDa) with interdisulfide bonding. Amino acid sequences of these different subunits confirm that all of them belong to the mas-like protein of crayfish. Moreover, this mas-like protein was found in hemocytes in a biologically inactive form. After recognition and binding to microorganisms, a proteolytic enzyme cleaves the intact mas-like protein at a specific site, LDL/YR. According to amino acid sequences of the five bands, we can predict a structure for the intact and processed forms of the mas-like protein (Fig. 2B). The intact form of the mas-like protein contains seven putative disulfide-knotted motifs and three putative N-linked glycosylation sites, which are located at positions N92, N433, and N739 within the open reading frame. Two of the disulfide-knotted motifs are involved in interdisulfide bonding. The 134- and 129-kDa proteins are isomers encoded by one cDNA, and the difference in molecular masses are most likely results from glycosylation. The 134-kDa protein consists of the 65-, 47-, 33-, and 29-kDa subunits and the 129-kDa protein consists of 63-, 47-, 33-, and 29-kDa subunits. Obviously, the 65- and 63-kDa subunits are not processed to completion into 47- and 29-kDa subunits, because these
two molecules always exist in a fairly high concentration. The molecular masses of the processed five bands of the mas-like protein determined by SDS-PAGE do not correspond exactly to the deduced amino acid sequences, and this is also possibly a result of glycosylation of the 47- and 33-kDa subunits and the high amount of cysteines in the other subunits. Moreover, because the 65- and 63-kDa proteins showed identical N-terminal sequences, but had slightly different molecular masses on SDS-PAGE, we propose that glycosylation or cleavage at C-terminal part of the 65-kDa protein might produce a 63-kDa subunit. The structure of the mas-like protein and how the molecule is processed upon binding to bacteria and yeast are shown in Fig. 2. The processed form of the mas-like protein was unable to bind to any microorganisms, not even to LPS or β-1,3-glucans (Fig. 6).

*E. coli* coated with the mas-like protein and injected into crayfish hemolymph were more rapidly cleared and removed from the hemolymph by immune responses such as phagocytosis and encapsulation than noncoated control bacteria (Fig. 8). These results indicate that the crayfish mas-like protein have dual functions, as an LPS and β-1,3-glucan recognition protein and as a cell adhesion and/or opsonic protein. The intact crayfish mas-like protein recognizes invading Gram-negative bacteria and fungi, and upon binding the mas-like protein is cleaved by a proteolytic enzyme to generate the cell adhesion and opsonic activities, which will greatly accelerate the rate of phagocytosis.

Interestingly, the processing of the crayfish mas-like protein is similar to that of human HGF (26). HGF is a growth factor with a molecular mass of 82 kDa and is composed of a large α-subunit and a small β-subunit. This protein is processed to a mature form by cleavage between R494 and V495 with an unknown trypsin-like enzyme. The mature β-chain has extensive homology (37%) with a serine proteinase domain of plasminogen, but the histidine and serine residues of the proteinase active site are replaced by glutamine and tyrosine, respectively. The β-chain of HGF shows 24.5% amino acid sequence identity with the 33-kDa subunit of the crayfish mas-like protein (Fig. 9). The α-chain of HGF contains four kringle motifs, whereas the 45-kDa subunit of the mas-like protein has seven repeats of putative disulfide-knotted motifs, all of which shows similarity to those of D. melanogaster mas (18) and A. gambiæ ispl5 (28). However, these two cysteine repeated motifs in HGF and mas-like protein have low amino acid sequence similarity.

Human azurocidin, which is also a serine proteinase homologue, is a multifunctional protein with antimicrobial activity against Gram-negative and Gram-positive bacteria and *Candida albicans* (21–23) as well as LPS (24) and heparin binding activity (33, 34). The crayfish mas-like protein is functionally similar to human azurocidin, for example, in its LPS-binding activity, but the crayfish mas-like protein does not have any antibacterial activity as does azurocidin (data not shown). The whole amino acid sequence of human azurocidin shows 23.4% identity to the 33-kDa crayfish mas-like protein (Fig. 9).

To date, several serine proteinase homologue proteins have been characterized from different animals, and these proteins with inactive serine proteinase-like domains show biologically important functions, such as cell adhesion activity (17), LPS binding activity (24), and antimicrobial activity (21–23). Although the serine proteinase homologue proteins do not have proteinase activity, after proteolytic processing they will participate in the immune system of invertebrates and vertebrates. In this context, the mas-like protein plays a critical role in crayfish innate immunity as a pattern recognition protein that recognizes Gram-negative bacteria and yeast and participates in the clearance of microorganisms.

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