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Identification and characterization of a QM protein as a possible peptidoglycan recognition protein (PGRP) from the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

In an attempt to identify a peptidoglycan recognition protein (PGRP) in *Penaeus (Penaeus) monodon*, *in vitro* pull-down binding assays were used between shrimp proteins and purified peptidoglycan (PG). By gel electrophoresis and mass spectrometry followed by Mascot program analysis, proteins from shrimp hemocyte peripheral membrane proteins showed significant homology to records for a QM protein, actin and prophenoloxidase 2 precursor (proPO2), while proteins from cell-free plasma showed significant homology to records for a vitellogenin, a fibrinogen related protein (FREP) and a C-type lectin. Due to time and resource limitations, specific binding to PG was examined only for recombinant PmQM protein and PmLec that were synthesized based on sequences reported in the Genbank database (accession numbers FJ766846 and DQ078266, respectively). An *in vitro* assay revealed that hemocytes would bind with and encapsulate agarose beads coated with recombinant PmQM (rPmQM) or rPmLec and that melanization followed 2 h post-encapsulation. ELISA tests confirmed specific binding of rPmQM protein to PG. This is the first time that PmQM has been reported as a potential PGRP in shrimp or any other crustacean. The two other potential PGRP identified (FREP and the vitellin-like protein present in male *P. monodon*, unlike other vitellin subunits) should also be expressed heterologously and tested for their ability to activate shrimp hemocytes.

1. Introduction

Innate immunity plays an important role in the defence mechanism of both vertebrates and invertebrates. The main functions are to differentiate self from non-self and initiate highly regulated immune cascades such as the prophenoloxidase (proPO) activating system that finally eliminate pathogens (Cerenius and Soderhall, 2004). The non-self recognition process involves the binding of pathogen-associated molecular patterns (PAMPs) with specific shrimp humoral pattern recognition proteins (PRPs). Examples of PAMPs are β-1,3-glucan (BG), peptidoglycan (PG) and lipopolysaccharide (LPS) (i.e., microbial cell wall components that are conserved in pathogens but absent in the host). PRP–PAMP complexes trigger the proPO system resulting in melanogenesis accompanied by the production of antimicrobial substances such as quinones and melanin (Cerenius and Soderhall, 2004; Johansson and Soderhall, 1996).

PG is a bacterial cell wall component consisting of a glycan strand of alternating β(1-4) linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The pattern recognition proteins responsible for binding to PG and activating an immune response are called peptidoglycan recognition proteins (PGRPs) and they can be found in a wide range of organisms such as insects, mollusks, echinoderms and vertebrates, but not in nematodes or

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plants. PGRP was first discovered in the hemolymph and cuticle of *Bombyx mori* (the silk worm) Yoshida et al., 1996. As a consequence, other orthologs of PGRPs were later identified and cloned in other arthropods such as *Trichoplusia ni* (Kang et al., 1998) and *Drosophila melanogaster* (Werner et al., 2003). Subsequently, orthologs of PGRP were also discovered and cloned in mammals such as the mouse and humans (Kang et al., 1998). All PGRPs so far reported have at least one carboxy-terminal PGRP binding domain that shares a similar structure to bacteriophage and bacterial type 2 amidases (Dziarski and Gupta, 2006).

PG is an activator of the proPO system in crustaceans including shrimp (Cerenius and Soderhall, 2004). However, except for a recent publication about activation of the proPO system by PG through serine proteinase homologues (*Pl*-SPH1, and *Pl*-SPH2) and a lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) in *Pacifastacus leniusculus* (Liu et al., 2011), no PGRP has yet been reported from any crustacean. The aim of this work was to search for and characterize protein(s) that have ability to bind to peptidoglycan as the preliminary step in activation of an immune cascade. Both in-house and commercial preparations of PG were used in the pull-down assays. The candidate proteins were to be selected for heterologous expression in bacteria followed by confirmation of their binding to PG. Finally, expressed proteins were to be tested together with commercial peptidoglycan for their ability to activate shrimp hemocytes.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli, DH5α [(*sup*E44) Δ*lac*U169 (φ80 lacZ ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 thi-1 *rel*A1)] was used as the host for plasmid propagation. *E. coli* BL21 [pLys (DE3)] and C41 [(DE3) (F^- ompT hsdSB (rB^- mB^-) *gal dcm* (DE3)] were used as the hosts for expression of genes inserted in vectors. *E. coli* DH5α was cultured in LB medium (Difco™).

2.2. Shrimp

Living male broodstock of the giant tiger shrimp *Penaeus monodon* were purchased from the Shrimp Genetic Improvement Center (SGIC), Chaiya, Surathani Province. They were acclimatized under laboratory conditions (20–30 ppt salinity with ambient temperature of 27–28 °C) for at least 1 week prior to being used in experiments.

