Molecular Immunology 92 (2017) 1–11

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

A study on β-glucan binding protein (β-GBP) and its involvement in phenoloxidase cascade in Indian white shrimp *Fenneropenaeus indicus*

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ARTICLE INFO

Keywords:
Innate immunity
β-1, 3 glucan binding protein
Phenoloxidase
Serine protease

ABSTRACT

The present study reports the purification of novel immune molecule β-1, 3 glucan binding protein from the heamolymph of the Indian white shrimp, *Fenneropenaeus indicus* (*Fi*β-GBP). The purified *Fi*β-GBP had 95 kDa molecular weight in SDS-PAGE analysis. MALDI-TOF/TOF analysis revealed that the purified *Fi*β-GBP showed similarity to various crustacean proteins; 48 and 46% similarity was observed for β-1, 3 glucan binding protein of Chinese white shrimp *Fenneropenaeus chinensis* and banana shrimp *Fenneropenaeus merguiensis,* with MOWSE score of 3.11e + 12 and 2.05e + 8, respectively. The phenoloxidase activity (PO) of *Fi*β-GBP was evaluated and, in the presence of laminarin, PO activity increased significantly. Substrate specificity assay demonstrated that *Fi*β-GBP had the specific binding site for soluble or insoluble β-glucan (laminarin), since the PO activity increased in the presence of laminarin when compared to other sugars. Enzymatic activities revealed that the optimum temperature and pH for *Fi*β-GBP activating PO were 40 °C and pH 7–8. Moreover, even at 100 °C *Fi*β-GBP enhanced PO activity highlighting that *Fi*β-GBP was thermostable and thermophilic in nature. Among various divalent metallic ions, *Fi*β-GBP significantly promoted the PO activity in presence of Mg^{2+} and Ca^{2+}. The breakdown of para nitroanilide from N_{α} − Benzoyl-L-Arginine 4-Nitroanilide hydrochloride showed that serine protease activity was induced by *Fi*β-GBP and also increased concentration of *Fi*β-GBP evoked the activity. Furthermore, hemolytic activity tests revealed that PO reaction product induced RBC membrane damage and cell shrinkage. Lastly, Baclight bacterial viability assays showed maximum killing effect of PO reaction product on both Gram positive and Gram negative bacteria.

1. Introduction

The ability to distinguish self from non-self is a fundamental aspect in any immune system (Mogensen, 2009). In invertebrates, a set of conserved host proteins, known as pattern recognition proteins, plays a critical role in non-self recognition. Upon pathogen introduction, PRPs sense non-self pathogen associated molecular patterns (PAMPs), with surface markers of invading microbes and triggering the innate defense mechanism of invertebrates. Although, crustaceans lack true adaptive immune system, they have the ability to recognize invading microbes by plasma proteins or pattern recognition proteins. The innate immune system of invertebrates consists of humoral and cellular immunity. During pathogen introduction, cellular-mediated immune responses such as melanization, phagocytosis, encapsulation and nodule formation, can be triggered by PRPs (Nappi et al., 2004; Sideri et al., 2008). Apart from these, humoral immune responses include the activation of enzymatic reactions that regulate coaglulation and melanization of heamolymph, serine protease and release of antimicrobial peptides from immunocytes, production of reactive oxygen (ROS) and nitrogen species also play a pivotal role in pathogen elimination (Tsakas and Marmaras, 2010).

Till date, various PRPs have been isolated and purified from invertebrates, showing diverse functions, especially in crustaceans (Chen et al., 2016). The most important PRPs purified from invertebrates include β-1, 3 glucan binding protein (β-GBP) (Sivakamavalli and Vaseeharan, 2014a; Mohanty et al., 2015; Anjugam et al., 2016;

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http://dx.doi.org/10.1016/j.molimm.2017.09.013
Received 21 August 2017; Received in revised form 20 September 2017; Accepted 22 September 2017

Iswarya et al., 2017), lipopolysaccharide and β-1, 3 glucan binding protein (LGBP) (Lee et al., 2000; Chaosomboon et al., 2017), Gram negative bacteria binding protein (G-NBP) (Lee et al., 1996; Kim et al., 2000), peptidoglycan recognition protein (PGRP) (Yoshida et al., 1996), and C-type lectin (Wongpanya et al., 2017; Sun et al., 2008; Sivakamavalli and Vaseeharan, 2014b; Jayanthi et al., 2017), which make a complex with the proPO activating enzyme, and microbial cell wall compositions leads to activated inactive precursor proPO to active PO by a limited proteolysis. It was stated that ProPO system was enthused by β-glucan (β-G) surface markers of fungal cell wall and lipopolysaccharide (LPS) of bacterial cell wall. Eventhough, fungal β-G and LPS activate the proPO cascade, β-GBP amplify the mechanism (Vargas-Albores and Yepiz-Plascencia, 2000; Söderhäll and Cerenius, 1998). β-GBP can be able to recognize the pathogens followed by serine protease stimulation and elicited the innate defense system of invertebrates through a proPO cascade (Sivakamavalli et al., 2015).

In invertebrates, phenoloxidase (PO) was generated from the limited proteolysis of zymogen (prophenoloxidase) by a proPO-activating enzyme (PpAE) (Aspan et al., 1995), which is responsible for initiating the conversion of o-phenols to unstable quinones. Quinones prompted the melanization process leads to melanin synthesis for the deterioration of invading pathogens (Asokan et al., 1997; Jiravanichpaisal et al., 2006; Hellio et al., 2007). However, the melanization process can be activated by a mechanism known as protein–protein interactions (PPI). PpAE was generated through PPI between PRPs and proteases. During pathogen invasion, PRPs and PAMPs interact themselves, thereby dormant zymogen serine protease (SP) can be turn on into serine protease, owing to the triggering mechanism of serine protease activating factor (SPAF) (Liu et al., 2011; Sivakamavalli et al., 2016). After activation, it induces both humoral and cellular immune functions simultaneously (Lemaitre and Hoffmann, 2007).