2.3. Preparation of hemocyte peripheral membrane proteins

To obtain hemocyte peripheral membrane proteins, 175 ml of hemolymph was collected from the ventral sinus using a syringe containing ice-cold AC-1 anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM sodium citrate, 10 mM EDTA, pH 7.0) (Smith and Soderhall, 1983) at 1:1 volume ratio. Hemocyte pellets were collected by centrifugation at 800*g* for 10 min at 4 °C and washed once with AC-1 solution. Since peripheral membrane proteins are non-covalently linked to the membrane, they can be separated from it by dissociating soluble protein–protein interactions without total membrane disruption or cell lysis using reagents such as a concentrated salt solution (Hafiz, 2005). Thus, 46 ml of ice-cold 1 M NaCl was added to the isolated hemocytes followed by gentle rotation for 10 min at 4 °C. The suspension was then centrifuged at 500*g* for 10 min at 4 °C and the supernatant was collected. PMSF was added to a final concentration of 1 mM and supplemented with protease inhibitor mix (GE healthcare) before dialysis against PBS buffer pH 7.5 (binding buffer) at 4 °C overnight.

2.4. Preparation of hemolymph proteins

Hemolymph proteins were prepared by collecting 4 ml hemolymph from the ventral sinus using a syringe containing ice-cold PBS buffer pH 7.5 (binding buffer) at 1:1 volume ratio. The suspension was centrifuged at 800*g* for 10 min at 4 °C to remove the hemocyte pellets. The supernatant was collected. PMSF and protease inhibitor mix were added before ultracentrifugation at 200,000*g* for 2 h at 4 °C to precipitate hemocyanin. SDS-PAGE analysis of the collected clear supernatant contained a reduced amount of hemocyanin. The product was referred to as "hemolymph protein".

2.5. Preparation of peptidoglycan (PG)

Peptidoglycan was prepared according to Araki et al. (1972). The Gram-positive bacterium *Lactobacillus acidophilus* was cultured in MRS broth 37 °C for 24 h. Cells (wet weight 20 g) were harvested by centrifugation at 8000*g* for 10 min and resuspended in 50 ml of cold distilled water. The cell suspension was further ultrasonicated using an amplitude of 40% with pulse on 9 s and off 3 s for 8 min using a VCX-750 Vibra-Cell™ sonicator (Sonic & Materials, USA). The suspension was then centrifuged at 2200*g* for 10 min at 4 °C and the supernatant was collected for further centrifugation at 20,000*g* for 45 min at 4 °C. The sediment fraction was resuspended in 50 ml of cold 1 M NaCl before the addition of 27 ml of distilled water for heating at 100 °C for 20 min. After cooling, the suspension was mixed with 47 ml of 2 M sodium acetate buffer, pH 5.9 and incubated with 3.33 mg ribonuclease at 37 °C for 3 h. Then, the suspension was centrifuged at 20,000*g* for 1 h and the pellet was resuspended in 33 ml of 50 mM sodiumphosphate buffer, pH 7.5 for digestion with 3.33 mg of trypsin at 37 °C for 2 h. The suspension was centrifuged at 20,000*g* for 1 h at 4 °C and the precipitate was collected for treatment with 33 ml of 0.4% sodium dodecyl sulfate (SDS) at room temperature for 1 h. Finally the pellet was washed 6 times with cold distilled water, lyophilized and referred to as "cell wall of *L. acidophilus*".

Peptidoglycan was prepared by heating a suspension of the cell wall of *L. acidophilus* in 0.1 N HCl at 60 °C for 24 h before washing with distilled water followed by lyophilization. This final product was referred to as "purified peptidoglycan". When analysed by SDS PAGE, the purified peptidoglycan gave no protein bands (not shown), and it was therefore not included as a negative control in gels of proteins from the pull down assays.

2.6. Pull down detection of putative PG binding proteins

The *in vitro* pull-down binding assay was performed by incubating shrimp hemocyte peripheral membrane protein (7.5 mg) or hemolymph protein (15 mg) in PBS pH 7.5 with 0.3 mg of peptidoglycan in a 2.0 ml binding tube with gentle rotation at 4 °C for 20 h. A higher quantity of hemolymph protein was used in comparison with hemocyte peripheral membrane protein due to the relatively high proportion of hemocyanin in the hemolymph protein preparation. After washing the pellet with washing buffer (PBS pH 7.5 with 0.01% triton X-100) three times by centrifugation at 16,000*g* for 1 min, bound and unbound proteins were subjected to analysis by SDS-PAGE. The electrophoresed protein bands were visualized by Coomasie Blue R staining.