In penaeid shrimps, β-GBP is an abundant plasmatic protein found in circulating haemocytes. Our previous study on β-GBP from various crustaceans revealed that it is involved in various immune functions, including phagocytosis, encapsulation and agglutination (Anjugam et al., 2016; Iswarya et al., 2017). Moreover, β-GBP was involved in PO activity. In the current study, the immune molecule β-GBP was purified from the heamolymph of Indian white shrimp *Fenneropenaeus indicus* (H. Milne-Edwards) (Decapoda: Penaeidae). The identification of the purified protein as *Fi*β-GBP was carried out via MALDI-TOF/TOF analysis. Furthermore, we assessed the impact of temperature, pH, and metal ion concentrations on *Fi*β-GBP in PO activation, by various assays. In addition, the antibacterial and antibiofilm activity of PO reaction product was screened against Gram positive *Staphylococcus aureus* and Gram negative *Pseudomonas aeruginosa*. The hemolytic potential of PO reaction product was determined on human red blood cells (RBCs).

2. Materials and methods

2.1. Shrimp collection

Apparently healthy *F. indicus* shrimps with an average weight of 15 ± 22 g, normal body color and unbroken body shell without fester, were collected from the coastal area of Mandapam (Latitude 9.2814° N; Longitude 79.1375° E), Ramanathapuram district, Tamilnadu, India and transferred to the laboratory. Indian shrimps were acclimatized and maintained in 500 l fiberglass reinforced plastic (FRP) tanks containing filtered seawater (35% salinity, 28 °C) with regular aeration. Animals were fed with formulated shrimp diet (crude protein 32%; crude fat 5%; fibre 5%; moisture 8–10%; ash 17%; hydrochloride insoluble 10%) at 10% of their body weight.

2.2. Extraction of heamolymph

Heamolymph was collected from the ventral sinus in the first abdominal segment of shrimp into a 2 ml syringe containing an anticoagulant solution (30 mm sodium citrate, 0.45 M NaCl, 0.1 M glucose, 10 mM EDTA, pH 7.5). Gathered heamolymph was immediately centrifuged at 3300*g* for 20 min at 4 °C (Shanthi and Vaseeharan, 2014). The resultant plasma supernatant was stored at −86 °C for further anaysis.

2.3. Heamocyte lysate supernatant (HLS) preparation

After centrifugation of heamolymph, the obtained heamocyte pellet was suspended in sodium coagulate buffer and homogenized using glass piston. The homogenized haemocyte suspension was centrifuged at 35,000 rpm for 20 min at 4 °C. The resultant supernatant was stored as heamocyte lysate supernatant (HLS).

2.4. Purification of Fiβ-GBP

F. indicus β-GBP was purified according to the method by Sivakamavalli and Vaseeharan (2013) with minor modifications. In brief, laminarin coupled Sepharose CL–6B affinity column was implemented to purify the *F. indicus* β-GBP. The plasma supernatant was dialyzed extensively in double distilled water overnight. The resultant pellet was diluted in 50 mM Tris HCl, pH 7.5 and applied into affinity chromatography containing laminarin with Sepharose CL–6B as matrix previously equilibrated with TBS II buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4). The unbound protein was eluted from the column by equilibration buffer until the absorbance of the eluent was zero. The bound protein in a column was eluted by 0.1 M acetate buffer containing 2.5 M $MgCl_2$ and 1 M D-glucose and the fractions showed absorbance at 280 nm.

2.5. Molecular weight determination

The eluent containing *Fi*β-GBP was subjected to SDS-PAGE analysis for determining its molecular weight (Laemmli, 1970). For analysis, 12% separating gel and 4% stacking gel was used to separate the proteins according to their molecular weight. In brief, 10 µl of *Fi*β-GBP was mixed with same volume sample buffer (1 mM Tris HCl, pH 6.8; 10% SDS, 2 ml Glycerol, β-mercaptoethanol, 1% bromophenol blue, 10 ml ddH_2O) and boiled for 10 min. Prior to loading, the sample was cooled at room temperature. The gel was subjected to electrophoresis in Tris-glycine buffer (0.025 M Tris, 0.2 M Glycine, pH 8.3) at constant electricity for 3–4 h. After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB) R-250 for band visualization.

2.6. Mass spectrum analysis

MALDI is one of the widely used techniques to confirm the protein identity. Mass spectrum analysis of purified protein was carried out as described by Iswarya et al. (2017). The purified 95 kDa protein was excised from SDS-PAGE gel using a sterilized scalpel and subjected to trypsin digestion. In brief, the excised gel bands corresponding to *Fi*β-GBP were cut into small pieces and stored into sterilized Eppendorf tubes containing stain removal solution (100 mM ammonium bicarbonate: 50% acetonitrile (1:1)) vortexed for 30 min with 10 min interval. After complete removal of stain, the gel pieces were dehydrated with 100% acetonitrile (ACN). Followed by dehydration, the gel pieces were subjected to reduction and alkylation procedure. Trypsin solution was prepared in 40 mM NH_4HCO_3 and added to each Eppendorf tube containing the gel pieces and maintained at ice cold condition for absorption of trypsin. Subsequently, NH_4HCO_3 was added and incubated at 37 °C for 12–16 h. By adding 2–4% formic acid (FA), the digestion process was terminated. The peptides were extracted by washing gel pieces for four times with 5% FA in 50% ACN. The resultant peptides were analyzed by MALDI-TOF/TOF MS (Shimadzu, Biotek Axima Performance). The acquired mass spectra of peptides were searched for